POLYMORPHISMS IN CANDIDATE GENES FOR THE NITRIC OXIDE PATHWAY IN SICKLE CELL PATIENTS WITH ACUTE CHEST SYNDROME AND ASTHMA

By

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To my children, Lindsay and Lesley Duckworth, for their love, support, and patience. To my parents, Louis and Lorraine Doucette, for believing in me. To all who nurtured my intellectual curiosity making this milestone possible.

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LIST OF ABBREVIATIONS

ACS	acute chest syndrome
AAT	intronic repeat NOS1 gene
DNA	deoxyribonucleic acid
FENO	exhaled nitric oxide
ICS	inhaled corticosteroid
NO	nitric oxide
cNOS	constitutive nitric oxide synthase
eNOS	endothelial nitric oxide synthase (NOS3)
HWE	Hardy-Weinberg equillibrium
iNOS	inducible nitric oxide synthase (NOS2)
LABA	long-acting beta agonists
OR	odds ratio
PCR	polymerase chain reaction
nNOS	neuronal nitric oxide synthase (NOS1)
SABA	short-acting beta agonists
SCD	sickle cell disease
SNP	single nucleotide polymorphism
NCC	Nemours Children’s Clinic

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Sickle cell disease (SCD) is one of the most common genetic diseases, affecting one in 600 African Americans. Acute chest syndrome (ACS) is the leading cause of mortality and the second most common cause of hospitalizations in patients with SCD accounting for nearly half of premature deaths. A number of recent studies have reported that asthma may increase the risk of ACS in children with sickle cell disease. Nitric oxide is thought to play a key role in the pathogenesis of ACS. The main objectives of this study were to test the hypotheses that polymorphisms in candidate genes; Arginase 1, nitric oxide synthase (NOS) genes; NOS1 and NOS3, associate with ACS in SCD patients and to characterize the association between physician-diagnosed asthma and ACS. A total of 134 participants between 5-21 years of age with SCD were enrolled. Associations between acute chest syndrome and asthma with the following polymorphisms were explored: the AAT in intron 13 (formerly intron 20) of the NOS1; T-786C and G894T and the repeat polymorphism in intron 4 of NOS3; and ARG I *Pvu* polymorphism. African Americans (n=74) comprised a cohort of healthy controls owing to non-Hardy-Weinberg equilibrium (HWE) in some variants.

Physician-diagnosed asthma was determined by chart review, parental report, and medication use. Eighty five percent of participants with asthma had at least on episode of ACS

compared to 14.6 % of participants without ACS; adjusted odds ratio (OR) (95%CI) 5.46 (2.20,13.5), $P = < 0.0001$. Physician-diagnosed asthma correlated with the number of episodes of ACS ($P < 0.001$). The NOS1 AAT repeat polymorphism associated with the risk of ACS ($P = 0.001$) in patients without physician-diagnosed asthma. No associations were found between the NOS3 T-786C polymorphism and ACS. Carriers of the ARG I minor allele were less likely to have asthma, 22/79 (28%) compared to WT homozygotes 6/47 (13%); p = 0.04.

Findings from this study suggest that asthma is a major risk factor for ACS. The NOS1 AAT repeat polymorphism may contribute to ACS in SCD patients without asthma. Studies that further characterize the association between asthma, ACS, and NOS genes in children with sickle cell disease are warranted.

CHAPTER 1
INTRODUCTION

Sickle cell disease (SCD) is one of the most common genetic diseases, affecting one in 600 African Americans. Acute chest syndrome (ACS) is the leading cause of mortality and the second most common cause of hospitalizations in patients with sickle cell disease (SCD) accounting for nearly half of premature deaths (Platt et al., 1994; Stuart, & Setty 2001; Buchanan et al., 2004). Our current understanding of the pathophysiology and mechanisms leading to ACS in SCD is limited and remains unclear. In a large prospective study infection and pulmonary fat embolism were identified as causal in 38% of episodes, but in approximately 50% of cases no cause was determined (Vichinsky et al., 2000).

Nitric oxide (NO) is thought to play a key role in the pathogenesis of ACS (Gladwin et al., 1999). NO is formed through the hydrolysis of arginine to NO by nitric oxide synthase (NOS). Arginine acts as a substrate for both NOS and arginase. The arginase and NOS pathways can interfere with each other via substrate competition (Morris, 2002) (Fig 1-1). Recent studies suggest that asthma may be related to decreased NO bioavailability (de Boer et al., 1999; Meurs, Maarsingh, Zaagsma, 2003) rather than an overproduction due to inflammation (Kharitonov&Barnes, 2007). This may occur as a result of the increased activity of arginase (Meurs et al., 2002; Zimmermann et al., 2003). Notably, plasma concentrations of NO are reduced during ACS as a consequence of reduced NO bioavailability (Morris et al., 2006) (Morris, et al., 2004).

Compared to normal control subjects, arginine concentrations were lower and the activity of arginase, the enzyme that hydrolyzes arginine to ornithine and urea, was higher in patients with asthma (Morris et al., 2004). Addtionally, arginase expression was strongly induced by IL-4 and IL-13 in mice models of asthma and by Th 2 cytokines, which may contribute to NO

deficiency in asthma (Zimmermann et al., 2003). Wechsler et al (2000), identified a group of patients with asthma with low concentrations of FE_{NO} that was inversely related to the AAT repeat polymorphism in intron 13 on the NOS1 gene.

Recent studies demonstrate that administration of inhaled NO has beneficial effects in treating ACS (Stuart & Setty, 2001; Sullivan et al., 1999; Atz & Wessel, 1997). Sullivan et al (2001) reported lower concentrations of exhaled nitric oxide (FE_{NO}) in children who previously had ACS. Additionally, they reported that low FE_{NO} was associated with a repeat polymorphism in intron 13 (formerly called intron 20) on the NOS1 gene (Sullivan, 2001). Other NOS genes may also be associated with ACS. For example, the NOS3 T-786C polymorphism and increased susceptibility to ACS in females with SCD was reported (Sharan et al., 2004). Chaar et al., (2006) reported that the C-786 allele was associated with a decreased risk of ACS. Genes that are involved in the regulation of NO may be important in ACS because of the central role NO plays in airway inflammation and the pulmonary endothelium.

In addition to genetic factors, environmental factors may contribute to the susceptibility of patients with SCD to develop ACS. A number of recent studies have reported that asthma may increase the risk of ACS in patients with SCD (Boyd et al., 2004; Knight-Madden et al , 2005; Bryant, 2005; Nordness, 2005; Sylvester et al., 2007). These reports were based on studies that documented a link between SCD and airway hyperresponsiveness, lower airway obstruction, reversibility, abnormal pulmonary function tests and the fact that corticosteroids and bronchodilators, drugs commonly used in asthma, were beneficial in ACS (Santoli et al., 1998; Koumbourlis et al., 2001) (Klings et al., 2006).

The purpose of this study was to characterize the association between asthma and SCD. The second aim of this study was to determine associations between polymorphisms in candidate

genes for the nitric oxide pathway. These genes code for enzymes that utilize arginine as a substrate and regulate NO production. Therefore it is possible that genetic variants that regulate NO production could contribute to ACS and asthma in SCD.

Background and Significance

Acute Chest Syndrome and Nitric Oxide

ACS is a common complication of sickle cell anemia. ACS is the second most common cause of hospitalization in patients with sickle cell disease and is the leading cause of premature deaths (Platt, 1994) (Vichinsky et al., 1997, 2000). It is characterized by the presence of a rapidly progressing multi-lobe infiltrate, cough, hypoxemia and dyspnea. The etiology of ACS is multifactorial and remains unclear. Our current understanding suggests that ACS may be a form of acute lung injury that progresses to acute respiratory distress syndrome. This injury is thought to be precipitated by sloughing of blood in the pulmonary microvascular resulting in pulmonary infarction, fat embolisation, and infection (Vichinsky et al 1996) (Scully et al ,1997). There is compelling evidence to support the central role of NO in the initiation of the pathophysiological process in ACS. Stuart and Setty assessed plasma NO metabolites in 36 patients with SCD and 23 age-matched controls. They found that serum concentrations of NO metabolites were decreased during acute chest syndrome with values lower than in controls and in patients at steady state (Stuart & Setty, 1999). Hammerman and colleagues (1999) exposed cultured pulmonary endothelial cells to the plasma of patients with sickle cell disease and acute chest syndrome and found that within two hours of exposure there were increases in NOS 3 protein and NOS 3 enzyme activity. They suggested that alterations of NO production and metabolism contribute to the pathogenesis of ACS.

Nitric Oxide Synthase Enzymes

Nitric oxide synthase enzymes are expressed in the lung parenchyma. NOS 1 and 3 are constitutive and regulated by intracellular calcium concentration. NOS 2 is induced under inflammatory conditions, such as asthma, and is independent of calcium levels. Studies in pig lungs suggest that exhaled nitric oxide originates at the alveolar surface rather than from the pulmonary circulation and may be derived from NOS 3 expressed in the alveolar walls of normal lungs (Kobzik et al., 1993). Airway epithelial cells express both NOS 1 and 3 and therefore contribute to NO levels in the lower respiratory tract (Shaul et al., 1994; Asano et al., 1994). Using exhaled nitric oxide (FE_{NO}) as a marker of NO production, Sullivan et al (2001) found that the concentration of FE_{NO} was lower in children with SCD who had previously suffered from acute chest syndrome as compared to children with sickle cell disease with no history of acute chest syndrome and healthy controls. Additionally they demonstrated that the FE_{NO} levels are significantly correlated with the number of NOS1 AAT repeats. Given that FE_{NO} in healthy controls is likely produced by NOS 1 and 3 at basal concentrations, the decreased level in patients with ACS as compared to healthy controls suggest genetic variations in these genes. It is tempting to speculate that the low FE_{NO} seen in SCD patients with ACS may have a genetic origin and possibly may be due to polymorphisms in NOS genes. Finally, a study in 97 mild asthmatic patients revealed that the size of the AAT repeat polymorphism on intron 13 of the NOS 1 gene was significantly related to FE_{NO} (Weschler et al., 2000). A certain fraction or phenotype of asthmatics had low $FE_{NO.}$ Collectively these findings suggest and support the hypothesis that genetic variation in NOS genes may contribute to airway disease predisposing patients with SCD to ACS.

Nitric Oxide, Airway Inflammation, ACS, And Asthma

The importance of NO as a marker for airway inflammation in asthma is well documented (Barnes, 1995; Batra, et al 2007). Exhaled nitric oxide levels serve as a useful marker for assessing asthma severity and medication compliance (Naprawa et al., 2005). Asthma is the most common chronic disease of childhood in the United States. The incidence of asthma in the African American population surpasses that of Caucasians, 17% vs 6% respectively (Yeatts & Shy, 2001) (Boyd et al., 2006). Differences in asthma related symptoms complicate the treatment and management of this disease in patients with SCD who may also have ACS. For example, patients with SCD who have recurring airway dysfunction may not be identified as asthmatic in that they are not evaluated for this condition. Sub specialists and Pediatricians may focus on the child's SCD and manage upper respiratory conditions on a case-by-case basis. This may leave the patients more vulnerable to an increased risk for ACS.

Significance of Research

The information gained from the proposed research may identify candidate genetic variants in the NO pathway that increase the risk of patients with SCD to develop ACS. More specifically, this research may determine whether polymorphisms in NOS genes and other genes that encode proteins that regulate NO predispose patients with sickle cell disease to episodes of acute chest syndrome. If the NOS genotype is suggestive of decreased NO production in the lung, early intervention with treatment modalities such as oral arginine may prevent ACS and thereby reduce morbidity and mortality associated with this complication. Findings from this research may lead to screening of patients with SCD for the genetic variants associated with ACS early in life resulting in aggressive management and particular attention to associated lung diseases such as asthma which may put them at heightened risk for pulmonary complications.

Research Aims and Hypotheses

Specific Aim 1

To characterize the relationship between ACS and asthma in children with SCD.

Hypothesis

Asthma is a risk factor for ACS.

Specific Aim 2

To explore associations among polymorphisms in candidate genes of the nitric oxide pathway and their association with asthma, ACS and SCD.

Hypothesis

Polymorphisms in candidate genes of the nitric oxide pathway predict asthma and ACS in children with SCD.

Significance to Nursing

This study will explore associations between candidate genes in the NO pathway and the incidence of asthma and acute chest syndrome in a sickle cell disease population. Gaining insight into mechanisms of chronic disease that may impact complications associated with SCD is vital to nursing care. Outpatient Sickle Cell Disease clinics are generally managed by nurses. The nurse is often the first contact when patients experience pain or respiratory distress. Asthma is the leading chronic illness in children, can be life threatening, and is more prevalent in the African American population. Recent retrospective studies suggest that patients with SCD who also have a history of asthma may be at increased risk for acute chest syndrome. Knowledge regarding polymorphisms in NOS genes and historical evidence of asthma, thereby predisposing patients to ACS, may heighten awareness and result in aggressive management and treatment for asthma related symptoms.

Theoretical Framework

Sickle Cell disease is the most common genetic disorder among African Americans. Acute Chest syndrome is a leading cause of death in patients with SCD. The incidence of asthma is greater in African Americans compared to Caucasians (Lugogo & Kraft, 2006). Racial disparities in the morbidity and mortality associated with asthma can be staggering suggesting that African Americans may receive substandard care with regard to diagnosis and treatment (Ford, & McCaffrey, 2006). The reasons for this are unclear but may involve many factors including; access to care, payment, and, educational resources. Theoretically, it stands to reason that patients with SCD who also have asthma may be more susceptible to the complication of ACS.

There is compelling evidence to support the central role of NO in the initiation of the pathophysiological process in acute chest syndrome. Stuart et al (1999) found that serum NO concentration metabolites were decreased during ACS with values lower than patients in steady state, without ACS. Decreased NO production in patients with ACS may be the final common pathway leading to ongoing hypoxemia, pulmonary hypertension, and acute lung injury. Patients prone to develop ACS have decreased levels of FE_{NO} even during periods of stability (Sullivan et al., 2001). This may be due to genetic variations in candidate genes that regulate NO, which predispose patients to reduced NO production and recurring episodes of ACS. A growing body of research suggests that decreased expression of NO synthase genes resulting in decreased NO production is deleterious to the lung and may lead to acute chest syndrome (Hammerman et al., 1999). Identifying these patients before developing ACS may decrease the morbidity and mortality associated with this condition.

To date few modifiable risk factors for ACS have been identified. Several retrospective studies have reported that having asthma may increase the risk of developing ACS in the SCD

population. Wechsler et al. identified a group of patients with asthma with low concentrations of FE_{NO} that was inversely related to the number of AAT repeats in intron 13 on the NOS1 gene. This study did not include patients with SCD (2000). A study in a SCD population reported that the number of AAT repeats in intron 13 of the NOS 1 gene associated with FE_{NO} levels; lower levels associated with a higher number of repeats (Sullivan, 2001)

The overall objective of this study was to determine whether patients with ACS and SCD have one or more genetic polymorphisms in candidate genes for the nitric oxide pathway that may predispose them to ACS. More specifically, the study will compare allele frequencies and genotype distributions of polymorphisms in NOS 1, NOS 3, and Arginase 1 genes in children with sickle cell disease and ACS with age, gender, and asthma. Additionally this study will explore associations between asthma and ACS.

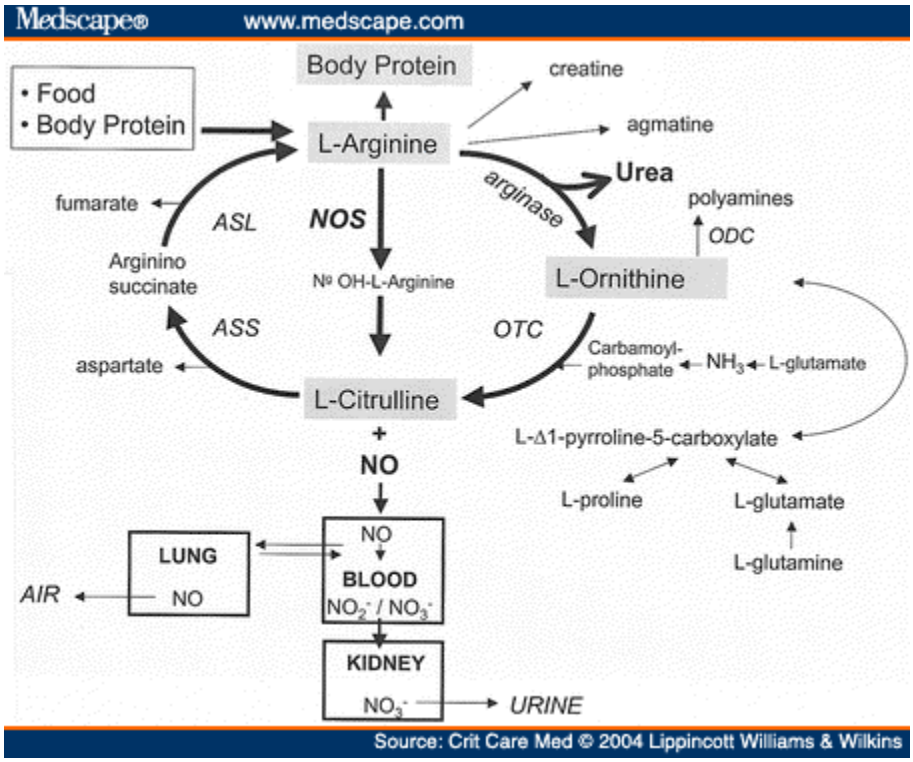


Figure 1-1. Metabolic pathways for Arginine, Arginase, Nitric oxide synthase, and Nitric oxide. Reprinted with permission by Lippincott, Williams & Wilkins.

CHAPTER 2
MATERIALS AND METHODS

This chapter is divided into three sections. The first section presents subject characteristics, sampling method, and eligibility criteria. Second, the methods and procedures with specifics on study design, protocol, and data collection are presented. Finally a description of data management and statistical analyses of the two aims are reviewed.

Subjects

Sample and Setting

A convenience sample of 134 African American subjects with SCD were selected and recruited from the Sickle Cell Disease Clinic, Emory University School of Medicine, Atlanta, GA, and from the Hematology Clinics at the Nemours Children's Clinic, Jacksonville and Orlando, Florida. All African American patients with SCD meeting eligibility criteria were offered participation in the study.

Informed consent and children's assent were obtained in accordance with the requirements and guidelines of the Institutional Review Boards at the participating centers. (Appendix A)

Inclusion Criteria

- African American
- ≥ 5 years to 21 years of age
- Sickle Cell Disease diagnosis (HbSS, HbSC or HbSβ)

Exclusion Criteria

Prematurity of birth resulting in Bronchopulmonary Dysplasia or Respiratory Distress Syndrome

Blood transfusion within the past 30 days

Methods And Procedures

Study Design

A prospective descriptive correlation study design was utilized to investigate the association between polymorphisms in NOS genes and the incidence of ACS and asthma in children with sickle cell disease. Participants with a positive history of ACS served as cases, those without served as controls. The diagram (Figure 2-1) illustrates the study protocol used in this study.

Consent

Informed consent and children's assent was obtained in accordance with the requirements and guidelines of the Institutional Review Boards at The University of Florida, Nemours Children's Clinic, and Emory University. Participants and their guardians were approached in person by the study coordinator or principal investigator. The purpose, risks, and benefits of the study were explained and reviewed in detail. The participants right to withdraw from the study at any time without penalty was discussed.

Demographic Information

General demographic information was collected from the participant and guardian following informed consent. (Appendix B)

Medical History

A brief medical history checklist was completed by the study coordinator or PI following informed consent (Appendix C).

Acute Chest Syndrome Diagnosis

The diagnosis of ACS was determined by history and chart review. ACS was defined by the presence of multilobar infiltrates by chest radiograph and history of cough, hypoxemia, and dyspnea. Patients with at least one episode of ACS were classified as cases. Patients with SCD

without a history of ACS were classified as controls. The age at which participants experienced their first episode of ACS was recorded.

Asthma Diagnosis

Participants were classified as having asthma if it was diagnosed by a physician and if they were currently prescribed one or more of the following asthma medications: inhaled short-acting beta agonists (SABA), inhaled corticosteroids (ICS), long-acting beta agonists (LABA), a leukotriene receptor antagonists (LTRA).

Isolation of Genomic DNA

Isolation of DNA was accomplished by a published, non-invasive method (Lum & LeMarchand, 1998). At least one hour after eating, subjects rinsed their mouths with water, then swished Scope mouthwash vigorously for one minute and emptied their oral contents into a Sarstedt 50 ml Centrifugation tube with a screw cap for closure. Alternatively the study participants could swish mouthwash for shorter duration on consecutive occasions until the subject had swished mouthwash for one minute. The tubes were coded with the study ID number, and mailed to the Cell and Molecular Biology Laboratory, Nemours Children's Clinic, in Jacksonville Florida. Alternatively mouthwash samples were stored at –20 to –70 ° C and sent to Nemours in bulk shipment.

Mouthwash samples were centrifuged at 2700 rpm for 15 minutes. The supernatant was poured off. Approximately 0.7ml of $T_{10}E_{10}SDS_{0.5\%}PK_{100\ g/ml}$ was added to the concentrate. The specimen was placed into a labeled and serum separator tube, and put in 50 ^{o}C incubator overnight. 0.7ml of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, pH 8.0) was added to the serum separator tube, and centrifuged at 2700 rpm for 10 minutes. This step was repeated. The supernatant was then placed in a labeled 2ml centrifuge tube. Approximately 0.7ml of 3M Sodium Acetate and 0.7ml of Isopropyl was added to the sample and then centrifuged at 14000

rpm for 5 minutes. The supernatant was drawn off, 1 ml of 70% alcohol was added and the sample was centrifuged again at 14,000 rpm for 2 minutes. The final specimen was suspended in 10mM of Tris, pH 7.5 and rehydrated overnight.

Genotyping

DNA was extracted as previously described and the quantity of DNA determined by spectrophotometry. (Lum & LeMarchand, 1998). Aliquots of DNA containing 500 ng were prepared and stored at -20°C in a secure and locked freezer, and labeled with the participant's code number. Forward and reverse primers specific to polymorphic loci in the NOS 1 and NOS3 genes were used to isolate regions of interest. Polymerase chain reaction was utilized to identify patient genotype.

Below is a listing of the various known polymorphic loci in the NOS 1 and NOS 3 genes and the ARG 1 gene. (Grasemann Yandava, & Drazen, 1999). In previous studies the location of the AAT repeat was reported as intron 20 (Sullian et al., 2001; Wechsler, et al., 2000). However, according to homosapiens chromosome 12 genomic contig NT_009775, the AAT repeat polymorphism locates in the region of intron 13 (complement 8267923-8271203) of the human NOS1 gene. Oligonucleotides were synthesized by Operon Technologies (Alameda, CA, USA). The restriction enzymes were purchased from New England Biolabs (Ipswhich, MA, USA).

NOS 1 (neuronal NOS)

ATT intronic repeat: intron 13 (complement 8267923 - 8271203)

- Forward primer: 5'CTGGGGCAATGGTGTGT-3'
- Reverse primer: 5'GAGTAAAATTAAGGGTCAGC-3'

NOS 3 (endothelial NOS) (Sharan et al., 2000)

-786 T to C polymorphism: (rs2070744)

- Forward primer: 5'GCATGCACTCTGGCCTGAAGTG-3'
- Reverse primer: 5'CAGGAAGCTGCCTTCCAGTGC-3'
- 223BP PCR product

Exon 7 G894T = Glu298Asp polymorphism: (rs1799983)

- Forward primer: 5'CTGGAGATGAAGGCAGGAGAC-3'
- Reverse primer: 5'CTCCATCCCACCCAGTCAATC-3'
- 267 BP PCR product

Intron 4 Deletion/Insertion of 27 BP

- Forward primer: 5'AGGCCCTATGGTAGTGCCTT-3'
- Reverse primer: 5'TCTCTTAGTGCTGTGGTCAC-3'
- Amplified by PCR on 6% polyacrylamide gels

ARG1 gene (Pvu II polymorphism) (rs17599586)

- 5'ATCTGAGGTAATAGAGAAGC 3'
- 5'TGAAAGTAGTACAGACAGAC 3'

Statistical Analysis

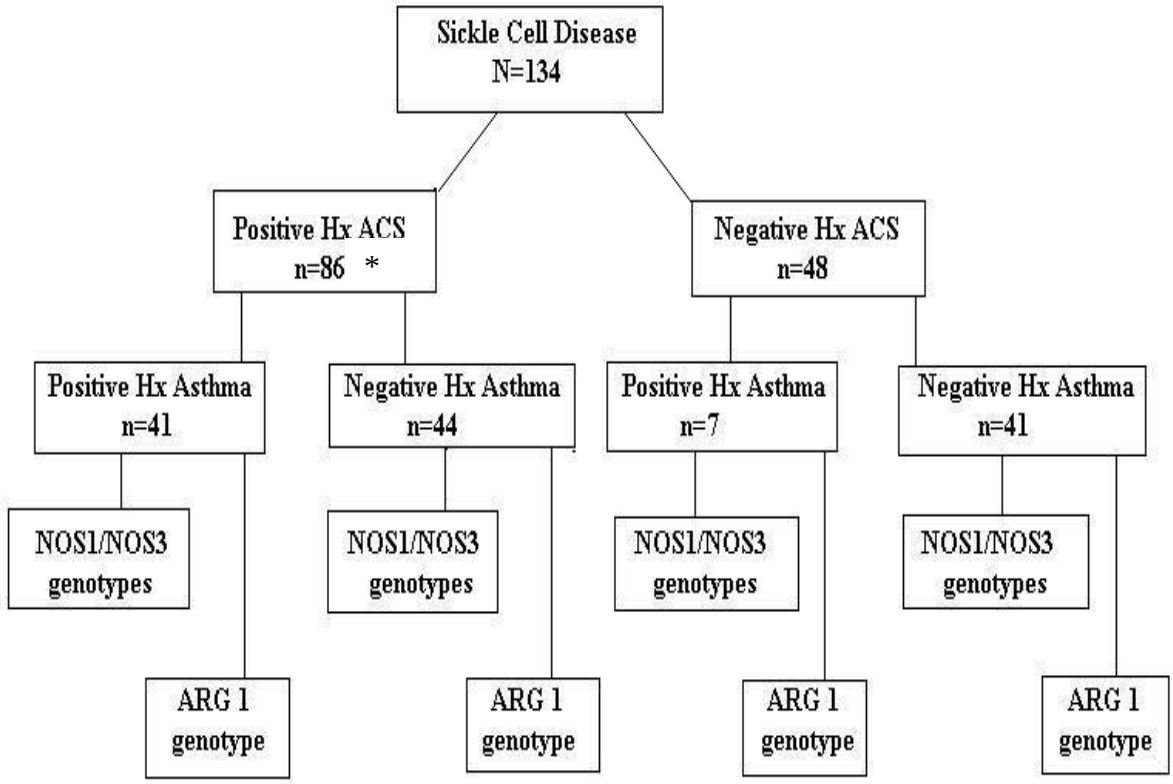
Data analysis was performed using SPSS version 11.0. The statistical significance of differences in allele frequencies and genotype distributions were determined by calculating odds ratios and by using chi square analysis. The Hardy –Weinberg equilibrium (HWE) was examined using the Markov chain method with a program for population genetics data analysis (Genepop, School of Biomedical Sciences, Curtin University of Technology, France) as well as

chi square goodness of fit tests. Differences in age among groups were determined using one-way ANOVA with Bonferroni correction for multiple comparisons. Association between groups with gender, allele, genotype, and the number of AAT repeats on NOS 1 were assessed using chi-square test. The strength of associations between disease risk and genotype were evaluated with Mantel-Haensze common odds ratios (OR) and 95% confidence intervals. The relationship between risk of ACS and the number of AAT repeats in intron 13 of the NOS 1 gene in patients with and without asthma was determined by simple linear regression. Associations between the incidence of asthma and episodes of ACS were determined by logistic regression analysis with age and gender as covariates.

The Hardy-Weinberg law is commonly used for calculating genotype frequencies from allele frequencies. This law is the cornerstone of population genetics. In population genetics, the Hardy–Weinberg principle is a relationship between the frequencies of alleles and the genotype of a population. The occurrence of a genotype, perhaps one associated with a disease, stays constant unless matings are non-random or inappropriate, or mutations accumulate. Therefore, the frequency of genotypes and the frequency of alleles are said to be at "genetic equilibrium". Genetic equilibrium is a basic principle of population genetics (Nussbaum, McInnes, & Willard, 2004). This sudy tested for Hardy Wienberg Equillibrium (HWE) with 74 healthy African American control subjects. The reasons for testing HWE were to establish that the allele frequencies in the study participants were consistent with an Arican American population, and secondly to rule out genotyping error. Interestingly, the NOS 3 polymorphysims were not in HWE when compared to the healthty control cohort. This suggest that polymorphisms in this gene may be realted to the disease process. This was not however confirmed with the results. In fact, associations were found between the AAT repeat

polymorphism in the NOS1 gene and the arginase 1 gene, neither of which showed differences with regard to HWE.

Figure 2-1. Diagram of study protocol.



*History of asthma was not reported for one participant

CHAPTER 3
LITERATURE REVIEW

Sickle Cell Disease

Sickle cell disease is an inherited blood disease that is characterized by defective hemoglobin. It is one of the most prevalent genetic disorders and is the most common genetic disease in the African American population. The genetic mutation associated with sickle cell disease occurs in approximately one in every 600 African American births. This disease affects millions worldwide and approximately 72,000 people in the United States (Platt, 1994). The clinical course of the disease varies from patient to patient. Some patients have mild symptoms while others are severely affected. The reasons for this are unclear.

Sickle cell anemia is caused by an abnormal type of hemoglobin called hemoglobin S. Hemoglobin is a protein inside red blood cells that carries oxygen. Hemoglobin S, however, distorts the red blood cells shape. The fragile, sickle-shaped cells deliver less oxygen to the body's tissues, and can break into pieces that disrupt blood flow (Goldman, 2004). Hypoxia enhances the sickled erythrocytes adherence to both the macrovascular and microvascular endothelium. The pulmonary microcirculation is particularly vulnerable to deoxygenation (Stuart & Setty, 1999).

Sickle cell anemia is inherited as an autosomal recessive trait. This means it occurs in someone who has inherited hemoglobin S from both parents. Sickle cell disease is much more common in certain ethnic groups, significantly affecting African Americans. Someone who inherits hemoglobin S from one parent and normal hemoglobin (A) from the other parent will have sickle cell trait. Someone who inherits hemoglobin S from one parent and another type of

abnormal hemoglobin from the other parent will have another form of sickle cell disease, such as thalassemia (Goldman, 2004).

Patients with sickle cell disease require continuous treatment, even when they are not having a painful crisis. The purpose of treatment is to manage and control symptoms, and to try to limit the frequency of crises. Supplementation with folic acid, an essential element in producing red blood cells, is required because of the rapid red blood cell turnover. Analgesics and hydration are mainstay treatments for patients during a sickle crisis. Treatment of pain is critical. Non-narcotic medications may be effective, but many patients require narcotics. Hydroxyurea was found to help some patients by reducing the frequency of painful crises and episodes of acute chest syndrome. It also been shown to decrease the need for blood transfusions. Newer drugs are being developed to manage sickle cell anemia. Some of these drugs work by trying to induce the body to produce more fetal hemoglobin (in an attempt to decrease the amount of sickling), or by increasing the binding of oxygen to sickle cells. To date, there are no other commonly used drugs available for treatment (Hoffman, 2005).

Acute Chest Syndrome

Acute Chest Syndrome is a common complication of sickle cell disease and is the leading cause of premature death in this population (Platt, 1994) (Stuart et al., 1994) (Buchanan et al., 2004). ACS is characterized by the presence of multi-lobe infiltrates, cough, dyspnea, hypoxia, and often chest pain. The pathophysiology of ACS is unclear but recent research indicates that NO plays a central role in airway pathology associated with this condition (Hammerman et al., 1999). This is not surprising when we take into account that the lung contains all three forms of nitric oxide synthase (NOS), NOS 1, 2, and 3. Kharitonov et al (1995) demonstrated that oral administration of L-arginine to healthy subjects increased exhaled nitric oxide (ENO) levels.

Recent studies have shown that L-arginine levels are low in patients with sickle cell disease (Lopez et al., 2003 Enwonwu et al., 1990), and decreased to an even greater extent in patients who have sickle cell disease with evidence of ACS. (Morris et al ,2000).

Asthma

Asthma is a disease involving chronic inflammation of the airways. Airway inflammation is a consistent finding in patients with mild, moderate, and severe asthma. Numerous studies have reported elevated exhaled nitric oxide levels in patients with asthma (Kissoon et al., 1999) (Kharitonov, 1994,1996) (Piacentini et al., 1999). The following definition of asthma is the accepted definition as proposed in the National, Heart, Lung and Blood Institutes (NHLBI's) National Asthma and Prevention Program (NAEPP) Expert Panel Report: Guidelines for the Diagnosis and Management of Asthma Update – 2002:

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocyutes, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night and in early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperressponiveness to a variety of stimuli (National Asthma Education and Prevention Program Expert Panel, (2003).

Asthma is one of the most common chronic diseases of industrialized nations and its prevalence continues to increase throughout the world. Statistics form the Centers for Disease Control reveal that the incidence of asthma in the United States is between 8-9 %. Asthma is the leading chronic illness in children and the number one cause of school absences. Overall, mortality rates for asthma have declined since 1995, however mortality rates continue to be 3

times higher for African American males as compared to white males, and 2.5 times higher for black females when compared to white females (Fagan et al., 2000). Over the past 10-15 years research has led to a greater understanding of the mechanisms of asthma, thereby reducing mortality and improving the quality of life for many patients suffering with asthma. However, reasons for disparities in mortality among African Americans continue to elude scientists. Recent studies have reported a relationship between asthma and ACS (Boyd et al., 2004) (Knight-Madden et al., 2005 (Bryant, 2005) (Nordness et al., 2005). Many of the symptoms related to ACS are also seen in asthma; dyspnea, cough, decreased oxygen saturation. Surprisingly, the prevalence of asthma in these studies ranged from 45 to 53%, a striking increase over the incidence of asthma generally seen in African Americans. What remains unclear is direction of the causality of the relationship between ACS and Asthma. Do patients with asthma have more episodes of ACS, or are patients with a history of ACS more likely to develop asthma?

Few studies address polymorphisms in NOS genes and how they may impact airway disease, specifically asthma, in patients with SCD. Given that asthma is the leading chronic illness in children and more prevalent in African Americans, it is important to explore relationships among candidate genes in the NO pathway, asthma, sickle cell disease, and acute chest syndrome. Recent studies citing the importance of arginase in asthma pathogensis additionally warrant further research (Vercelli, 2003).

Nitric Oxide

Nitric oxide (NO) plays a major role in lung physiology and airway disease. This ubiquitous gas is an unstable free radical that serves as a mediator for several physiological events including vascular and airway smooth muscle tone, bronchodilation, and airway inflammation (Barnes, 1995). Nitric oxide may also be necessary for ciliary action (Jain et al., 1993) and is thought to aid in maintaining sterility in the lower respiratory tract due to its

antimicrobial properties against pathogens including viruses and mycobacterium (Xia & Zweir, 1997). Endogenous NO is produced from the amino acid L-arginine by the enzyme NO synthase which has three isoforms. Two constitutive forms (cNOS); neuronal (nNOS or NOS1) and endothelial (eNOS or NOS3) are found in small quantities and serve basal metabolic functions. The third isoform, inducible (iNOS or NOS2) is mediated by inflammatory cytokines and endotoxin and plays a major role in the inflammation seen in asthma. All three of these isoforms are found in the respiratory tract (Kobzik et al., 1993; Robbins et al., 1994).

Historical Events Leading to the Discovery of Nitric Oxide

Nitric oxide was discovered by Joseph Priestley in 1772 as a clear, colorless gas. In 1980. Furchgott (1980) discovered that endothelial cells produce endedothelium relaxing factor (EDRF) in response to acetylcholine . In 1987 Moncada & Higgs (1987) and Ignarro et al (1987) discovered that EDRF is nitric oxide. Within a years time Moncada reported that NO is synthesized from the amino acid L-arginine (Palmer, Ashton & Moncada, 1988). Nitric Oxide was proclaimed as molecule of the year on the cover of Science magazine in 1992. (Koshland, 1992). Six years later, the importance of the nitric oxide discovery was recognized by awarding the Nobel Prize in Physiology and Medicine to Furchgott, Ignarrao, and Murad (Williams, 1998). By 1993 NO had been implicated in the pathogenesis of a multitude of diseases including, hypertension, septic shock, and dememntia (Moncada & Higgs, 1993). The next ten years led to multiple publications related to NO, on average over 6000 papers per year addressing all areas of medicine, including diabetes, wound healing, neurotransmission, cancer, immune function, infection, eye disease, and respiratory function (Yetik-Anacak & Catravas, 2006). Presently it is difficult to find a disease that is not associated with nitric oxide. This is amazing in that nitric oxide was considered as nothing more than an irritant and pollutant twenty years ago (Moncada & Higgs, 1993).

During the mid 1990's several studies demonstrated that nitric oxide plays a key role in physiological regulation of airway disease (Gaston & Jonsen , 1994; Shaul et al., 1994; Barnes, 1995; Kharitonov et al 1994,1995,1996; Massaro et al., 1995, 1996). More specifically, numerous studies reported increased exhaled nitric oxide levels in patients with asthma (Alving Weitzberg, & Lundberg, 1993; Kharitonov et al., 1994, 1996; Barnes, 1995; Massaro et al., 1995). Nitric oxide is released by a variety of pulmonary cells including epithelial cells, eosinophils, and macrophages (Yates, 2001). It is believed that elevated nitric oxide in the airways is generated by inducible nitric oxide synthase (iNOS) mediated by inflammatory cytokines and endotoxin (Asano et al.,1994).

There is compelling evidence to support the central role of NO in the initiation of the pathophysiological processes in acute chest syndrome. Stuart and Setty (1999) observed that serum concentration of NO metabolites were decreased during episodes of ACS with values lower than controls and patients in steady state. Recent studies have shown that L-arginine levels are low in patients with sickle cell disease (Lopez et al., 2003; Enwonwu, 1990), and decreased to an even greater extent in patients who have sickle cell disease with evidence of ACS (Morris et al., 2000). These findings support the key role of NO in the pulmonary endothelium and airway inflammation.

Nitric Oxide – Mechanisms of Action

Nitric oxide (NO) is a simple free radical gas. NO reacts with oxygen to form nitrite and nitrates. Endogenous nitric oxide is produced from the amino acid L-arginine by the enzyme NO synthase, which has three isoforms (Nathan, & Xia, 1994) (Fig1-1). Two constitutive forms (cNOS); neuronal (nNOS or NOS1) and endothelial (eNOS or NOS3) and are found in small quantities and serve basal metabolic functions. The third isoform, inducible (iNOS or NOS2) is mediated by inflammatory cytokines and endotoxin and plays an important role in the

inflammation seen in asthma. All three of these isoforms are found in the respiratory tract (Kobzik, 1993;Robbins, 1994). Synthesis of NO by cNOS is thought to be responsible for vasodilator tone associated with regulation of blood pressure, neurotransmission, respiratory function, cardiac contractility, and also plays a role in platelet aggregation (Nathan & Xia, 1994;Yates, 2001; Hammerman et al., 1999). NOS1 and NOS3 are regulated by intracellular calcium concentration, whereas NOS2 is induced under inflammation independent of calcium concentration. Agonists such as stress, bradykinin, acetylcholine, and histamine may activate cNOS resulting in the release of pico molar levels of NO. Conversely, iNOS is generated by cytokines present in the airway and produce nano molar levels of NO (Yates, 2001).

NO impacts vascular homeostasis in a variety of ways. Levels of NO may inhibit smooth muscle cell proliferation, platelet aggregation, and platelet and monocyte adhesion to the endothelium. Low levels, or decreased bioavailabilty of NO may lead to hypertension, coronary artery disease, peripheral artery disease, sickle cell, or stroke (Puddu et al., 2005).

Nitric oxide acts as a vasodilator, bronchodilator, neurotransmitter, and mediator of inflammation in the lung (Barnes, 1993). Due to its role in smooth muscle relaxation, NO showed promise as a bronchodilator. A study in guinea pigs demonstrated that NO will limit methacholine-induced bronchoconstriction however the effect is short-lived and requires high levels of inhaled NO (Dupuy et al., 1992). A study in asthmatics revealed that inhalation of NO has a small effect on airway caliber and resistance, thus not showing much promise as a therapeutic agent (Frostell e tal, 1993). Smooth muscle exists in both the bronchi and pulmonary vasculature within the lung. Numerous studies demonstrate that NO plays a key role in pulmonary arterial vasoconstriction (Dinh-Xuan, 1992; Leeman & Naecje, 1995; Yetik-Anacak

& Catravas, 2006). Basal levels of NO in pulmonary endothelial cells maintain dilation of the pulmonary vascular bed (Pepko-Zaba et al., 1991).

NO is produced in the airways by inflammatory cells, most notably eosinophils, macrophages, epithelial cells, and mast cells, all of which are relevant to asthma (Gustafsson, 1998). Epithelial cells stimulated by cytokines result in the induction of iNOS producing high quantities of NO (Robbins, 1996). NO generated from epithelial cells may be a physiological defense against infection and could influence susceptibility to airway disease given its antimicrobial properties Decreased levels of NO in the airway may increase susceptibility to infection (Hart, 1999). NO reacts with thiols to form S-nitorsothiols (Stamler et al., 1992). These compounds have bronchodilator activity and may also contribute to airway homeostasis by their antimicrobial and anti-inflammatory properties (Gaston, 1994)

Exhaled Nitric Oxide and Airway Disease

Airway inflammation plays a central role in the pathogenesis as well as symptomology of asthma (Shelhamer et al.,1995; Obyrne, 1996) Exhaled nitric oxide (FENO) levels are elevated in patients with asthma, however, there is a substantial amount of variance (Massaro et al., 1995, 1996; Kissoon et al., 1999; Rosias et al., 2004; Storm et al., 2004). Repeatable noninvasive measurement of inflammation would be useful in order to assess severity and guide treatment in patients with asthma. Measurement of exhaled nitric oxide levels is an exciting recent development that may provide an indication of the degree of airway inflammation in asthmatics as opposed to traditional pulmonary function test, which are indirect measures of airway flow. Customary monitoring techniques for assessing asthma severity include peak expiratory flow rates, spirometry, and responses to medications. Despite the importance of inflammation in asthma, monitoring airway inflammation is not routine. This is due in large part to the fact that only invasive techniques such as bronchoscopy can directly sample lung tissue and fluids for the

presence of inflammatory cells and mediators. Sputum examination is noninvasive and is one of the few methods available that can produce valuable information from the lower respiratory tract (Busse, 1998). Sputum sampling in children may be impractical in that it is time consuming, expensive, and requires cooperation from the child. Additionally, these measures may only reflect the severity of disease at the time of measurement. (Ratnawati & Thomas, 2005).

The great advantage of FENO measurement is that sample collection is noninvasive and can be performed repeatedly (Kissoon et al., 1999; Barnes, 1996). There are several analyzers now commercially available that have the capability to measure FENO. Most of the studies done to date measure nitric oxide using a chemiluminescence analyzer which detects the photochemical reaction between NO and ozone in the analyzer. (Kharitonov et al., 1994,1996; Kissoon et al., 1999; Smith et al., 2005) The beauty of this method is the ability to measure FENO directly in line to the analyzer, in real time, or indirectly by obtaining an exhaled air sample in a balloon to be later analyzed at a more convenient time. Levels of FENO are reported in parts per billion (ppb). Until recently reported values for FENO have varied widely most likely due to significant differences in sampling technique. Major differences relate to exhalation flow rate and nasal contamination (Kissoon et al., 1999). The American Thoracic Society has published guidelines for FENO measurement in adults and children (American Thoracic Society, 1999). Portable devices are currently being developed for at home monitoring of FENO in patients with asthma.

Exhaled nitric oxide is reduced in patients receiving anti-inflammatory treatment (Massaro et al., 1995; Kharitonov, et al., 1996, 2007). It is believed that glucocorticoids prevent the induction of inducible NOS (iNOS/NOS2) by cytokines in epithelial cells. (Kharitonov et al., 1996). Measuring FENO may be useful for monitoring whether anti-inflammatory therapy is

adequate as well as patient compliance. A recent paper in The New England Journal of Medicine reported that in patients with chronic, persistent asthma, treatment with inhaled corticosteroids could successfully be titrated with the use of FE_{NO} measurements. Thus FE_{NO} measurements may help to minimize the potential long-term side effects related to inhaled corticosteroids (Smith et al., 2005; Deykin, 2005). With new technology currently available, FE_{NO} measurements are easy to perform, can be reproduced accurately, and provide immediate results on which the primary care provider can act.

While several studies have described the value of FE_{NO} measurement as a useful indicator of airway inflammation and asthma (Cicutto & Downey, 2004; Karitonov et al ,2007; Zeidler, Kleerup, Tashkin, 2004; Smith et al., 2005), few studies have addressed the variance of these levels in patients with asthma or acute chest syndrome. Genetic variation may contribute to the variability in exhaled nitric oxide levels. A limited number of studies have reported the contribution of genetic variants in candidate genes for the NO pathway and how they may correlate with exhaled nitric oxide and asthma.

Storm and colleagues (2003) identified a strong association between a NOS3 gene variant, G893T, and the variability of FENO levels in patients with asthma. FENO levels were lowest in subjects with the TT genotype and were significantly higher in subjects with either the GT or GG genotype. As mentioned previously, the number or trinucleotide repeats (AAT) in the NOS1 gene correlated with FENO values in patients with asthma however varied depending on genotype (Wechsler et al., 2000). Grasemann et al (2003) investigated FENO in both NOS1 and NOS3 genes. Specifically they studied the number of AAT repeats in intron 13 (formerly intron 20) of the NOS1 gene and the 894G/T mutation in the NOS3 gene. They found no genetic association between FENO levels and the NOS1 gene. However, they did report that females

with 12 or more AAT repeats in NOS3 had lower FENO levels compared to females with fewer than 12 AAT repeats, suggesting that gender and/or genetic variants in NOS3 may affect exhaled nitric oxide levels. Collectively, these findings may offer a plausible explanation for differences in asthma phenotype and may explain the variance in FENO values.

To date the only study reporting and association between FENO and NOS genotype in patients with sickle cell disease and acute chest syndrome is that by Sullivan and colleagues (2001). As mentioned, they found an inverse correlation between the number of repeats in NOS1 and FENO levels. Further studies are warranted exploring associations between NOS genes, variability in FENO, asthma, and SCD.

Associations between NOS Genetic Variants and ACS

Nitric oxide (NO) is thought to play a key role in the pathogenesis of ACS (Gladwin et al., 1999). Plasma concentrations of NO are reduced during ACS as a consequence of reduced NO bioavailability (Morris et al., 2006) (Morris et al., 2004). Recent studies demonstrate that administration of inhaled NO has beneficial effects in treating ACS (Stuart & Setty, 2001; Sullivan et al., 1999). Identifying genetic variants in NOS genes may be beneficial in predicting susceptibility for developing ACS. Hammerman and colleagues (1999) investigated the theory that alterations in endothelial cell production and metabolism of NO products might be associated with ACS. They measured NO products from cultured pulmonary endothelial cells exposed to plasma from sickle cell patients during crisis. They found that within two hours of exposure there were increases in NOS3 protein and NOS3 enzyme activity suggesting that an increase of toxic NO metabolites might contribute to the cellular and tissue damage seen in ACS.

In an attempt to clarify the genetic differences associated with the phenotypic diversity in patients with SCD, Vargas et al (2006) analyzed three polymorphisms in the eNOS gene; the single-nucleotide polymorphism (SNP) T-786C in the promoter region, the SNP E298D in exon

7, and a 27-bp-repeat VNTR in intron 4. They found no associations between E298D or VNTR and SCD. Interestingly, they did report that all patients homozygous for the –786C variant had a tendency to develop a more severe clinical course. Limitations to this study include the small sample size, n=73. A recent retrospective study by Sharan et al (2004) investigated eNOS polymorphisms; E298D and T-786C, in patients with SCD. They concluded that the D298 allele was not associated with SCD, however the C-786 allele was strongly associated with the risk of ACS in female subjects.

Sullivan et al (2001) tested the hypothesis that exhaled nitric oxide levels (FE_{NO}) are altered in subjects with SCD who have had at least one episode of ACS. They also tested the hypothesis that the number of AAT repeats in intron 13 (formerly intron 20) of the NOS1 gene correlates with FE_{NO}. They reported that (FE_{NO}) levels in patients who have a history of ACS are approximately one-third those observed in healthy controls and in patients with SCD who have not had ACS. Additionally, they found that low FE_{NO} was associated with a repeat polymorphism in the NOS1 gene. More specifically, they identified that high numbers of repeats are inversely correlated with FE_{NO} levels. It is tempting to speculate that the low FE_{NO} seen in SCD patients with ACS may have a genetic origin and may be due to polymorphisms in NOS genes.

Grasemann et al., (1999) demonstrated significant differences in allele frequencies and genotypes of the NOS1 gene among ethnically diverse populations. The number of AAT repeats in intron 20 (currently noted as intron 13) of the NOS1 gene ranged from 7-16 in Causcasians and African Americans. Individuals homozygous for allele 10 were more common among Caucasians ($p = 0.0004$), whereas those homozygous for allele 14 were more common among African Americans ($p<0.05$). These findings suggest that ethnicity may have an impact on

variants in the NOS1 gene and that these differences may be misinterpreted if not addressed in future studies. This study did not report whether or not participants had a history of asthma.

NOS genes that are involved in the regulation of NO may be important in ACS because of the central role NO plays in airway inflammation and the pulmonary endothelium.

NOS Genes and Association with Asthma

There is a plethora of research in the literature describing the key role of NO in the airway epithelium. Patients with asthma have increased NO production in their airways (Kharitonov, Yates, & Robbins, 1994; Massaro et al., 1996; Barnes, 1996; Piacentini et al., 1999). NOS genes are located throughout the genome at 7q35-36 (NOS3) (Robinson et al., 1994), 12q24 (NOS1) (Xu et al., 1993), and 17q12 (NOS2) (Marsden et al., 1994), all candidate loci for asthma (Collaborative Study on the Genetics of Asthma, 1997; Daniels, 1996; Ober et al., 1998).

Nitric Oxide Synthase 1 (nNOS)

Genome-wide searches have established linkage between asthma and the NOS1 (nNOS) gene (Collaborative Study on the Genetics of Asthma, 1997; Barnes, 1996; Ober et al., 1998). Grasemann et al (2000) showed a genetic association between a polymorphism in the NOS1 gene and asthma using a case control design. They demonstrated that frequencies for allele 17 and 18 of a CA repeat in exon 29 of the NOS1 gene were significantly different between 490 asthmatic and 350 control subjects. To confirm their findings they genotyped and additional 1131 control subjects and verified that the frequencies of alleles 17 and 18 were nearly identical to those found in their original control group. This study in particular is impressive given the sample size, case-control design, and reproducibility. Findings from this study provide support for NOS1 as a candidate gene for asthma.

A study in 97 mild asthmatic patients revealed that the size of the AAT repeat polymorphism on intron 13 (formerly intron 20) of the NOS 1 gene was significantly related to

exhaled nitric oxide (FE_{NO}) (Weschler et al., 2000). These findings are extremely important given that FE_{NO} is now widely accepted as a marker for airway inflammation in patients with asthma. Results from this study may help explain the phenotypic variability among asthmatics. In a genetic association study, Gao and colleagues (2000) tested whether variants of NOS1, NOS2, and NOS3 were related to asthma. Neither NOS2 nor NOS3 variants showed any association with asthma. They did however find an association between variants in NOS1 and asthma. More specifically they described significant differences in 183 bp allele frequencies between control and asthmatic subjects. Homozygous 183 bp alleles were strongly associated with asthma.

Nitric Oxide Synthase 2 (iNOS)

NO derived from NOS2 (inducible NOS or iNOS) is involved in inflammatory diseases of the airways (Barnes, 1995). NOS2 has been shown to be upregulated in asthmatics and is a substantial source of NO in the airways (Xia, 1992). Konno et al investigated whether the 14-repeat allele (CCTTT) of the NOS2 gene influences the development of atopy and asthma. Their findings suggest that the CCTTT repeat polymorphism is associated with atopy but not with asthma. A recent study in 230 families with asthma investigated the genetic association of iNOS repeats with asthma. Four repeats were identified; (CCTTT)n promoter repeat, intron 2(GT)n repeat, intron 4 (GT)n repeat, and an intron 5 (CA)n repeat. This study is the first to identify repeat polymorphisms in the iNOS gene and their association with asthma. Individuals carrying allele 4 of the promoter repeat had high serum IgE and nitric oxide levels, characteristic of asthma. Individuals carrying allele 3 of the intron 4 (GT)n repeat had elevated blood eosinophils and increased asthma severity (Batra et al., 2006).

Given the role of NOS2 in airway inflammation further genetic studies are warranted exploring variants in the gene and how they may contribute to asthma pathology.

Nitric Oxide Synthase 3 (eNOS)

Endothelial nitric oxide (eNOS) is expressed in the airway and pulmonary epithelium and serves an important role in vasodilator tone (Shaul et al., 2002; Vallance & Moncada, 1989). Genome screenings have identified gene linkages to eNOS and asthma. (Holgate, 1997; Lee et al 2000) demonstrated an association in polymorphisms of eNOS and angiotensin coverting enzyme in patients with asthma. A more recent study also looking at polymorphisms of eNOS and angiotensin coverting enzyme in patients with asthma found no relationship among polymorphisms of NOS3 and asthma (Yildiz et al., 2004). Finally, a study in 163 patients with asthma found no relationship between the tandem repeat polymorphism in intron 4 and the (G894T) variant of the NOS3 gene with atopic asthma (Holla et al., 2002). Although widely discussed, there is a lack of research in the literature demonstrating as association among polymorphisms in the NOS3 gene and asthma.

Nitric Oxide and Acute Chest Syndrome

Most of the morbidity associated with sickle cell disease stems from vaso-occlusive crisis (Platt, 1994) (Vichinsky et al., 2000). Nitric oxide is a vasodilator (Busse, 1998). Could a polymorphism in nitric oxide synthase genes interfere with or inhibit nitric oxide production? Several studies are beginning to address this. Morris et al. (2000) found that there may indeed be a relationship between L-arginine and the NO pathway. Their study attempted to sort out the issue of substrate deficiency or substrate depletion. Sickle cell patients may experience lengthy periods of vaso-occlusion, creating a constant demand for vasodilation mechanisms, i.e. NO production. This overwhelming need or utilization may deplete the quantity of the substrate, L-arginine, thereby decreasing overall NO production (Morris et al., 2000). Interestingly, Morris's group studied 36 patients at steady state (period of wellness) and during vaso-occlusive crisis (VOC). During steady state, L-arginine levels were normal. L-arginine levels were decreased

during periods of vaso-occlusive crisis and acute chest syndrome. These findings suggest that there may be arginine depletion in response to demand, as opposed to intrinsic substrate deficiency. (Morris et al., 2000). A similar study looked at L-arginine levels during VOC when sickle cell patients presented to the emergency department. They studied 50 adult patients and found arginine levels were significantly low compared to steady state (Lopez et al., 2003). These findings indicate that L-arginine levels are diminished during periods of exacerbation.

L-Citrulline is an amino acid and a precursor for arginine (Fig 1-1). Waugh et al. (2001) demonstrated that giving l-citrulline 0.1g/kg orally twice daily elevated plasma arginine levels, and increased symptoms of wellness in children with SCD. The oral citrulline supplements were well tolerated and without side effects.

Arginase Genes and Asthma

Characterization of an asthma phenotype will likely be related to a complex interaction of genes and their polymorphic variants. The pathogenetic mechanisms of and contributing genetic factors in asthma continue to elude scientists. In a murine model, Zimmerman et al (2003) found that among signature asthma genes, there was over expression of the genes encoding for the uptake and metabolism of arginine, a basic amino acid, by arginase. Additionally their results demonstrated regulation of arginase by IL-4 and IL-13, cytokines that activate inflammatory pathways seen in asthma. Microarray analysis in murine models of asthma found high levels of arginase I and arginase II activity in association with IL-4 and IL13 overexpression (Vercelli, 2003). Notably, arginine acts as a substrate for both arginase and NO synthase. The arginase and NO synthase pathways may interfere with each other by way of competition for arginine (Vercelli, 2003). Much of the literature regarding NO in asthma has focused on iNOS and centered on the proinflammatory role of NO. Meurs and colleagues report that a deficiency of NO caused by increased arginase activity and altered arginine levels is a contributing factor in

the pathogenesis of asthma (Meurs, 2003). Using global micorarray analysis, Zimmermann et al (2006) reported that asthmatic conditions involve metabolism of arginine by arginase. They report that arginase I and arginase II genes are key regulators of processes associated with airway tone and lung inflammation. Collectively these studies indicate the need for further investigation of Arginase I and Arginase II genes and how they may relate to asthma and airway disease.

Impact of Genetics

In April 2003, sequencing of the human genome was completed. The consequences of this landmark event will have a dramatic impact on the ability to understand the mechanisms of disease and develop treatments specifically tailored to a patient's genetic profile (Collins et al., 2003). Sickle cell disease (SCD) is one of the most common genetic diseases, affecting one in 600 African Americans. Despite its Mendelian inheritance the disease is phenotypically highly variable. For example, some affected by the disease suffer from recurrent vaso-occlusive crisis and die at a young age, while others seem minimally affected and enjoy a normal life span (Buchanan,, Debaun, & Steinberg, 2004). Hence the need for identification of risk factors and genetic variants that may predict outcomes and reduce mortality.

Acute chest syndrome (ACS) is the leading cause of mortality and the second most common cause of hospitalizations in patients with sickle cell disease (SCD) accounting for nearly half of premature deaths (Platt, 1994; Stuart & Setty, 2001; Buchanan, Debaun, & Steinberg, 2004). Our current understanding of the pathophysiology and mechanisms leading to ACS in SCD is limited and remains unclear. There is paucity in the literature describing associations between ACS, airway disease, and genetic variation.

Genetic Influence

Studies investigating the influence of genetic variation in candidate genes in the NO pathway are not unprecedented. The gene that encodes NOS 1 in humans is located on the long

arm of chromosome 12 in the region 12q24.2. Multiple genome wide screening studies in different ethnic populations have shown linkage of this region to asthma (Grasemann et al., 1999; Barnes, 1996; CSGA, 1997; Ober et al., 1998). Grasemann and colleagues (1999) found significant differences in allele frequencies and genotypes of the NOS1 gene among ethnically diverse populations. They studied 305 American-Caucasian and 105 African –American healthy subjects. The number of AAT repeats in intron 13 (formerly intron 20) ranged from 7-16. The overall distribution of alleles differed significantly between groups. Individuals homozygous for allele 10 were more common among Caucasians whereas those homozygous for allele 14 were more common in African-American subjects. A study in 97 mild asthmatics revealed that the size of the AAT repeat polymorphism on intron 13 (formerly intron 20) of the NOS 1 gene was significantly associated with FE_{NO} (Weschler, 2000). The NOS3 gene is located at 7q35-36. A recent study in SCD patients found a functional single nucleotide polymorphism (SNP) in the NOS3 gene, T-786C, that associated with increased susceptibility to acute chest syndrome in females (Sharan et al., 2004).

Analysis Of Ethical, Social, Political, Economic, and/or Cultural Issues

Asthma presents an enormous burden to both the individual and healthcare system. It is estimated that 20 million persons in the United States suffer from asthma and asthma accounts for more than 5.000 deaths annually (National Asthma Education and Prevention Program, 2002). In the last two decades there has been a rise in asthma hospitalizations and asthma mortality (Akinbami & Schoenforf, 2002). Mortality associated with asthma peaked in 1998 and has decreased over the past few years. This increase is more pronounced in African American, and people of low socioeconomic background (Mannino et al.,1998). According to the National Health Interview Survey, nine million children under the age of 18 years have been diagnosed with asthma (13%), children in poor families (15%) were more likely to be diagnosed with

asthma, 4 million children (6%) had an asthma attack within the last 12 months, African American children were more likely to have had an asthma attack in the last 12 months, and children in fair or poor health were more than six times as likely to have had and asthma attack in the past 12 months (National Center for Health Statistics, 2003). This is staggering given that the National Institutes of Health and the international Global Initiatives for Asthma have focused on asthma treatment and asthma management (National Heart, Lung and Blood Institute, 2003).

A recent study conducted by the American Lung Association Asthma Clinical Research Centers reported that the number of asthma episodes was highest in children less than 10 years of age. Additionally they found that African American ethnicity and a past history of severe asthma were risk factors for poor asthma control (McCoy, et al., 2006). Minority groups with diverse ethnic backgrounds experience disparities in asthma care and management resulting in increased mortality (Coultas, Gong, & Grad, 1993). Lieu et al (2002) found that African American children had worse asthma status compared to Caucasian children based on the American Academy of Pediatrics Children's Health Survey (AAP), experiencing a greater number of symptom-days, and an increased number of school absences. Additionally they discovered that African American and Latino children were less likely to be using inhaled corticosteroid medication compared to Caucasians. Notably, this cross sectional study included 1000 subjects between the ages 2-16 years with a diagnosis of asthma, participating in a managed care Medicaid plan. Zoratti and colleagues (1998) examined patterns of asthma care in 464 African Americans and 1609 Caucasians with participating in a managed care program. Compared with Caucasians, African Americans had fewer visits to asthma specialists, filled fewer prescriptions for inhaled steroids, and were more likely to visit the emergency room for treatment of asthma.

These findings suggest ethnic differences in asthma related health care within a managed care plan and suggest that financial barriers to health care are not the only cause for disparities.

As noted by Joseph et al (2005), it is important to distinguish race as a risk marker for asthma as opposed to a risk factor. Risk markers imply a relationship between race and a measured variable, whereas risk factors include ancestry, more specifically genetic variations that associate with disease. Despite asthma guidelines, medical advances, and managed care programs; ethnic disparities in the morbidity and mortality of asthma persist. This is not to suggest that adherence to guidelines would minimize disparities but rather supports the role of genetic variation.

Limited understanding of etiology of ACS

As stated previously, pulmonary disease manifested as ACS is a common complication of sickle cell anemia. ACS is the second most common cause of hospitalization in patients with sickle cell disease and is the leading cause of premature deaths (Platt, 1994;Vichinsky, 1996, 2000). ACS is characterized by the presence of a rapidly progressing multi-lobe infiltrate, cough, hypoxemia and dyspnea. The etiology of ACS is multifactorial and remains unclear. Our current understanding suggests that ACS may be a form of acute lung injury that progresses to acute respiratory distress syndrome. This injury is thought to be precipitated by sloughing of blood in the pulmonary microvascular resulting in pulmonary infarction, fat embolisation, and infection (Vichinsky, 1996; Scully et al., 1997). There is compelling evidence to support the central role of NO in the initiation of the pathophysiological process in ACS. Stuart and Setty assessed plasma NO metabolites in 36 patients with SCD and 23 age-matched controls. Serum concentrations of NO metabolites were decreased during acute chest syndrome with values lower than in controls and in patients at steady state (Stuart et al 1999). Hammerman and colleagues (1999) exposed cultured pulmonary endothelial cells to the plasma of patients with sickle cell disease and acute

chest syndrome and found that within two hours of exposure there were increases in NOS 3 protein and NOS 3 enzyme activity. They suggested that alterations of NO production and metabolism contribute to the pathogenesis of ACS. In a 30-center study, Vichinsky et al (2000) studied 671 episodes of ACS in 538 patients with SCD to determine the cause. They reported ACS is precipitated by fat embolism and a variety of lung infections including; chalmydia, mycoplasm, and legionella. More importantly their results show that in more than 50% of cases the cause of ACS was undetermined. They conclude that the etiology for ACS remains unclear. Surprisingly, the incidence of asthma in this study is reported to be 2%, significantly lower than what is generally seen in the African American population.

Similarities and Differences Between Asthma and ACS with Regard to Symptomology and Lung Function

A number of recent studies have reported that asthma may increase the risk of ACS in patients with SCD (Boyd et al., 2004; Knight-Madden et al., 2005; Bryant, 2005; Nordness et al., 2005). These reports were based on studies that documented a link between SCD and airway hyperresponsiveness, lower airway obstruction, reversibility, abnormal pulmonary function tests and the fact that corticosteroids and bronchodilators, drugs commonly used in asthma, were beneficial in ACS (Santoli et al., 1998; Koumbourlis et al., 2001; Klings et al., 2006). Several papers are cited in the literature describing pulmonary function in patients with sickle cell disease (Koumbourlis et al., 2001; Sylvester et al., 2004, 2006; Klings et al., 2006). Exposure to repeated vaso-occlusive crisis undoubtedly has an impact on the pulmonary vasculature resulting in airway damage. This type of injury results in restrictive airway disease as opposed to the obstructive airway disease seen in asthmatics. Multiple episodes of vaso-occlusive crisis and recurrent episodes of ACS may result in irreversible airway damage (Sylvester, 2004, 2006; Klings, 2006).

A recent study in children with SCD was conducted to determine whether children with SCD have restrictive lung disease and if so whether this abnormality increases with age. Sixty-four children with SCD and 64 ethnic matched controls, ages 5-16 years were studied. Compared to controls, children with SCD had lower mean forced expiratory volume (FEV 1), lower forced vital capacity (FVC), and lower peak expiratory flow (PEF). The effect of age on lung function differed significantly between the two groups. Findings from this study demonstrate that children with SCD have a restrictive airway disease pattern and this abnormality increases with age. A limitation of this study is that episodes of acute chest syndrome were not reported for children with SCD (Sylvester et al., 2004).

In 2006, this same group of investigators tested the hypothesis that children with SCD and a positive history of acute chest syndrome would have worse lung function compared to children with SCD and no history of acute chest syndrome. Forty subjects were enrolled, 20 positive for ACS, and 20 negative for ACS. The mean total lung capacity and residual volumes were significantly higher in children who had no history of ACS. They did not however demonstrate any differences in bronchodilator reversibility tests. Their findings suggest that children with SCD and a positive history for ACS have significant differences in lung function as compared to children with SCD and no history of ACS. These differences are consistent with restrictive airway disease often seen in adults with SCD (Sylvester, 2006).

A cross-sectional study of 310 adults with SCD found that 90% of subjects showed a restrictive airway disease pattern. Additionally, they reported that the presence of restrictive airway disease was associated with a more severe clinical course (Klings et al., 2006).

Koumbourlis and colleagues investigated the prevalence and reversibility of lower airway obstruction in children with SCD ages 5-18 years. Interestingly they found that obstructive

disease, reversible in nature, precedes the development of restrictive airway disease. The limitations of this study include; small sample size, limited historical information, and subjective information with regard to a history of asthma. These children were not identified as having physician-diagnosed asthma (Koumbourlis et al., 2001)

Asthma is a chronic inflammatory disease of the airways. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night and in early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperressponiveness to a variety of stimuli (National Asthma Education and Prevention Program Expert Panel, (2003).

ACS is characterized by the presence of multi-lobe infiltrates, cough, dyspnea, hypoxia, and often chest pain (Hammermann, 1999). Many of the symptoms related to ACS are also seen in asthma; dyspnea, coughs, and decreased oxygen saturation. Differentiating between asthma and ACS may be difficult in patients with SCD. Further research is needed to establish an objective assessment for primary care providers who are managing patients with SCD and airway disease.

CHAPTER 4
RESULTS

The primary aim of the study was to characterize the association between physician-diagnosed asthma and acute chest syndrome. The secondary aim of this study was to test the hypothesis that polymorphisms in candidate genes for the NO pathway associate with ACS in SCD patients.

Descriptive Results

A total of 134 children with SCD participated. Forty-eight patients with SCD had no history of having ACS (36%) (controls), 86 at least one episode of ACS (64%) (cases). Fifty percent of cases had either 1 or 2 episodes. No differences in age or sex were observed between cases and controls (Table 1). Ninety percent (n=121) of patients with SCD were homozygous for βS globin (HbSS); 6% (n=8) were heterozygous (HbSC); 3% (n=4) patients had sickle beta thalassemia (HbSβ); and genotype data on one patient was missing. On average the age for the first episode of ACS was 4.4 years; the median age was 3.5 years; and the range was <1 to 17 years old. There was no relationship between age and the number of ACS episodes (data not shown). The prevalence of physician-diagnosed asthma in our study was 36.1% (48 of 133; asthma status was not recorded in one participant). There was 100% concordance between physician-diagnosed asthma and asthma reported by patients or guardians. All participants with physician-diagnosed asthma were taking inhaled SABA; 68.8 % were on ICS (33/48); 2 and 3 participants were on LABA (salmeterol) and LTRA (montelukast) respectively.

Genotyping

The success rate of genotyping ranged between 95 and 100% (average rate was 97.6%). Table 2 compares the minor allele frequencies of the polymorphisms in SCD and healthy controls. In SCD participants, the NOS1 AAT repeat polymorphism and the ARGI variant were

in HWE. The NOS3 polymorphisms were not in HWE, which was in contrast to healthy controls, suggesting that genotyping of these variants was not in error, but was probably due to the presence of disease in SCD patients. The minor allele frequency of the AAT repeat (< 12 repeats) was significantly higher in healthy controls compared to SCD patients, 0.21 vs. 0.091; p< 0.001). The distribution of alleles carrying the number of AAT repeats in patients with SCD was skewed to the right compared to healthy controls (χ^2=122; p< 0.0001) (Figure 2). The data demonstrate that using a cut-off of < 12 repeats as suggested by Wechsler et al. in a mostly Caucasian asthmatic cohort is reasonable for patients with SCD.

Asthma and Acute Chest Syndrome

Among SCD patients with physician-diagnosed asthma, 85.4% had at least one episode of ACS compared to 14.6% of controls (odds= 5.85); whereas cases and controls were about evenly distributed among participants without asthma (odds = 1.07). The adjusted OR (95%CI) is 5.47 (2.20,13.5), p = <0.0001 (Table 3). Figure 2 shows that the proportion of SCD patients who had physician-diagnosed asthma was related to the number of episodes of ACS. The intercept of the regression line was 0.178; the slope of the regression line was 0.097; and the correlation coefficient, R, was 0.89, indicating that 79% (R2*100) of the variability in the proportion of SCD patients with physician-diagnosed asthma is accounted for by the number of episodes of ACS (p=0.001). Seven of 8 patients with 7 or more episodes of ACS had physician-diagnosed asthma (one patient failed to report asthma status, his medical history was missing and he was on asthma medications). Neither age nor gender contributed to the relationship (data not shown).

Genetic Associations

The association between the risk of ACS and the number of AAT repeats for the NOS1 gene in SCD patients is shown in Figure 3. The risk of ACS in participants without physician-diagnosed asthma and who were carrying alleles with < 12 AAT repeats (n = 16 alleles; 11.6%)

was relatively high, 0.69, then declined to a risk of about 0.4 at 12 to 15 repeats (n = 87 alleles, 63%), followed by an increased risk at higher numbers of repeats (n=35 alleles, 25.4%). The r^2 for the regression was 0.76; and the p values for coefficients of quadratic regression line: x and x^2, were 0.024 and 0.024, respectively. For SCD patients with physician-diagnosed asthma, the mean ± SD risk of ACS was higher than in non-asthmatics: 0.87 ± 0.09 vs. 0.49 ± 0.12; p = 0.001, and was not associated with AAT repeat numbers. No associations were found between the risk of ACS and the T-786C NOS3 polymorphism in either the asthma or the no-asthma cohorts or by sex (Data not shown). A modest association was found between the A2/A1 ARG1 polymorphism and asthma (Table 4-4). Carriers of the A1 allele (A1 homozygotes and heterozygotes) were less likely to have asthma, 22/79 (27.8%), compared to A2 homozygotes, 6/47 (12.8%) (Fisher's exact test: = 3.98; p = 0.04).

Table 4-1. Characteristics of self-identified African Americans with sickle cell disease who had at least one episode of acute chest syndrome (cases) and individuals with no episodes of acute chest syndrome (controls).

Characteristic	Cases (ACS)	Controls (No ACS)
Number	86	48
Mean ± SD age, years	12.6 ± 4.64	14 ± 8.9
Mean ± SD age at 1st ACS episode, years=	4.4 ± 3.6	--
Percent female	49.3	50.0
Percent on Hydroxyurea		
Current	5.8	10.4
Ever	12.7	16.6
Percent on chronic PRBC* transfusion	18.6	12.5

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*Packed Red Blood Cells. =Data from 70 participants

Table 4-2. Comparison of Hardy-Weinberg Equilibria (HWE) and minor allele frequencies of NOS 1, NOS 3 and ARG I polymorphisms in 134 patients with sickle cell disease (SCD) and 74 healthy controls

Gene	Polymorphism (reference SNP)	Minor Allele Frequency	
		Sickle Cell Disease participants	Healthy Controls
NOS 1	AAT repeats in intron 13 (WT, ≥ 12; minor, < 12)	0.091	0.21
NOS 3	T-786C (rs2070744) =	0.098	0.155
	G894T (rs1799983) =	0.14	0.121
	27 bp repeat in intron 4 =		
	A=4 repeat	0.303	0.277
	B=5 repeats	0.363	0.291
	C=6 repeats	0.059	0.014
ARG1	A2/A1 ♥(rs17599586)	0.133	0.142

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= indicates polymorphisms not in HWE in patients with SCD

♥ A1 is the T allele; which is not cut by *Pvu II*; A2 is the C allele, which is cut by *Pvu II*.

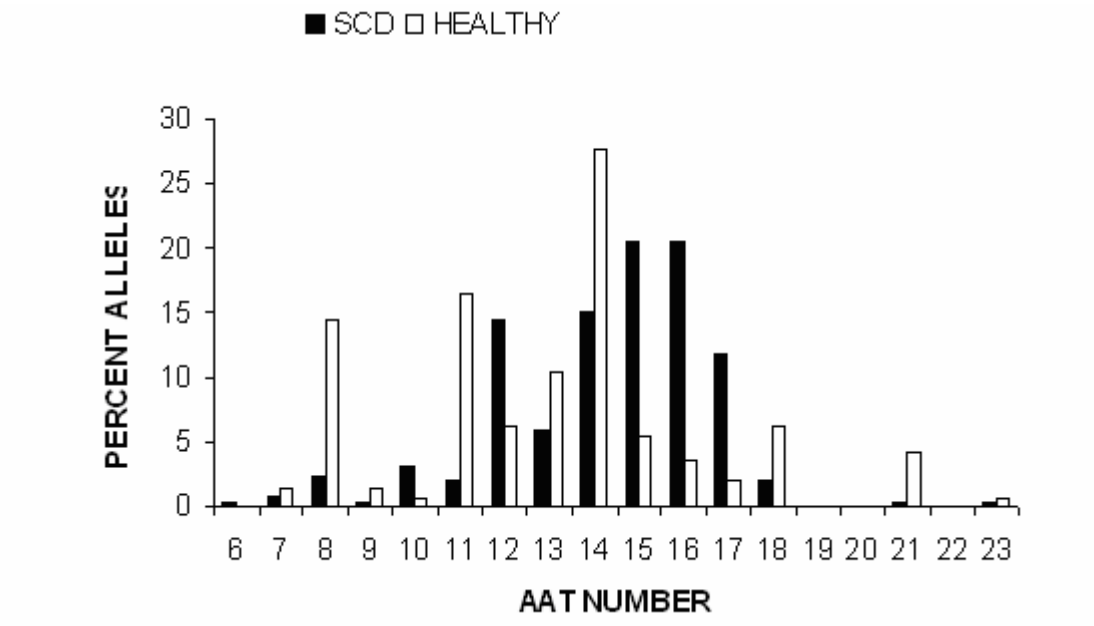

Figure 4-1. Comparison of distributions of alleles carrying AAT repeats in intron 13 on NOS 1 gene in healthy, 73 self-identified, healthy African Americans (n=146 alleles) and in 127 African Americans with sickle cell disease patients (n=254 alleles). Reprinted with permission by Wiley-Liss. Duckworth, L. et al. (2007). *Pediatric Pulmonology*, 42(4), 335

Table 4-3. Influence of physician-diagnosed asthma on the risk of having at least one episode of acute chest syndrome in patients with sickle cell disease (cases) compared to no physician-diagnosed asthma (controls). (p value represents the chi square difference between the asthma and no asthma groups)

	Group, number (%)	
	Physician-diagnosed Asthma	No asthma
Cases (ACS)	41 (85.4)	44 (51.8)
Controls (no ACS)	7 (14.6)	41 (48.2)
Total	48 (100)	85 (100)
Adjusted Odds Ratio (95%CI)	5.46 (2.20,13.5)	1.07
p value	p <0.0001	

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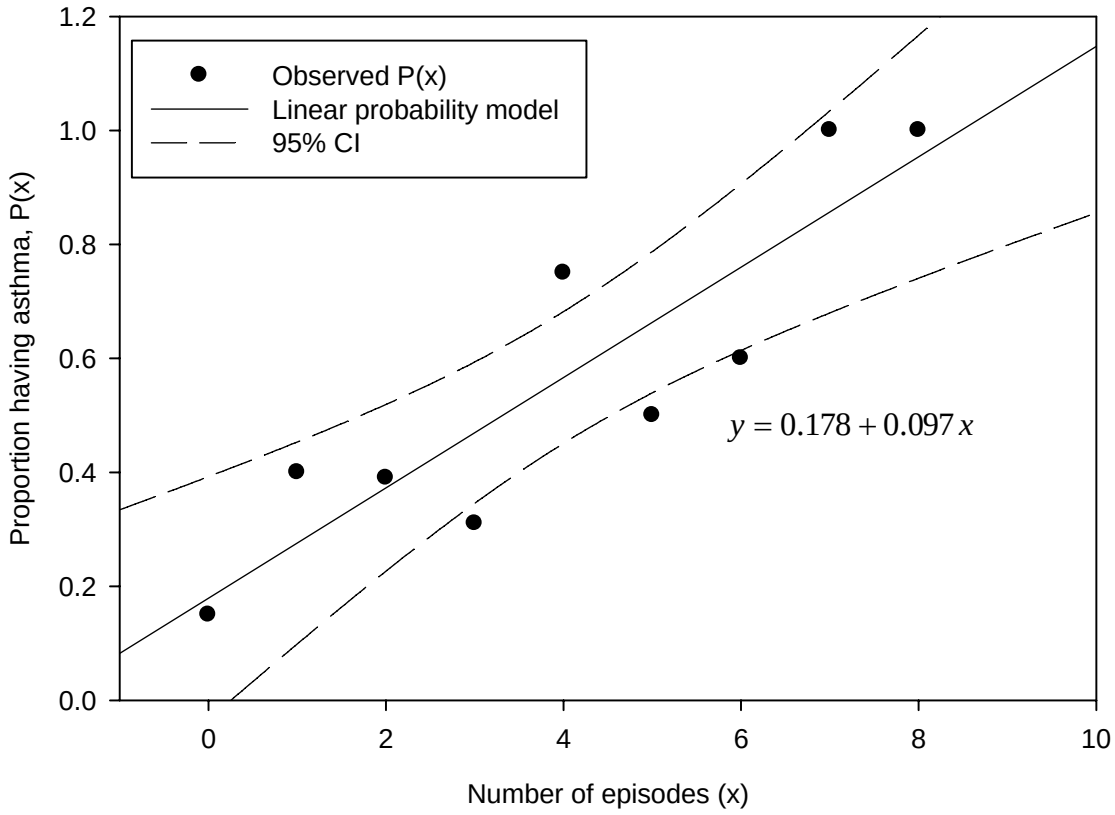

Figure 4-2. Prevalence of physcian-diagnosed asthma and acute chest syndrome episodes. The proportion of SCD patients having physician-diagnosed asthma was plotted against the number of episodes of acute chest syndrome in children with sickle cell disease. Reprinted with permission by Wiley-Liss. Duckworth, L. et al. (2007). *Pediatric Pulmonology*, 42(4), 334

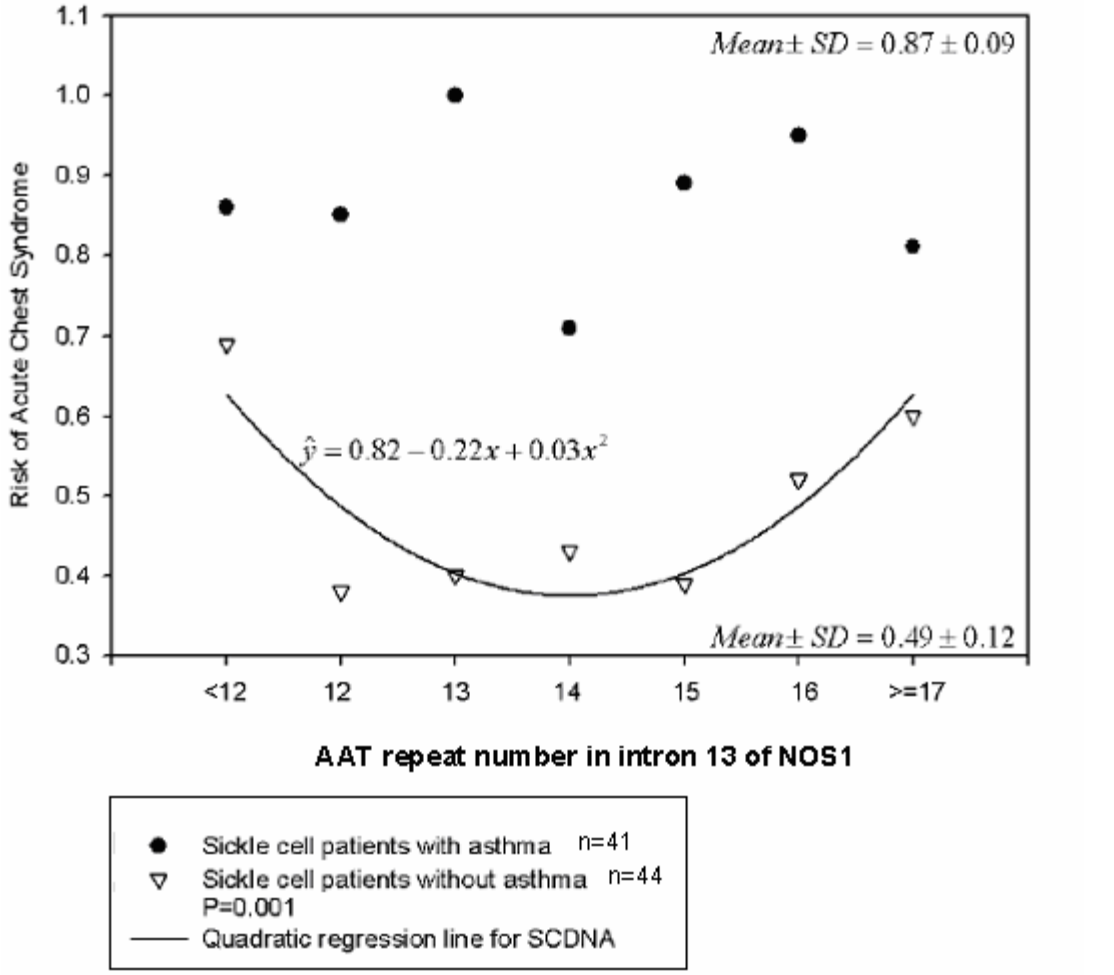

Figure 4-3. Risk of ACS and NOS 1 AAT repeats in intron 13. The risk of ACS (1-[controls/(cases+controls)]) is plotted against the number of NOS1 AAT repeats in patients with SCD with physician-diagnosed asthma (closed circles) and without physician-diagnosed asthma (SCDNA). Reprinted with permission by Wiley-Liss. Duckworth, L. et al. (2007). *Pediatric Pulmonology*, 42(4), 334.

Table 4-4. Association between ARG1 A2/A1 polymorphism and asthma among SCD patients.

Genotype	Number of SCD patients		Odds[=]
	Asthma	No asthma	
A1 carriers	6	22	0.27
A2 homozygotes	41	58	0.71

[=]Statistic likelihood ratio Fisher's exact test: 3.98; p= 0.04

CHAPTER 5
DISCUSSION

Acute chest syndrome is the leading cause of mortality and the second most common cause of hospitalizations in patients with sickle cell disease accounting for 25% of premature deaths (Platt, 1994; Stuart & Setty, 1999; Buchanan et al., 2004). The prevalence of ACS in children with SCD in our study was 64%, which was in reasonable agreement with a previous stiudy[4] that reported prevalence rates of 61% in 0 to 9 year olds, and 46% in 10 to 19 year olds, suggesting that our sample was representative of the SCD population. In the present study, we identified physician-diagnosed asthma as an important risk factor for ACS in patients with SCD. The prevalence of physician-diagnosed asthma in SCD in our study was 36% (48 of 133 participants), and is in good agreement with those reported in previous studies, which ranged between 17% and 53% (Boyd et al., 2004; Knight-Madden et al., 2005; Bryant, 2005; Nordness et al., 2005) Among SCD patients with physician-diagnosed asthma, 85.4% had at least one episode of ACS compared to 14.6% for participants who did not experience an episode of ACS (OR = 5.47; $p < 0.0001$). The prevalence of physician-diagnosed asthma in non-ACS participants is in excellent agreement with the prevalence of asthma in African American children without SCD (Yeatts & Shy, 2001; Fagan et al., 2001; Koumbourlis et al., 1997). Importantly, our data also show that the proportion of patients with SCD who have physician-diagnosed asthma increases linearly as the number of ACS episodes increase (Figure 2). To our knowledge our study is the first to show this relationship, and, given the mortality and morbidity associated with ACS, underscores the importance of coordinated pulmonary and sickle cell hematology care in diagnosing asthma in SCD. These data are also important because they point to the testable hypothesis that the aggressive treatment of asthma may reduce the mortality and morbidity of ACS in SCD patients.

The results in Figure 2 imply that proportion of patients with physician-diagnosed asthma is caused, at least in part, by the number of ACS episodes experienced by patients with SCD. The fact is we cannot determine causality from our data. It is possible that ACS may cause asthma, or that having asthma increases the risk of ACS, or that causality is bidirectional.

The results of this study support a genetic component for both ACS and for asthma in SCD. The higher number of AAT repeats may act to decrease the activity of NOS1 enzyme leading to reduced production and availability of NO, which can increase the risk of ACS and possibly asthma (Weschler et al., 2000). Arginase activity is increased in asthma, leading to reduced arginine availability, which can exacerbate NO deficiency in SCD patients. If the A2 allele of ARG1 leads to high expression of arginase I compared to the A1 allele, then A2 homozygotes would utilize arginine to a greater extent than A1 carriers, resulting in less arginine for NOS 1 to convert to NO.

Study Limitations

A major limitation of the present study is how asthma was defined. We used a diagnosis of asthma by a pediatrician, self- or guardian-reported asthma and drug use to define asthma. Self- or guardian-reported asthma was in complete agreement with physician-diagnosed asthma, and all participants with physician-diagnosed asthma were on SABA, two-thirds were on ICS, which supports the idea that they had true asthma. Additionally, we only offered study participation to those who presented for their clinic appointments. This may have biased the study in that children with ACS may be more likely to keep their scheduled appointments compared to SCD patients who are stable. Earlier studies show pulmonary function abnormalities in children and adults with SCD. However, the data are conflicting in that some studies in children with SCD have obstructive lung disease (Koumbourlas et al., 2001; Leong et al., 1997), while others have found restrictive lung disease (Sylvester et al., 2006). Adults with

SCD have restrictive lung disease owing to repeated episodes of pulmonary vaso-occlusion, which increases with age (Klings et al., 2006). Moreover, SABA and ICS are often prescribed to patients with sickle cell lung disease. Although our study confirms the results of previous studies that physician-diagnosed asthma is associated with ACS, and further shows that the risk of physician-diagnosed asthma is strongly associated with the number of ACS episodes, it is not clear that participants with physician-diagnosed asthma in our study had true asthma as defined by conventional methods. Clearly, further studies are warranted to determine if obstructive lung disease and asthma increase the risk of ACS in children with SCD.

In a pilot study of non-asthmatic children with SCD, we reported that FE_{NO} levels were reduced in individuals with ACS compared to those without ACS and to a cohort of healthy African American children (without SCD) (Sullivan et al., 2001). We also reported that levels of FE_{NO} were inversely related to the allelic sum of AAT repeats in intron 13 of NOS1 gene, which suggested a genetic link for ACS and led us to hypothesize that this repeat variant associated with ACS. A specific aim of the present case-control study was to determine the association between the AAT repeat polymorphism and ACS. We found that the risk of ACS in individuals without physician-diagnosed asthma was reduced in alleles with 12 to 15 repeats compared to alleles with < 12 or with alleles carrying 16 or more repeats. These data support the hypothesis that the risk of ACS in SCD patients without asthma is associated with the AAT repeat polymorphism, thereby implicating a genetic basis this disease. However, the association we found, if true, was modest and may represent of false-positive association owing to small numbers. The confounding influence of physician-diagnosed asthma on the risk of developing ACS (Figure 3) reduced our numbers and our power to detect a true genetic association. Thus we conclude that a larger study is warranted to replicate our findings.

The association between ACS and two polymorphisms in the NOS3 gene: E298D and T-786C, has been reported in 87 African Americans with SCD (Sharan et al., 2004). The C-786 allele was associated with an increased risk of ACS in females (n=45; relative risk=8.7). We were not able to replicate these data in the present study. The reasons for this are unclear, but may be related to small number of patients in our study who did not have physician-diagnosed asthma. Whether or not participants in the study by Sharan et al had physician-diagnosed asthma is not clear.

The etiology of ACS is not completely understood but is known to involve infection, pulmonary infarctions and pulmonary fat embolism (Vinchinsky et al., 2000; Vinchinsky et al 1994). Free fatty acids released by the hydrolysis of phospholipids in embolized fat can cause acute lung injury (Styles et al., 1996). Isoenzymes of phospholipase A2 (PLA2) hydrolyze phospholipids at the *sn*-2 position to generate lysophospholipids and free fatty acids (Dennis, 1994). Both cytosolic PLA2 and secretory PLA2 (sPLA2) function to generate arachidonic acid from phospholipids in inflammatory cells (Balsinde et al., 1994; Calabrese et al., 2000). Plasma concentrations of secretory phospholipase (sPLA2) are elevated in ACS) (Styles et al., 1996) and have been proposed as accurate markers of ACS in patients in SCD crises (Styles et al., 2000; Naprawa et al., 2005). Asthma is accompanied by increased production of arachidonic acid and enhanced activity of cysLT (Calabrese et al., 2000). Thus, it may be postulated that the asthma phenotype associated with ACS in SCD may be leukotriene-dependent, and therefore may be responsive to the leukotriene modifiers: 5-lipoxygenase inhibitors or leukotriene receptor antagonists. Additionally, earlier studies have shown that corticosteroids may provide some benefit to patients with ACS (Bernini et al., 1998), although associated with rebound vasoocclusive pain when stopped abruptly. Aggressive treatment with moderate to high doses

of inhaled corticosteroids and a leukotriene modifier (5-liopoxygenase inhibitor or LTRA) in SCD patients with physician-diagnosed asthma and SCD may reduce the mortality and morbidity associated with ACS.

Conclusions

In summary, our study confirmed that physician-diagnosed asthma is an important risk factor for ACS in SCD patients, and further demonstrated that the incidence of physician-diagnosed asthma was highly correlated with the number of episodes of ACS. Our study suggests that the NOS1 AAT repeat polymorphism may contribute to the risk of ACS in patients without physician-diagnosed asthma, and that the A2/A1 ARGI polymorphism may contribute to the incidence of asthma in SCD patients. Further studies are warranted to determine if aggressive treatment of physician-diagnosed asthma reduces the risk of ACS in SCD, and if the AAT repeat polymorphism contributes to ACS. It is important to note that our sample is representative of the general African American population in that 14.6% of children with physician diagnosed asthma who did not have a history of ACS is consistent with the incidence of asthma nationally.

Implications for Clinical Practice

Findings from this study suggest that asthma may be a significant risk factor in children with SCD for developing ACS. Quite often in primary care settings an asthma diagnosis is not assigned before the age of 2 years. More specifically we often see related diagnoses such as reactive airway disease, coughing, or upper airway congestion. In children with SCD the average age for the first ACS episode is between 2 to 4 years of age. Provider education regarding the association between asthma and ACS may heighten awareness of asthma related symptoms in infants and young children with SCD and result in more aggressive airway management. Additionally, educating the caregivers of patients with SCD regarding asthma symptoms and

their association with ACS may lead them to seek prompt medical attention for coughing episodes and upper airway congestion, symptoms which may otherwise be viewed as the common cold.

Sickle cell disease is clearly a genetic disease. Asthma on the other hand is a complex, multifactorial disease. Children suffering from SCD who also have asthma may have several specialists managing their care, for example a pulmonologist, hematologist, and primary care provider. Enhancing communication between providers may lead to better outcomes for these patients.

CHAPTER 6
FUTURE WORK

Several recent studies have reported that asthma may increase the risk for ACS in patients with SCD (Boyd, 2004; Bryant, 2005; Knight-Madden, 2004; Nordness, 2005, Sylvester et al., 2007). If indeed children with concomitant asthma and SCD have increased episodes of ACS, studies investigating aggressive asthma treatment may have an affect on the mortality and morbidity associated with this disease. Treatment with bronchodilators and inhaled steriods are the mainstay of asthma management and are consistent with asthma guidelines (National Asthma Education and Prevention Program Expert Panel, 2002). Likewise, patients with SCD and airway compromise are routinely treated with bronchodilators and inhaled steroids. (Vichinsky, 2006; Handelman, 1991; Mehta, 2006). Unlike preventative treatment in asthma, these medications are used primarily as supportive care strategies in patients with SCD.

To date there are no randomized clinical trials exploring aggressive asthma management in patients with SCD. Additionally, no studies have been reported that associate NOS genotypes, asthma, and SCD. It is interesting to hypothesize that aggressive asthma treatment in patients with SCD may prevent or reduce the incidence of ACS. More intriguing is the possibility that identifying genotypes associated with asthma and ACS early in life, in conjunction with aggressive asthma management, may reduce mortality in patients with SCD.

Montelukast, a leukotriene receptor antagonsist, is frequently prescribed for the treatment and management of asthma in adults and young children (Knorr, 2001) (Biernacki, 2005) Montelukast is widely accepted and commonly prescribed by pediatricians given its safety profile and compliance. Recent updates to the national asthma guidelines suggest that combination therapy with Montelukast and inhaled corticosteroids may improve asthma control.

Potential future studies could target patients with SCD and physician diagnosed asthma early in childhood prior to the first episode of ACS. The first episode of ACS usually occurs between 2-5 years of age (Knorr, 2001). Subjects would be randomized to receive either aggressive asthma management with moderate to high doses of inhaled corticosteroids (ICS) and a leukotriene receptor atagonist or standard of care, then followed over a period of 3-5 years. Subjects would be monitored for asthma symptoms, episodes of ACS, and genotyped for the AAT repeat polymorphism in the NOS1 gene. In addition, exhaled nitric oxide collection and pulmonary function testing would be employed to assess and monitor airway inflammation. Given that exhaled nitric oxide is an accepted measure of airway inflammation in asthma, utilizing this biomarker as a predictor or diagnostic tool to measure airway inflammation, may be useful when managing sickle cell disease patients.

Randomized clinical trials are warranted to determine if aggressive treatment of physician diagnosed asthma reduces the risk of ACS in SCD, and to determine whether polymorphisms in candidate genes in the nitric oxide pathway contribute to ACS. Collaboration among sickle cell centers for large clinical trials will be especially important for genome wide association studies related to SCD and asthma. Studies of this nature require large numbers of patients in order to identify genetic associations. Finally, stressing the importance for cooperation between pulmonary and hematology clinics can only benefit future studies involving SCD and asthma.

APPENDIX A
CONSENT FORM

THE NEMOURS CHILDREN'S CLINIC
JACKSONVILLE, FLORIDA
INFORMED WRITTEN CONSENT

You are being asked to volunteer in a research study. This form will explain the study. It is important that you understand the study before deciding to be in it. You may ask the people in charge of the study who are listed on this page questions about the study at any time.

WHAT IS THE TITLE OF THIS STUDY?

Polymorphisms of Nitric Oxide Synthase Genes in Sickle Cell Patients with Acute Chest Syndrome

WHO ARE THE PEOPLE IN CHARGE OF THIS STUDY?

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(800) SOS-KIDS

WHO CAN I TALK TO ABOUT MY RIGHTS AS A STUDY SUBJECT?

Tim Wysocki, Ph.D.
Chairperson
Nemours-Florida IRB
Nemours Children's Clinic
807 Children's Way
Jacksonville, FL 32207
(904) 390-3698

WHAT IS THE PURPOSE OF THIS STUDY?

Some patients with sickle cell anemia may develop a condition called acute chest syndrome (ACS). This condition is believed to be caused by the sickle red blood cells clogging the blood vessels in the lungs. After repeated episodes of ACS, the blood pressure in the blood vessels between the heart and lungs can remain high permanently. This condition is called pulmonary hypertension.

The doctors who take care of children with sickle cell anemia have noticed that certain children have a greater tendency to develop repeated episodes of acute chest syndrome while others do not develop this complication. The reason for this is not clear. Likewise, intensive care doctors who care for children with the acute chest syndrome have noticed that these children respond dramatically to an inhaled gas called nitric oxide (NO) when they are extremely ill. NO is a gas that is made in the cells that line the pulmonary (lung) blood vessels and is normally made in our own bodies. We believe that children with sickle cell anemia who are prone to ACS may not make enough NO in their pulmonary (lung) blood vessels. Recent research has shown

that patients with ACS have lower amounts of NO in the air they exhale when compared to sickle cell patients who have not had ACS.

We believe that sickle cell patients with ACS produce very low quantities of NO in the pulmonary (lung) system. We believe that this may be because some children have different types of the protein (amino acid) that produces NO than others. This protein is called nitric oxide synthase (NOS). Humans have three different types of the enzyme that produces NO. We want to examine whether or not there is a link between tendency to suffer from ACS, and the type of enzyme the patient has. This can be determined by studying your DNA (Studying genes or DNA is becoming more common in clinical research studies, but is still in an early stage. We know that certain genes make you tall or short. Certain genes give you brown or black hair). This will be important to know because if there is a genetic or inherited component that determines how severe the sickle cell disease will be it could be important for screening and lead to treatment that may help avoid complications in groups that are at high risk of having ACS.

WHAT IS THE PURPOSE OF COLLECTING THESE SAMPLES FOR DNA ANALYSIS?

You/your child are being asked to take part in this research study because you/your child have sickle cell disease, or are a healthy volunteer. Similarly, certain genes are associated with sickle cell disease, and may be associated with whether or not you/your child may develop ACS. Studying DNA from people who have sickle cell disease may help us better understand the importance of those genes and how they are involved in causing ACS.

You/your child are being invited to provide a sample of your/your child's buccal cells (cells from your/your child's mouth) to test for DNA or genes related to sickle cell disease. Participation is voluntary. You/your child do not have to provide a sample for DNA analysis.

We would also like to store some of your/your child's DNA in a bank (storage facilities) so that it may be used in future studies of sickle cell disease.

WHO IS SPONSORING THIS STUDY?

This study is being sponsored by The American Lung Association who will pay Nemours Children's Clinic for conducting this study.

WHO CAN BE IN THIS STUDY?

You may participate in this study if you have sickle cell disease. You may or may not have had an ACS episode. You do no have to have sickle cell disease to participate, however, you may participate as a healthy volunteer. You must be African American to participate.

HOW MANY OTHER PEOPLE WILL BE PARTICIPATING IN THIS STUDY?

This study will involve approximately 300 children, ages 6 and older, and adults from the Jacksonville, Florida and Atlanta, Georgia area. This study lasts only as long as it takes for you/your child to provide a buccal cell sample.

WHAT ARE THE PROCEDURES FOR THIS STUDY?

Buccal Samples

In order to provide a buccal sample, you/your child must not have eaten for one hour. Then, you/your child will gargle a mouthful of water for the purpose of washing and spit out the water. You/your child will receive a small bottle of mouthwash (Scope) and an empty tube, which will be labeled with an identification number. You/your child will vigorously gargle a mouthful of mouthwash for one minute and spit the gargled mouthwash into the coded tube. If you/your child find it hard to gargle the mouthwash for one minute, you/your child can gargle the mouthwash for shorter times, and repeat the procedure until you/your child have gargled for a total of one minute (For example, you/your child can gargle a mouthful of mouthwash for 20

seconds, and gargle another mouthful of mouthwash for 20 seconds and repeat the procedure one more time). The gargled mouthwash contains buccal cells from your/your child's mouth.

WHAT HAPPENS AFTER THE SAMPLES ARE COLLECTED?

The buccal cells will be processed and your/your child's DNA will be stored in our laboratory at the Nemours Children's Clinic in Jacksonville, FL. You/your child will not be notified of individual results and no results will appear in your/your child's medical records.

For those patients who have sickle cell disease, information from the medical record may be collected, for example, how many episodes of acute chest syndrome you have had, and number of hospitalizations.

WHAT ARE THE POTENTIAL RISKS OR DISCOMFORTS?

Any treatment has potential risks. The most common risks of the treatment used in this study are listed below. In addition, there is always the risk of very uncommon or previously unknown side effects.

DNA Testing

Even though we will be careful to not reveal the results of the DNA testing on your/your child's sample, there is a very small chance this information could accidentally become known to you, your child, your doctor, or others. Presently we know of no risk to you/your child if the genetic results become known.

Gargling with Mouthwash

You/your child may experience burning or tingling in your/his/her mouth from gargling with the mouthwash for one minute. You/your child can minimize burning or tingling by gargling for shorter periods of time on more occasions until he/she has gargled for one minute.

WHAT ARE THE POTENTIAL BENEFITS TO ME/MY CHILD OR OTHERS?

There is no direct medical benefit to you/your child for participating in this study. Although you/your child may not benefit directly from this research, there may be a benefit to society, in general, from the knowledge gained in connection with your/your child's participation in this study.

IS BEING IN THE STUDY VOLUNTARY?

Being in this study is totally voluntary. Anyone who does take part in the study can stop being in it at any time. There will be no change to the medical care given to anyone who decides not to be in it or who stops being in it. The researchers will destroy the samples obtained from anyone in the study if they are asked to do so.

WHAT ARE ALTERNATIVE TREATMENT OR PROCEDURES?

There are no alternative procedures for this study other than you/your child can choose not to participate.

WHAT HAPPENS IF MY CHILD DEVELOPS PROBLEMS FROM BEING IN THE STUDY?

In the event that your child suffers any injury directly resulting from these studies, you may contact any of the investigators listed on the front of this form. In the event that your child's participation in this study results in a medical problem, treatment will be made available. No other compensation of any type is available through Dr. Sylvester, Dr. Lima, Dr. Kissoon, Dr. Hsu Dr. Sullivan, Laurie Duckworth or Nemours Children's Clinic. Nemours Children's Clinic will not pay for treatment if your child suffers any injury related to this study.

You are responsible for reporting any adverse effect(s) to the investigator in charge as soon as possible.

WHAT HAPPENS IF I DECIDE FOR MY CHILD NOT TO PARTICIPATE OR TO WITHDRAW MY CHILD FROM THE STUDY?

If you decide that you do not want you/your child's samples to be studied any longer and you wish your/your child's samples to be destroyed, you can notify the investigators listed on the front of this form.

You understand that your consent for you/your child to participate in this study is given voluntarily. You may withdraw yourself/your child from or decide not to participate in the study at any time without prejudice. If you decide for yourself/your child to no longer participate in this study, it will not affect your/your child's future health care at Nemours Children's Clinic.

CAN I/MY CHILD BE TAKEN OUT OF THE STUDY WITHOUT MY CONSENT?

Presently, there is no known reason for taking you/your child out of this study without your consent. However, the study investigators can remove your sample from the study at any time, for any reason(s) deemed appropriate.

WHAT ARE THE COSTS FOR BEING IN THE STUDY?

There will be no charge to you or your insurance company for the tests involved in this study.

HOW WILL PEOPLE BE PAID FOR BEING IN THIS STUDY?

You will not be paid for participating in this study. You will not receive, either now or in the future, compensation, financial benefits, or any royalties which result from information obtained from this study.

WILL I BE TOLD OF NEW FINDINGS WHILE THE STUDY IS IN PROGRESS?

Participants will be told of any significant new findings developed during the course of this study that may relate to their willingness to continue participation in the study..

HOW WILL THE INFORMATION COLLECTED FROM AND ABOUT PEOPLE IN THE STUDY BE PROTECTED?

All information will be maintained on a confidential basis. Your/your child's identity will be protected to the extent permitted by law. Care will be taken to preserve the confidentiality of all information. You/your child understand that a record of your/your child's progress while in this study will be kept in a confidential file at Nemours Children's Clinic. The samples will be coded with a unique identifying number and stored in a secure location. The confidentiality of any central computer record will be carefully guarded and no information by which you/your child can be identified will be released or published. However, information from this study will be submitted to the American Lung Association and the Food and Drug Administration (FDA). (It may be submitted to governmental agencies in other countries where the study drug combination may be considered for approval.) Medical records which identify participants and the signed consent form can be inspected by

- The sponsoring drug company or designee
- The U.S. Food and Drug Administration
- The U.S. Department of Health and Human Services
- Governmental agencies in other countries; and
- The Nemours Florida Institutional Review Board (a group of people who carefully review the study activities and are responsible for protecting the safety and the rights of the volunteers).

Because of the need to release information to these parties, absolute confidentiality cannot be guaranteed. The results of this research project may be presented at meetings or in publications; however, your child's identity will not be disclosed in those presentations.

WHO CONTROLS AND OWNS GENETIC MATERIALS?

The sample that you/your child provide will be stored for 10 years at Nemours Children's Clinic.

You/your child's DNA samples will remain in possession of the Nemours Children's Clinic and stored in Jacksonville, FL. The results of this genetic research might be valuable for commercial and/or intellectual property (for example, patent) purposes. If you decide to participate in this genetic research, you are giving your/your child's sample to Nemours Children's Clinic. Nemours Children's Clinic retains sole ownership of the research results, and of any use or development of the research records (including your/your child's sample) consistent with this consent. You will not receive any financial benefit that might come from the research results.

WILL I HAVE ACCESS TO THE GENETIC INFORMATION?

You/your child will not be notified of individual results from DNA tests and no results will appear in your/your child's medical records.

HOW ELSE MIGHT THESE SAMPLES BE USED?

The samples obtained in this study will be analyzed for genes of related to sickle cell disease and other related diseases. Samples will not be shared with other investigators. Your/your child's DNA samples will not be sold. It is possible that we may wish to contact you/your child for further study. If you do not want yourself/your child to be re-contacted for further study, please indicate by checking the box below:

☐ *I do not wish (my child) to be re-contacted*
☐ *I do wish (my child) to be re-contacted*

PARENT'S (LEGAL REPRESENTATIVE) STATEMENT OF CONSENT

By signing this form, you have not waived any of the legal rights which your child otherwise would have as a participant in a research study.

The signing of this consent does not absolve doctors from responsibility for proper medical care at all times.

My signature indicates that I consent and authorize Drs. Lima, Kissoon, Sullivan and whomever they may designate as their assistants including Nemours Children's Clinic, its employees and its agents to perform upon _____(Name of Patient) the research described above.

I am making a decision whether or not to have my child or myself participate in this study. I have read, or had read to me in a language that I understand, all of the above, asked questions and received answers concerning areas I did not understand, and willingly give my consent for my/my child's participation in this study. Upon signing this form, I will receive a signed and dated copy.

_____	_____	_____	_____
Name of Participant (Print)	Birthdate	Signature of Participant	Date

If participant is less than 18 years of age the parent/legal representative must give consent:

_____	_____	_____
Name of Parent/Legal Representative	Signature of Parent/Legal representative	Date

_____	_____	_____
Name of Witness	Signature of Witness	Date

I the undersigned, certify that to the best of my knowledge the subject/parent/legal representative signing this consent had the study fully and carefully explained. He/she clearly understands the nature, risks and benefits in his/her child's participation in this project.

_____	_____
Signature of Investigator/Designee	Date

CHILDREN'S INFORMED ASSENT (for 6 to 12 year olds)

You are being asked to be in a research study. Before you decide whether you want to be in it, we want to tell you about it so you can ask any questions you have about it.

The doctor in charge of the study is Dr. Lima. This doctor would like to find out if you have certain genes that influence whether or not you develop acute chest syndrome (a lung disease associated with sickle cell disease). Genes come from your mother and father and help make up what you look like and how your body works.

If you decide to be in the study, here is what will happen. For this study, you will be asked to gargle with a mouthwash.

To gargle, you will first wash out your mouth with water. You will then gargle some mouthwash for one minute and spit the mouthwash into a tube. You can also choose to gargle the mouthwash for shorter periods of time more than once until you have gargled mouthwash for one minute.

You may feel burning or tingling from the mouthwash.

You don't have to do the study if you don't want to. If you are in the study, you can stop being it at any time. Nobody will be upset at you if you don't want to be in the study or if you want to stop being in the study. The doctors and their assistants will take care of you as they have in the past. If you have any questions or don't like what is happening, please tell the doctor or assistant.

Your parent or guardian knows about this study. You have had the study explained to you and you have been given a chance to ask questions about it. By writing your name below, you are saying that you know what will happen to you in the study and that you want to be in it.

_____	_____
Child's Signature	Signature of Witness
_____	_____
Researcher's Signature	Date

APPENDIX B
DEMOGRAPHIC INFORMATION

Name: MR#:

Study # DOB: AGE: SEX: M F

RACE:

Date enrolled: Date of DNA Collection:

Date of Consent:

APPENDIX C
MEDICAL HISTORY

Date of birth _____

Hb type SS SC S beta thel S other

Baseline pulse ox

Premature birth yes no

Asthma yes no

Reactive airway disease yes no

On any long term Antisickling therapy yes no

Episodes of ACS

Dates_____, _____, _____, _____, _____,

_____. _____, _____, _____

Medications

PRN Albuterol
ICS _____
Singulair _____
Salmeterol_____

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BIOGRAPHICAL SKETCH

Laurie Duckworth, native Floridian, grew up in Miami, Florida. She received her Bachelor of Science in Nursing with honors from Florida State University in 1983 and began her career as a registered nurse in the burn unit at Jackson Memorial Hospital, Miami, Florida. In 1985 she relocated to Jacksonville, Florida and was employed as a pediatric intensive care nurse. In 1987 she joined Nemours Children's Clinic, Jacksonville, Florida as a registered nurse and clinic coordinator. For the past 17 years she has served as a clinical research coordinator in the Biomedical Research Department with a focus on pulmonary disorders, specifically asthma and acute chest syndrome. She enrolled in the accelerated BSN to PhD program at the University of Florida in 2001 and completed her Masters of Science in Nursing degree and received her license as a nurse practitioner in 2003. She is a member of Sigma Theta Tau, Council for the Advancement of Nursing Science, Florida Nurse Practitioners Association, Association of Clinical Research Professionals, and the Florida Nurse's Association.

The summer of 2003 she was awarded a competitive grant from the National Institutes of Nursing Research, National Institutes of Health for a fellowship in genetics and completed a minor degree in genetics at Georgetown University. In 2006 she presented at the National Congress for Nurse Scientists in Washington, D.C. As a nurse scientist, she realizes the importance of how she may contribute to society through scientific research. She strongly believes that dissemination of knowledge through research in a multidisciplinary setting will enhance and contribute to evidence-based practice.