Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Olufolake Adisa Date

The Role of Heme and Heme Degradation in the Severity of Acute Chest Syndrome in Children with Sickle Cell Disease

By

Olufolake Adisa
Master of Science
Clinical Research

_____

Clinton H. Joiner
Advisor

_____

Amita Manatunga
Committee Member

_____

Amit Shah
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

The Role of Heme and Heme Degradation in the Severity of Acute Chest Syndrome in Children with Sickle Cell Disease

By

Olufolake Adisa

MBBS, University of Ilorin, 2000

Advisor: Clinton H. Joiner, MD PhD

An abstract of

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

in Clinical Research

2017

Abstract

The Role of Heme and Heme Degradation in the Severity of Acute Chest Syndrome in Children with Sickle Cell Disease

By Olufolake Adisa

Acute chest syndrome (ACS), a leading cause of morbidity in sickle cell disease (SCD) occurs most commonly in children aged 2 to 4 years, but only a minority having recurrent and severe episodes. A severe rapidly-progressive ACS phenotype is described in adults, but rare in children. Infection, fat emboli, infarction, heme and microthrombosis have been implicated in ACS pathogenesis, however in the largest ACS study; an etiologic factor was not identified in a majority (46%). Heterogeneity in the incidence and severity of ACS, coupled with the lack of established biomarkers that predict its occurrence or severity prompted our study of children with SCD, aged 2-20years, admitted to Children's Healthcare of Atlanta with ACS (n=52), to identify modifiable biochemical predictors of ACS severity.

We defined ACS as a new pulmonary infiltrate on chest radiograph, associated with fever, cough or other respiratory symptoms, and classified ACS events into severe and non-severe. Severe ACS was defined as ACS with respiratory failure (≥3L of oxygen to maintain saturations ≥92%), within 48hours of diagnosis. Our severe ACS group (n=7) had lower oxygen saturations, more red cell transfusions, intensive care admissions, and longer hospitalizations. ACS diagnosis associated with a drop in hemoglobin (Hb), suggesting hemolysis and release of heme into the circulation. Total plasma heme (TPH), plasma free heme (PFH), bilirubin and the major heme degradation enzymes; Hemopexin (Hpx) and Heme Oxygenase-1 (HO-1) were measured in both acute (within 48hours of ACS diagnosis) and follow-up (≥4weeks from ACS diagnosis) phases. Using the Wilcoxon signed-rank test, we found that HO-1 ($p=0.0652$) and Hpx ($p=0.0009$) were higher at ACS diagnosis, while Hb ($p<0.0001$), TPH ($p=0.0488$), PFH and bilirubin were higher at follow-up. At ACS diagnosis, PFH related positively with Hpx ($r=0.3383$, $p=0.0163$) but negatively with HO-1 ($r=-0.4426$, $p=0.0026$). Using multivariable regression analysis, Hpx and the difference in Hb at ACS diagnosis were associated with increased odds of severe ACS ($P=0.0042$, OR; 0.307, CI=0.056, 0.918). Our findings coupled with preclinical studies in sickle mice, where respiratory failure was averted after the onset of ACS-like symptoms following treatment with recombinant Hpx, suggest that Hpx is a predictor of ACS severity.

The Role of Heme and Heme Degradation in the Severity of Acute Chest Syndrome in Children with Sickle Cell Disease

By

Olufolake Adisa

MBBS, University of Ilorin, 2000

Advisor: Clinton H. Joiner, MD PhD

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

in Clinical Research

2017

ACKNOWLEDGEMENTS

I am indeed grateful for the opportunity to complete my training and the guidance, mentorship and support I received with this project from my clinical and research mentors; Clinton Joiner, Solomon Ofori-Acquah and Michael DeBaun. I am also grateful to my thesis readers; Amita Manatunga and Amit Shah, and the professors in the MSCR program for their guidance with this project. The Aflac Cancer and Blood Disorders Center Sickle Cell Disease Research Team, led by Leann Schilling played an enormous role in helping me with my HOP-ACS research study recruitment and I am very appreciative of all their hard work and commitment to the HOP-ACS study. I am pleased and thankful to all the patients and families who participated in this study with the hope to find targeted and improved therapies for acute chest syndrome. I am also grateful to Fang Tan and David Archer in the Aflac Cancer Center and Blood Disorders Center Sickle Cell Disease Core Lab and to Rimi Hazra in the Solomon Ofori-Acquah Lab in Pittsburgh. To Nancy Murray in the ACTSI, who has spent countless hours working with me and supporting me with my data analysis, I am immensely grateful. Lastly, I want to thank my daughters; Ife and Ini Agbeja for understanding and allowing me time to complete this project. I dedicate it to the memory of my late husband, Tolu Agbeja whose search for a cure was halted by the morbidity and mortality of severe acute chest syndrome.

TABLE OF CONTENTS

A. INTRODUCTION

Sickle Cell Disease (SCD) is an inherited chronic hemolytic and inflammatory disease that affects an estimated 100,000 people in the United States (US) and millions more around the world [1, 2] and is associated with significant morbidity and shortened lifespan[3, 4]. There is currently no widely available cure for SCD and it continues to exert a global burden on the society [1]. The manifestations of SCD vary broadly, and include acute and chronic pulmonary complications.

Acute chest syndrome (ACS) is an acute pulmonary complication that accounts for the second most common reason for hospital admissions [5] and is a leading cause of morbidity and mortality in patients with SCD [3]. It is defined as a new pulmonary infiltrate on chest x-ray associated with fever and varying signs of respiratory distress[6], however various clinical definitions and classification for ACS severity[7] have been proposed since it was first described by Charache in 1979 [8]. Multiple national and cooperative studies of SCD have been done including the Cooperative Study of Sickle Cell Disease [3, 5, 9], followed by the National Acute Chest Syndrome Study Group (NACSSG) to identify the incidence, risk factors, causes, course and outcome of ACS [6]. ACS occurs most commonly in children aged 2 to 4 years [10], although only a minority of children have recurrent and severe episodes of ACS with a significantly higher (4-fold) incidence among patients 20 years and older suggesting heterogeneity in the incidence and severity of ACS. A recent study describe a phenotype of ACS: Rapidly Progressive ACS (RPACS), that is well known clinically to occur more in adults with SCD (21% versus 2.1% in children in their population), and progresses to respiratory

failure within 24hours [11]. RPACS was preceded by thrombocytopenia and associated with multiorgan failure in this population [11]. Currently most children with SCD presenting to the hospital with a new infiltrate on CXR are admitted to the hospital with no distinction between a viral lower respiratory infection and the well-known clinically severe ACS phenotype. This study focused on identifying modifiable and innate biochemical predictors of ACS severity in children with SCD, by studying children with SCD admitted to all 3 hospitals of Children's Healthcare of Atlanta with a confirmed diagnosis of ACS, and using a modification of the ACS severity criteria proposed by Chaturvedi and others that is more applicable in children.

The largest study of ACS by the NACSSG studied 671 episodes of ACS in 538 patients with SCD and noted that 72% of these patients were initially admitted to the hospital for vaso-occlusive crises and developed ACS at a mean of 2.5 days following admission. Infection (28%), fat emboli (8.8%) and infarction (16%) have been implicated in ACS pathogenesis; however in a large majority (46%) of cases studied, no known etiologic factors were identified [6]. Intravascular sickling [12], microvascular in situ thrombosis [13], secretory phospholipase A2[14], over expression of endothelial VCAM-1 are other clinical events that have been associated with development of ACS[15]. These studies and related investigator-initiated clinical research [14, 16] have identified potential etiological factors of ACS, and they have helped in the development of supportive therapies, but no study has demonstrated a definite causal role for any factor in ACS. Early work exploring the pathophysiology of ACS focused on its vaso-occlusive aspects and assumed that hemolysis played a lesser role [17]. However more recent work has

explored the contribution of hemolysis in SCD pathology, and emerging data implicates hemolysis, and more specifically heme, in the development and severity of ACS [18].

The diagnosis of ACS is commonly associated with a sudden drop in hemoglobin (Hb) concentration, suggesting acute hemolysis [6, 12, 19, 20], which may result in the release of potentially dangerous amounts of heme into the circulation [21, 22]. Heme is an inflammatory agonist [23] and a major source of oxidative stress in SCD. Normally, excess heme is removed from the circulation by hemopexin (Hpx) for degradation in the liver [24] by heme oxygenase-1 (HO-1) into several byproducts including biliverdin, which is subsequently reduced to bilirubin [24]. Patients with SCD have been shown to have very low levels of Hpx [25], therefore resulting in accumulation of excess heme in the plasma. In a pilot study of 81 children with SCD, we found that the concentration of excess unscavenged plasma heme (PFH) and total bilirubin, measured at steady state was significantly higher among subjects with a history of multiple ACS compared to age-matched controls without a prior history of ACS [26]. PFH and not total plasma heme was associated with increased odds of vaso occlusive (VOC) pain episodes and ACS [26], providing a strong rationale for us to study the role of heme and heme degradation markers in the acute setting. In support of our findings, a polymorphism in the HO-1 gene promoter has been associated with a reduced incidence of ACS [27], however that polymorphism is present in only 4% of African Americans and cannot account for all the variations in ACS incidence and severity.

Our preliminary findings coupled with those involving the HO-1 promoter clearly implicate the heme degradation pathway in the pathogenesis of ACS incidence and

severity. The objective of this study is to define the relationship between circulating heme and the heme degradation pathway in ACS pathogenesis and severity by establishing a measurable biomarker that is predictive of severe ACS risk with the future goal to improve prognosis. We will test the overall hypothesis that **"Unscavenged circulating heme increases the risk of severe ACS in children with SCD".** If successful, then we would have identified a marker to facilitate early intervention and management of ACS, and thereby reduce morbidity and mortality in SCD.

B. BACKGROUND

Sickle cell Disease (SCD) is a debilitating genetic blood disorder that causes premature death in the United States and sub-Saharan Africa. One in every 365 African Americans is born with SCD and it affects over 1000,000 individuals in the US. The manifestations of SCD are protean. An emerging paradigm in SCD is the existence of 2 overlapping but characteristically distinct sub phenotypes: hemolytic and vaso-occlusive sub phenotypes, each driven by distinct mechanisms [17]. Complications of SCD driven by vaso-occlusion include: recurring vaso-occlusive episodes or pain crises, acute chest syndrome and osteonecrosis, while complications driven by increased rate of intravascular hemolysis include: pulmonary hypertension, stroke, and priapism and leg ulceration. Acute and chronic pulmonary complications remain the leading cause of morbidity and mortality in SCD. [3]

Acute Chest Syndrome (ACS) is the most common form of acute lung injury in SCD. It is the second most common reason for hospital admission for patients with SCD [5], the leading cause of admission to an intensive care unit [28] and accounts for as many as 25% of deaths in SCD [3] with a death rate of 1.8% in children and 4.3% in adults [9]. The Cooperative Study of Sickle Cell Disease (CSSCD) showed that ACS occurs more frequently in young children and among individuals with homozygous sickle cell anemia (HbSS) [5]. The highest incidence of ACS, 25.8 episodes per 100 patient-years, occurs in children, ages 2 to 4 years with HbSS, whereas the incidence is 8.8 episodes per 100 patient-years in adults with HbSS [10]. The mean length of hospitalization for all patients with ACS is about 7 days, but children stay in the hospital about 3 days less than adults

[10]. The NACSSG established in 1993, defined ACS as a new pulmonary alveolar consolidation involving at least one complete lung segment associated with acute symptoms such as fever, chest pain, tachypnea, wheezing, and cough [6]. Fever and cough are common clinical findings in young children with ACS, but chest pain, shortness of breath, chills, a productive cough, and hemoptysis are more common in older individuals. About two-thirds of patients with ACS are hypoxemic and the signs and symptoms of ACS may precede the appearance of an infiltrate on a chest radiograph by as much as 24-48 hours. Lower and middle lobes are affected more often than upper lobes in adults, although upper lobe disease is more common in children. Bilateral infiltrates or involvement of multiple lobes predicts severe disease. When severe, ACS is analogous to acute respiratory distress syndrome and this clinically severe form of ACS is well known to occur more in adults. With the significant variation in the presentation and outcomes of ACS in children and adults with SCD, lack of a standard ACS definition and severity criteria, a recent study describes a severe rapidly progressive ACS phenotype in adults (21% in adults versus 2.1% in children) that is more frequently associated with acute kidney injury (68.8%), hepatic dysfunction (75%), altered mental status (43.8%), multiorgan failure (93.8%) and death (6.3%) when compared to adults with ACS that is not rapidly progressive [11]. Rapidly progressive ACS was described as ACS resulting in respiratory failure within 24 hours, an attempt to describe the temporal relationship between the onset of symptoms and the abrupt decline in respiratory status; however they described respiratory failure as decreased oxygen saturation requiring at least 3L of oxygen to maintain oxygen hemoglobin saturation at least 90% or intubation and mechanical ventilation [11]. Although respiratory failure in defined by the need for

supplemental oxygen to maintain oxygen hemoglobin saturation at least 90%, normal oxygen saturation in room air is >95% and clinically supplemental oxygen is indicated for oxygen saturations <93% in room air in children with SCD, followed by blood gas analysis[29]. This raises concern with the applicability of this severity criterion in children with SCD, hence our modification.

There are many presumed causes of ACS. The NACSSG studied 671 episodes of ACS in 538 patients with SCD and noted that 72% of these patients were initially admitted to the hospital for vaso-occlusive crises and developed ACS at a mean of 2.5 days following admission. While 80% of patients had fever at the time of diagnosis of ACS only 28% had an established infectious etiology. About 8.8% were associated with fat emboli, 16% with infarction but the majority (46%) had no known associated factors. Despite the gravity of this complication, the precise etiology of ACS remains largely unknown, emphasized by the lack of optimal therapy and preventive strategies. Targeted therapies are clearly needed for this common and serious complication. Until recently, ACS had been described in the literature as a vaso-occlusive complication of SCD [17] associated with high steady state hemoglobin (Hb) concentrations. However, multiple studies have reported the sudden and significant drop in Hb concentration below patients' baseline, as well as an increase in markers of hemolysis immediately preceding the diagnosis of ACS [6, 12, 19, 20], suggesting an associated acute hemolysis of sickle red blood cells. Sprinkle et al reported an average drop of 1.6g/dl in Hb concentration for pediatric SCD patients admitted to the hospital with the diagnosis of ACS [20]. Vaso-occlusion and infection (known to be associated with ACS) can also cause acute intravascular

hemolysis, releasing excess Hb and heme into the circulation. Intravascular hemolysis over time produces a state of endothelial dysfunction, vascular proliferation, pro-oxidant and proinflammatory stress. Adequate mechanisms for the effective and efficient removal of free Hb from the plasma are critical to prevent long terms consequences of hemolysis and attenuating or preventing ACS. Measuring plasma Hb, plasma haptoglobin, indirect bilirubin, lactate dehydrogenase (LDH) and reticulocyte counts are indirect measures of the severity of hemolysis. Plasma haptoglobin scavenges free Hb delivering the Haptoglobin-Hb complex to tissue macrophages [24]. In situations of excessive hemolysis such as is seen in SCD patients, serum haptoglobin levels are low to undetectable [25, 30]. Once plasma haptoglobin is exhausted, any excess Hb released into the circulation is immediately oxidized to methemoglobin [21] which in turn releases free heme into the circulation [22]. Free Heme (hemin) in plasma is an inflammatory agonist [23] and is alleged to be a major source of oxidative stress in SCD and other pathologic conditions associated with intravascular hemolysis. To effectively remove excess heme from the circulation, plasma free heme is bound up by specific plasma proteins primarily hemopexin which delivers the heme molecules to liver kupffer cells for conversion into biliverdin and eventually bilirubin.[24]. Heme Oxygenase-1 (HO-1) an acute phase cytoprotective enzyme is induced in response to excess heme. It is the rate-limiting enzyme in the degradation of heme into iron, carbon monoxide and biliverdin (which is subsequently reduced to bilirubin) [24]. HO-1 has anti-oxidative, anti-inflammatory, anti-apoptotic and anti-proliferative effects[31] and increased expression of HO-1 protein has been found in the lungs of patients with acute respiratory distress syndrome (ARDS) [32], asthma, cystic fibrosis, in monocytes of patients with systemic

inflammatory response syndrome (SIRS) [33], in bone marrow macrophages of patients dying from severe sepsis or septic shock [33] and in kupffer cells of patients with acute liver failure [34]. HO-1 induction has been shown to be protective in various inflammatory states such as hemorrhagic shock, ischemia/reperfusion injury (decreased leukocyte/endothelial interaction), transplantation (improved graft survival) and acute lung injury (less cytokines and neutrophils in bronchoalveolar lavage fluid after lipopolysaccharide exposure). Absence of HO-1 is rare in humans and a single reported case of HO-1 deficiency died in childhood [35, 36]. There are however polymorphisms of HO-1 promoter that result in low levels of HO-1 expression and increased risk of respiratory diseases, susceptibility to coronary artery disease and organ rejection after transplantation [37]. A polymorphic (GT)n repeat in the HMOX1 promoter regulates the promoter activity and gene expression [38-40]. In the only reported case of HO-1 deficiency both intravascular hemolysis and endothelial dysfunction were prominent. In SCD patients, the Hb and heme scavenging systems are overwhelmed; the endothelium is therefore exposed to higher levels of reactive oxygen species catalyzed by plasma Hb, heme and free iron. Endothelial dysfunction results in capillary leak evidenced by edema /infiltrate in the lungs of SCD patients with ACS. HO-1 is highly induced in response to various stresses and its expression in various tissues has been associated with anti-inflammatory and protective effects in clinical trials [41] but this has not been confirmed in SCD. Perhaps the magnitude of HO-1 increase could be associated with disease severity and thereby related to outcome, particularly in the background of an overwhelmed haptoglobin/hemopexin scavenging system in patients with SCD and ACS.

In previous studies, we found that intravenous injection of hemin (free heme) at doses previously reported to have no adverse effects in control mice, caused sudden death in transgenic SCD mice, while control littermates with sickle cell trait survived [18]. To mimic hemolysis and the release of free heme into the circulation of patients with SCD, we infused low dose hemin into transgenic sickle mice and controls, which resulted in acute intravascular hemolysis and a lethal acute lung injury similar ACS in patients with SCD. [18] Real time monitoring showed that death was preceded by respiratory distress, severe hypoxemia, worsening hypercapnia, acidosis, and confirmed by vascular congestion, edema, alveolar wall thickening and hemorrhage in post-mortem lungs of the sickle mice. Plasma Hpx levels was observed to decrease sharply in the sickle mice and low baseline plasma Hpx was a risk factor for this hemin induced acute lung injury in the mice. Hemopexin replacement therapy prior to hemin infusion averted the acute lung injury suggesting protective benefits in ACS patients with acute hemolysis, release of free heme as observed in the severe ACS phenotype; RPACS. Of note, RPACS was preceded by thrombocytopenia and associated with significantly higher rates of worsening anemia.[11]. TPH and more significantly unscavenged PFH, increased in the transgenic sickle mice with acute lung injury. [18]. In children with SCD, we found that higher baseline PFH is associated with increased odds of VOC and ACS after adjusting for age and gender.[26]

While all patients with SCD have the same amino acid substitution (valine by glutamic acid), there is a wide range of phenotypic manifestation of this disease, the penetrance and severity of specific complications of SCD as well as the risk factors for these

complications and the age of occurrence are highly variable. These studies implicate heme catabolism and the major enzymes in the heme degradation pathway, are involved in the pathogenesis and severity of ACS in children with SCD.

C. METHODS

Research Goal:

The objective of this study was to define the relationship between circulating heme and the heme degradation pathway in ACS pathogenesis and severity by establishing a measurable biomarker that is predictive of severe ACS risk with the future goal to improve prognosis. If successful, then we would have identified a biomarker to facilitate early intervention and management of severe ACS, and thereby reduce morbidity and mortality in SCD. We tested our hypothesis that **"Unscavenged circulating heme increases the risk of severe ACS in children with SCD"** using 2 interrelated specific aims:

[1]: To determine the association between plasma levels of individual heme degradation biomarkers; total plasma heme (TPH), plasma free heme (PFH), plasma HO-1, plasma Hemopexin (Hx) during ACS and compare with their corresponding plasma levels at steady state.

[2]: To determine the association between each individual heme degradation biomarkers [total plasma heme (TPH), plasma free heme (PFH), plasma HO-1, and plasma Hemopexin (Hx)] with severe ACS.

Study Design:

Our overall strategy was to study SCD patients admitted to the hospital with ACS to define the relationship between circulating free heme, markers of heme degradation and severe ACS, using a cross sectional study design. Eligible patients with a confirmed

diagnosis of SCD and ACS were recruited from the three hospitals of Children's Healthcare of Atlanta: Egleston, Scottish-Rite and Hughes Spalding, which provides care for over 1,800 children and adolescents with SCD in the metro Atlanta area. According to the National Acute Chest Syndrome Study Group, the incidence of ACS in our patient population is approximately 20%. Eligible SCD patients admitted to the hospital with ACS or who develop ACS during admission were enrolled on study for a 3-year period (2015-2018) in the HOP-ACS Study. Patients were recruited within 48 hours of ACS diagnosis and informed consent or assent (where applicable) was obtained to the HOP-ACS protocol approved by the Emory Institutional Review Board (IRB) [IRB00047246]. ACS was defined as a new pulmonary infiltrate detected by chest radiograph (CXR) and associated with any of the following symptoms alone or in combination; pain (particularly chest/back), fever, hypoxia and /or difficulty breathing.

Characteristics of the study population:

SCD patients admitted to the hospital with ACS or who develop ACS during admission from December, 2015 to 2018, were identified based on the following criteria:

- **Inclusion Criteria**
  - A confirmed diagnosis of ACS
  - All sickle cell genotypes at least 2 years old, both genders
  - Subjects will be stratified based on chronic packed red blood cell (PRBC) transfusion therapy
  - Subjects will be stratified based on Hydroxyurea (HU) therapy
  - Subjects will be stratified based on having a co morbid diagnosis of asthma

- **Exclusion Criteria**
  - Patients who do not meet inclusion criteria
  - Treatment with any investigational drug in the preceding 90days
  - Pregnancy
  - Inability to provide informed consent

Eligible patients were enrolled with the first ACS event within the study period and each ACS episode was considered an event. Patients can have multiple events within the 3-year period but for each event, the diagnosis of ACS was confirmed by a documented radiological report of a new infiltrate on chest radiograph. For each event, data and labs were collected for both acute and follow up (Dichotomous) states and entered into a secure web application; REDCap (Research Electronic Data Capture).

Sources of Data:

The outcome variable for this cross sectional study is Severe ACS. Each ACS episode was categorized as severe or non-severe.

- **Severe ACS**: Meets definition of ACS with respiratory failure (≥ 3L of oxygen to maintain saturations ≥ 92%) within 48hours from diagnosis
- **Non-Severe ACS**: Meets definition of ACS with or without hypoxia but <3L oxygen requirement

As standard of care in our institution, blood samples are routinely collected daily from SCD patients admitted to the hospital and at their routine follow up clinic visit. Following informed consent (obtained with the first event), 10-14ml of whole blood was collected once for **each** event during:

- Acute phase sample on day 0; within 48hrs of ACS diagnosis as defined by a new pulmonary infiltrate on chest x-ray
- Follow up sample on day F; Follow up in clinic at least 4 weeks from acute phase

Past medical records were reviewed to determine patient's genotype, confirmed by hemoglobin electrophoresis. Other variables collected include: Prior history of ACS and number if any, number of hospital admissions in the preceding 2 years for VOC or ACS, history of asthma and history of other known hemolytic phenotypes of SCD (priapism, AVN) if available. We also determined if subjects were currently on HU therapy and or Chronic PRBC transfusion therapy. Data was collected at set time points: Admission, ACS diagnosis and Follow up. Vital signs, pain score, minimum and maximum oxygen saturations determined by pulse oximetry and amount of oxygen if required were collected. Use of bi-level positive airway pressure (BiPAP), mechanical ventilation and ICU admission was collected as categorical variables at ACS diagnosis as well as use of HU therapy, narcotics, antibiotics and steroids. PRBC transfusions at ACS diagnosis was categorized into Yes (Simple and Exchange transfusion) and No. We also reviewed and collected (if available) standard of care laboratory studies at the set time points; complete blood counts with differentials (CBCD), reticulocyte count, complete metabolic panel (CMP), blood culture, blood gases, respiratory viral panel (RVP), most recent HbF %

particularly for patients on HU. The duration of hospital stay measured in days (LOS) was also collected.

The predictor variables for this study were age in years, the change in Hb concentration as measured by the difference in Hb (g/dl) between acute and follow-up states, total bilirubin, plasma HO-1, plasma Hpx, TPH and PFH.

The covariates for this study are sPO2, ICU admission, mechanical ventilation and LOS. Patients with severe ACS are hypoxic (required to meet severe-ACS definition), may require mechanical ventilation and or critical care in the ICU and tend to stay in the hospital longer. In the review of patient's records, missing data were not inputted and excluded in the analysis if unavailable.

Measurements:

Whole blood samples for the HOP-ACS research study were collected in EDTA anticoagulated tubes, and then centrifuged at 1500g, 4C for 10 min to collect plasma. Plasma was harvested and aliquoted into 400µl samples stored at -80C and later shipped to the Ofori-Acquah Laboratory in Pittsburgh for further analysis. Leukocytes were saved for HO-1 gene polymorphisms. In Pittsburg, to analyze for PFH, the unfractionated plasma was subjected to a second spin through a microcon YM-3 column (Millipore Corporation, Billerica, MA USA) at 20000g, 4C for 2 h to prepare a protein-depleted plasma fraction devoid of molecules with a molecular weight >3000 Daltons as described in our previous studies. Both TPH and PFH were determined using a chromogenic assay

according to the manufacturer's instructions (QuantiChrom heme assay kit, Bioassay Systems, CA) [26, 42]. We analyzed plasma samples for HO-1 and Hx concentrations using validated two- site enzyme linked immunosorbent assay (ELISA) kits (Kamiya Biomedical, Seattle, WA) according to manufacturer's instructions.

Analytic Plan

Exploratory Data Analysis was done using SAS version 9.4. Sample size calculation was based on our preliminary studies of mean plasma levels of HO-1 in patients with SCD and controls at steady state. The plasma concentration of HO-1 in healthy individuals is reported to be 2.3 ± 0.26 ng/ml, [43] similar to the mean in our control group with a normal Hb genotype of (2.6 ng/ml ± 0.2). We found that plasma HO-1 concentration in SCD patients was 5-fold higher (13 ng/ml ± 8.2, n=80), and is comparable only to those reported for critically ill patients, including those with ARDS [37, 39]. The standard deviation of plasma HO-1 in critically ill patients range 2 to 5[37], while the standard deviation in our cohort of SCD patients was 8.2. We calculated the power and sample size required for this study using a standard deviation of 8 in a one-sample t- test, using the StatMate software. A one-sample t-test was used since plasma HO-1 has not previously been studied in SCD patients stratified by ACS. This analysis showed that a total sample size of 100 has a 90% power to detect a difference in a hypothetical severe ACS group mean and non-severe ACS group mean of 2.61 with a significance level (alpha) of 0.05 (two-tailed). A smaller sample size will be needed if the difference between the mean plasma HO-1 activity in the two groups is larger than 2.61. Therefore, we planned to study fifty severe-ACS events.

Descriptive analyses of the continuous study population variables were performed using Wilcoxon rank-sum tests, a nonparametric test which allows us to compare the populations of severe and non-severe ACS patients. Similarly, we used Fisher's exact test for categorical variables. Next, we performed univariable analyses to examine the relationship between severe ACS and each potential risk factor independently, during the acute phase (ACS diagnosis) and also at follow-up. For categorical variables, the Fisher's exact test of association was used and logistic regression modeling the log-odds of severe ACS was used for continuous variables with the Firth's bias correction due to the small number of severe-ACS patients. An alpha of 0.05 suggests a significance level (alpha) of 0.05 (two-tailed), and therefore, a p-value less than 0.05 indicates an association. Odds ratios (OR) for severe ACS, and each risk factor were examined, and the 95% Confidence intervals (CI) for the odds ratios were reported as well. Next we conducted multivariable analyses used to estimate the risk of severe ACS using multiple covariates. Logistic regression models were fitted to assess the relationship between severe ACS (dependent variable) and the covariates: TPH, PFH, Bilirubin, HO-1, Hpx and Hb (independent variables). In order to account for the small number of events (Severe ACS), we utilized penalized maximum likelihood logistic regression but limited our final model to one covariate. We considered an alpha level of 0.05.

Next, we proceeded to model selection using variables that were significant from univariable analyses. We assessed the model structure and performed model diagnostics using the Hosmer-Lemeshow goodness of fit test. $P>0.05$ suggests a well-fitting model.

D. RESULTS

Sixty-six children with SCD meeting eligibility criteria were enrolled on the HOP-ACS research study within the study period of 13 months attained at the time of our analyses. Five patients had 2 ACS events and one patient had 3 ACS events. Thirty seven of the 66 patients enrolled had presented to the outpatient clinic for follow up and had research blood samples collected per research study protocol. We conducted analysis on 52 ACS events in 52 children with SCD, with each patient contributing one ACS event, due to our batched analyses and missing data. The patient with three ACS events had completed only one outpatient follow up visit prior to ACS recurrence. One patient with severe ACS only had analyses of heme biomarkers done on a research blood sample collected post PRBC transfusion and was therefore excluded from the analysis due to the potential for bias. Of the 52 ACS events, 7 (13.5%) met criteria for severe ACS and 45 non-severe ACS. Thirty-seven (71%) ACS events occurred in patients with sickle cell anemia (HbSS genotype), including 100% of the severe ACS events.

The median age for ACS in our population was 8.47 (range 2.98-18.33) years and patients with severe ACS were older (median 11.19 vs. 7.82 years), although the median age difference was not statistically significant ($p=0.086$) and age was not statistically significantly associated with severe ACS ($p=0.07$, OR; 1.16, CI=0.98, 1.38). We found no significant association of severe ACS with gender, hydroxyurea use, and history of abnormal and or conditional TCD. Nineteen patients did not have TCD reports due to age and genotype (TCD screening is recommended for patients with HbSS and HbSβ zero thalassemia genotypes). We assessed for a history of other hemolytic phenotypes of SCD; AVN and priapism, and found no association with severe ACS (Table I and II). Asthma a

comorbid chronic obstructive reversible airway lung disease associated with increased incidence of ACS and VOC [44], and increased mortality [45] in patients with SCD, occurred in about 29% of our study population but did not differ significantly amongst our ACS groups and also had no association with ACS severity. Two patients were on chronic monthly PRBC transfusions and still had ACS that was non severe. Overall, we found no significant associations between the demographic and medical history of our study population and ACS severity (Tables I and II).

Next, we examined the clinical characteristics of our study population at ACS diagnosis (Tables I and II), as expected based on the diagnostic criterion we used for severe ACS (amount of oxygen required), patients with severe ACS compared with non-severe ACS, had lower minimal sPO2 on room air (mean 84.1%, $p<0.0001$). ACS severity associated significantly with minimal sPO2 on room air, PRBC transfusion, ICU admission and hospital length of stay (LOS). Patients with severe ACS stayed 4 days longer in the hospital than non-severe ACS ($p=0.004$). There was no difference in the use of BiPAP therapy and mechanical ventilation; however, severe ACS associated with ICU admission ($p<0.0001$).

Univariable analyses to examine the relationship between severe ACS and each potential risk factor independently, during the acute phase (ACS diagnosis) and also at follow-up were performed. At ACS diagnosis, severe ACS was significantly associated with Hb [$p=0.0312$, OR; 0.56, CI; 0.30, 1.02] and Hpx [$p=0.0185$, OR; 0.035, CI; 0.001, 0.97] but not at follow up. HO-1 was associated with the odds of severe ACS at follow up [$p=0.0352$, OR; 01.03, CI; 0.99, 1.08] but not at ACS diagnosis.

To determine the difference between plasma levels of individual heme degradation biomarker (TPH, PFH HO-, Hpx and bilirubin) during ACS and their corresponding plasma levels at follow up, we compared paired biomarker medians using the Wilcoxon signed-rank test and found significant difference in the median Hb level, plasma concentrations of Hpx and TPH during ACS compared to follow up (Table VII). Plasma concentration of Hpx significantly increased during ACS compared with follow up ($p=0.0009$), while Hb ($p<0.0001$) and TPH (0.049) were decreased at ACS diagnosis compared to follow up.

Using Spearman correlation to assess for monotonic relationships and then Pearson' correlation to determine if the relationships are linear, we found that PFH relates positively with Hpx ($r=0.3383$, $p=0.0163$) but negatively with HO-1 ($r=-0.4426$, $p=0.0026$). Hemopexin and HO-1 inversely related with each other ($r=-0.3729$, $p=0.0127$). TPH and Bilirubin were significantly positively related to each other ($r=0.6985$, $p<0.0001$). These relationships are consistent with our preliminary findings in preclinical studies and give strength to our association studies.

Using multivariable analyses to estimate the risk of severe ACS using multiple covariates that were significant in our univariable analyses, logistic regression models were fitted to assess the relationship between severe ACS and the covariates; TPH, PFH, Bilirubin, HO-1, Hpx and Hb. In order to account for the small number of events (Severe ACS), we utilized penalized maximum likelihood logistic regression but limited our final model to two covariates; Hpx and change in Hb [$p=0.0042$, OR; 0.307, CI; 0.056, 0.918].

We assessed the model structure and performed model diagnostics using the Hosmer-Lemeshow goodness of fit test. P>0.05 suggests a well-fitting model.

E. DISCUSSION/CONCLUSIONS

Patients with severe ACS had lower oxygen saturations, required more PRBC transfusions, more ICU admissions and longer hospitalizations compared with patients with non-severe ACS. Most standard of care clinical laboratory values did not significantly differ between the severe ACS and non-severe ACS groups when compared during ACS and also at follow up, however Hb which has been shown in multiple studies to decrease suddenly at ACS diagnosis, associated with ACS severity in our study population.

Consistent with preliminary studies that suggest a protective role for Hpx at ACS diagnosis, PFH during ACS significantly relates linearly and positively with Hemopexin but negatively with HO-1. Our preliminary data showing increased plasma HO-1 in SCD patients at steady state was validated in this study, however the negative correlation with PFH suggests that lower levels of HO-1 indicate increased unscavenged heme may be the result of lower HO-1 induction but does not address the temporal relationship between both biomarkers. Bilirubin is the end product of heme degradation and is appropriately increased with increased TPH levels.

The odds of having severe ACS decreases by 35 fold for each 1 mg/ml increase in plasma Hemopexin during ACS diagnosis and differed significantly during ACS compared with follow up in paired samples. Our final model incorporates Hpx and change in Hb at ACS diagnosis in our small study population. Our study and preclinical studies in sickle mice (where respiratory failure was averted after the onset of ACS-like symptoms by

treating with recombinant Hemopexin) suggest that Hemopexin is a predictor of ACS severity and provides convincing evidence for future studies.

Limitations:

This observational cross sectional study was limited by the small sample size. Recruitment of about seven ACS events occurred per month however the primary outcome; severe ACS is not common in children. When severe ACS occurs in our population, the rapidity of progression limits patient and caretaker enrolment as patients are too ill to enroll on study. When patients have been previously enrolled, then attempts are made to obtain research blood sample s prior to PRBC transfusions if possible. Left over blood samples in the hospital laboratory obtained pre-transfusion are sometimes insufficient in volume and quality for research analysis. Our small sample size also limited stratification in the current model.

Future Directions:

Our results provide invaluable information regarding the direction of future studies. We plan to complete enrolment during the three year period of the study and increase our sample size for further analysis. Larger multi-institutional studies will be required to evaluate the relationship between change in hemoglobin, hemopexin and Severe ACS. The relationship between the age, change in Hb and Hemopexin during ACS should also be evaluated in future studies. If successful, then we would have identified innate and

biochemical predictors of severe ACS, thereby facilitating early intervention and management and ultimately decreasing morbidity and mortality in SCD.

F. REFERENCES

1. Weatherall DJ: **Genomics and global health: time for a reappraisal.** *Science* 2003, **302:**597-599.
2. Ashley-Koch A, Yang Q, Olney RS: **Sickle hemoglobin (HbS) allele and sickle cell disease: a HuGE review.** *Am J Epidemiol* 2000, **151:**839-845.
3. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP: **Mortality in sickle cell disease. Life expectancy and risk factors for early death.** *N Engl J Med* 1994, **330:**1639-1644.
4. Hassell KL: **Population estimates of sickle cell disease in the U.S.** *Am J Prev Med* 2010, **38:**S512-521.
5. Castro O, Brambilla DJ, Thorington B, Reindorf CA, Scott RB, Gillette P, Vera JC, Levy PS: **The acute chest syndrome in sickle cell disease: incidence and risk factors. The Cooperative Study of Sickle Cell Disease.** *Blood* 1994, **84:**643-649.
6. Vichinsky EP, Neumayr LD, Earles AN, Williams R, Lennette ET, Dean D, Nickerson B, Orringer E, McKie V, Bellevue R, et al: **Causes and outcomes of the acute chest syndrome in sickle cell disease. National Acute Chest Syndrome Study Group.** *N Engl J Med* 2000, **342:**1855-1865.
7. Ballas SK, Lieff S, Benjamin LJ, Dampier CD, Heeney MM, Hoppe C, Johnson CS, Rogers ZR, Smith-Whitley K, Wang WC, et al: **Definitions of the phenotypic manifestations of sickle cell disease.** *Am J Hematol* 2010, **85:**6-13.
8. Charache S, Scott JC, Charache P: **"Acute chest syndrome" in adults with sickle cell anemia. Microbiology, treatment, and prevention.** *Arch Intern Med* 1979, **139:**67-69.
9. Vichinsky EP, Styles LA, Colangelo LH, Wright EC, Castro O, Nickerson B: **Acute chest syndrome in sickle cell disease: clinical presentation and course. Cooperative Study of Sickle Cell Disease.** *Blood* 1997, **89:**1787-1792.
10. Platt OS: **The acute chest syndrome of sickle cell disease.** *N Engl J Med* 2000, **342:**1904-1907.
11. Chaturvedi S, Ghafuri DL, Glassberg J, Kassim AA, Rodeghier M, DeBaun MR: **Rapidly progressive acute chest syndrome in individuals with sickle cell anemia: a distinct acute chest syndrome phenotype.** *Am J Hematol* 2016, **91:**1185-1190.
12. Davies SC, Luce PJ, Win AA, Riordan JF, Brozovic M: **Acute chest syndrome in sickle-cell disease.** *Lancet* 1984, **1:**36-38.
13. Gladwin MT, Schechter AN, Shelhamer JH, Ognibene FP: **The acute chest syndrome in sickle cell disease. Possible role of nitric oxide in its pathophysiology and treatment.** *Am J Respir Crit Care Med* 1999, **159:**1368-1376.
14. Styles LA, Aarsman AJ, Vichinsky EP, Kuypers FA: **Secretory phospholipase A(2) predicts impending acute chest syndrome in sickle cell disease.** *Blood* 2000, **96:**3276-3278.
15. Stuart MJ, Setty BN: **Sickle cell acute chest syndrome: pathogenesis and rationale for treatment.** *Blood* 1999, **94:**1555-1560.
16. Styles LA, Schalkwijk CG, Aarsman AJ, Vichinsky EP, Lubin BH, Kuypers FA: **Phospholipase A2 levels in acute chest syndrome of sickle cell disease.** *Blood* 1996, **87:**2573-2578.
17. Kato GJ, Gladwin MT, Steinberg MH: **Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes.** *Blood Rev* 2007, **21:**37-47.

18. Ghosh S, Adisa OA, Chappa P, Tan F, Jackson KA, Archer DR, Ofori-Acquah SF: **Extracellular hemin crisis triggers acute chest syndrome in sickle mice.** *J Clin Invest* 2013.
19. Koren A, Wald I, Halevi R, Ben Ami M: **Acute chest syndrome in children with sickle cell anemia.** *Pediatr Hematol Oncol* 1990, **7:**99-107.
20. Sprinkle RH, Cole T, Smith S, Buchanan GR: **Acute chest syndrome in children with sickle cell disease. A retrospective analysis of 100 hospitalized cases.** *Am J Pediatr Hematol Oncol* 1986, **8:**105-110.
21. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, Balla G: **Pro-oxidant and cytotoxic effects of circulating heme.** *Blood* 2002, **100:**879-887.
22. Bunn HF, Jandl JH: **Exchange of heme among hemoglobins and between hemoglobin and albumin.** *J Biol Chem* 1968, **243:**465-475.
23. Wagener FA, van Beurden HE, von den Hoff JW, Adema GJ, Figdor CG: **The heme-heme oxygenase system: a molecular switch in wound healing.** *Blood* 2003, **102:**521-528.
24. Maines MD: **Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications.** *FASEB J* 1988, **2:**2557-2568.
25. Muller-Eberhard U, Javid J, Liem HH, Hanstein A, Hanna M: **Plasma concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases.** *Blood* 1968, **32:**811-815.
26. Adisa OA, Hu Y, Ghosh S, Aryee D, Osunkwo I, Ofori-Acquah SF: **Association between plasma free haem and incidence of vaso-occlusive episodes and acute chest syndrome in children with sickle cell disease.** *Br J Haematol* 2013, **162:**702-705.
27. Bean CJ, Boulet SL, Ellingsen D, Pyle ME, Barron-Casella EA, Casella JF, Payne AB, Driggers J, Trau HA, Yang G, et al: **Heme oxygenase-1 gene promoter polymorphism is associated with reduced incidence of acute chest syndrome among children with sickle cell disease.** *Blood* 2012, **120:**3822-3828.
28. Gladwin MT, Vichinsky E: **Pulmonary complications of sickle cell disease.** *N Engl J Med* 2008, **359:**2254-2265.
29. Blaisdell CJ, Goodman S, Clark K, Casella JF, Loughlin GM: **Pulse oximetry is a poor predictor of hypoxemia in stable children with sickle cell disease.** *Arch Pediatr Adolesc Med* 2000, **154:**900-903.
30. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO, 3rd, Schechter AN, Gladwin MT: **Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease.** *Nat Med* 2002, **8:**1383-1389.
31. Otterbein LE, Choi AM: **Heme oxygenase: colors of defense against cellular stress.** *Am J Physiol Lung Cell Mol Physiol* 2000, **279:**L1029-1037.
32. Mumby S, Upton RL, Chen Y, Stanford SJ, Quinlan GJ, Nicholson AG, Gutteridge JM, Lamb NJ, Evans TW: **Lung heme oxygenase-1 is elevated in acute respiratory distress syndrome.** *Crit Care Med* 2004, **32:**1130-1135.
33. Mohri T, Ogura H, Koh T, Fujita K, Sumi Y, Yoshiya K, Matsushima A, Hosotsubo H, Kuwagata Y, Tanaka H, et al: **Enhanced expression of intracellular heme oxygenase-1 in deactivated monocytes from patients with severe systemic inflammatory response syndrome.** *J Trauma* 2006, **61:**616-623; discussion 623.
34. Fujii H, Takahashi T, Matsumi M, Kaku R, Shimizu H, Yokoyama M, Ohmori E, Yagi T, Sadamori H, Tanaka N, et al: **Increased heme oxygenase-1 and decreased delta-aminolevulinate synthase expression in the liver of patients with acute liver failure.** *Int J Mol Med* 2004, **14:**1001-1005.

35. Kawashima A, Oda Y, Yachie A, Koizumi S, Nakanishi I: **Heme oxygenase-1 deficiency: the first autopsy case.** *Hum Pathol* 2002, **33:**125-130.
36. Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S: **Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency.** *J Clin Invest* 1999, **103:**129-135.
37. Saukkonen K, Lakkisto P, Kaunisto MA, Varpula M, Voipio-Pulkki LM, Varpula T, Pettila V, Pulkki K: **Heme oxygenase-1 polymorphisms and plasma concentrations in the critically ill patients.** *Shock* 2010.
38. Hirai H, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, Suzuki S, Shibahara S, Sasaki H: **Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines.** *Blood* 2003, **102:**1619-1621.
39. Sheu CC, Zhai R, Wang Z, Gong MN, Tejera P, Chen F, Su L, Thompson BT, Christiani DC: **Heme oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome.** *Intensive Care Med* 2009, **35:**1343-1351.
40. Yamada N, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, Sasaki H: **Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema.** *Am J Hum Genet* 2000, **66:**187-195.
41. Buchanan G, Vichinsky E, Krishnamurti L, Shenoy S: **Severe sickle cell disease--pathophysiology and therapy.** *Biol Blood Marrow Transplant* 2010, **16:**S64-67.
42. Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M, et al: **Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria.** *Nat Med* 2007, **13:**703-710.
43. Bharucha AE, Kulkarni A, Choi KM, Camilleri M, Lempke M, Brunn GJ, Gibbons SJ, Zinsmeister AR, Farrugia G: **First-in-human study demonstrating pharmacological activation of heme oxygenase-1 in humans.** *Clin Pharmacol Ther* 2010, **87:**187-190.
44. Boyd JH, Macklin EA, Strunk RC, DeBaun MR: **Asthma is associated with acute chest syndrome and pain in children with sickle cell anemia.** *Blood* 2006, **108:**2923-2927.
45. Boyd JH, Macklin EA, Strunk RC, DeBaun MR: **Asthma is associated with increased mortality in individuals with sickle cell anemia.** *Haematologica* 2007, **92:**1115-1118.

G. TABLES AND FIGURES

Table I: Demographic and baseline clinical characteristics of the study population comparing differences between the severe and non-severe ACS groups

Study Population	**All Patients (n=52)**	**Severe ACS (n=7)**	**Non-Severe ACS (n=45)**	**p value**
Genotype n(%) - SS - SC - Sβ0 Thal - Sβ+ Thal	37 (71.15) 12 (23.08) 1 (1.92) 2 (3.85)	7 (100) 0 0 0	30 (66.67) 12 (26.67) 1(2.22) 2 (4.44)	0.3896
Age (years), mean (SD)	9.65(4.6)	12.64 (4.6)	9.18 (4.4)	0.0862
Male Gender n (%)	28 (53.85)	5 (71.43)	23 (51.11)	0.4300
Hydroxyurea (Yes) n (%)	29 (55.77)	4 (57.14)	25 (55.56)	1.0000
TCD n (%) - Normal - Conditional - Abnormal - None recorded * n=19 were N/A due to age and genotype	 5 (75.6) 5 (15.15) 0 2 (6.06)	 4 (80.0) 0 1(20) 0	 21 (63.64) 5 (17.86) 0 2 (7.14)	0.2092
Hx of Asthma (Yes) n (%)	15 (28.85)	2 (28.57)	13 (28.89)	1.0000
Hx of AVN (Yes) n (%)	2 (3.85)	0	2 (4.44)	1.0000
Hx of Priapism (Male=28, Yes) n (%)	7 (25)	2 (40)	5 (21.74)	0.5737
Hx of Chronic PRBC (Yes) n (%)	2 (3.85)	0	2 (4.44)	1.0000
Respiratory Rate n=51, mean (SD)	36.4 (11.5)	43.7 (12)	35.2 (11.1)	0.0786
Minimal sPO2 in room air (%) n=51, mean (SD)	92.9 (5.2)	84.1 (4.9)	94.3 (3.6)	**<0.0001*** **
Maximum Temp (C) n=51, mean (SD)	38.9 (1.2)	39.5 (0.7)	38.8 (1.2)	0.1100
PRBC Transfusion (Yes) n (%)	20 (47.62)	6 (85.71)	14 (40)	**0.0408**
Creatinine (mg/dl) n=29, mean (SD)	0.48 (0.3)	0.51 (0.3)	0.47 (0.2)	0.9588
BiPAP Therapy n=48 (Yes) n (%)	15 (31.25)	3 (50)	12 (28.57)	0.3598
ICU Admission (Yes) n (%)	5 (9.62)	5 (71.43)	0	**<0.0001*** **
Mechanical Ventilation n=50 (Yes) n (%)	1 (2)	1 (14.29)	0	0.1400
LOS (days) n=49, mean (SD)	4 (3)	7 (5)	3 (4)	**0.0042****

*p<0.05, **p<0.01, ***p<0.001

Descriptive analyses of the study population were performed using Wilcoxon rank-sum tests to compare the populations of severe and non-severe ACS for continuous variables and Fisher's exact test for categorical variables.

Table II: Association of severe ACS with demographic and baseline clinical characteristics of the study population

Study Population	**All Patients (n=52)**	**Severe ACS (n=7)**	**Non-Severe ACS (n=45)**	**p value**
Genotype n(%) - SS - SC - Sβ0 Thal - Sβ+ Thal	37 (71.15) 12 (23.08) 1 (1.92) 2 (3.85)	7 (100) 0 0 0	30 (66.67) 12 (26.67) 1(2.22) 2 (4.44)	0.3896
Age (years), median (IQR)	8.47 (7.45)	11.19 (8.68)	7.82 (6.97)	0.0703
Male Gender n (%)	28 (53.85)	5 (71.43)	23 (51.11)	0.4300
Hydroxyurea (Yes) n (%)	29 (55.77)	4 (57.14)	25 (55.56)	1.0000
TCD n (%) - Normal - Conditional - Abnormal - None recorded * n=19 were N/A due to age and genotype	 5 (75.6) 5 (15.15) 0 2 (6.06)	 4 (80.0) 0 1(20) 0	 21 (63.64) 5 (17.86) 0 2 (7.14)	0.2092
Hx of Asthma (Yes) n (%)	15 (28.85)	2 (28.57)	13 (28.89)	1.0000
Hx of AVN (Yes) n (%)	2 (3.85)	0	2 (4.44)	1.0000
Hx of Priapism (Male=28, Yes) n (%)	7 (25)	2 (40)	5 (21.74)	0.5737
Hx of Chronic PRBC (Yes) n (%)	2 (3.85)	0	2 (4.44)	1.0000
Respiratory Rate n=51, median (IQR)	36 (15)	46 (16)	34 (12)	0.0777
Minimal sPO2 in room air (%) n=51, median (IQR)	94 (7)	87 (9)	94.5 (4.5)	**<0.0001**
Maximum Temp (C) n=51, median (IQR)	39.3 (2)	39.5(0.8)	39.1(2.1)	0.1671
PRBC Transfusion (Yes) n (%)	20 (47.62)	6 (85.71)	14 (40)	**0.0408**
Creatinine (mg/dl) n=29, median (IQR)	0.4 (0.3)	0.4 (0.4)	0.4 (0.3)	0.6240
BiPAP Therapy n=48 (Yes) n (%)	15 (31.25)	3 (50)	12 (28.57)	0.3598
ICU Admission (Yes) n (%)	5 (9.62)	5 (71.43)	0	**<0.0001***
Mechanical Ventilation n=50 (Yes) n (%)	1 (2)	1 (14.29)	0	0.1400
LOS (days) n=49, median (IQR)	4 (3)	7 (5)	3 (4)	**0.0084**

*p<0.05, **p<0.01, ***p<0.001

To define the relationship between severe ACS and the demographic and clinical characteristics of our study population, we performed univariable analyses, using Fisher's exact test for association for categorical variables and logistic regression modelling the log odds of severe ACS with clinical covariates that are for continuous variables with the firth's bias correction. An alpha of 0.05 suggests a significance level (alpha) of 0.05 (two-tailed), and therefore, a p-value less than 0.05 indicates an association.

Table III: Association of severe ACS with laboratory characteristics of the study population at ACS diagnosis

Study Population	**All Patients (n=52)**	**Severe ACS (n=7)**	**Non-Severe ACS (n=45)**	**p value (LRT)**	OR ($CI._{95}$)
WBC Count (10^3/µl), median (IQR)	13.3 (8.11)	15.24 (11.45)	13.24 (7.18)	0.2136	1.05 (0.97, 1.13)
Hb (g/dl), median (IQR)	8.5 (2.5)	7.6 (0.6)	8.8 (2.6)	**0.0312***	**0.56 (0.30, 1.02)**
Hct (%), median (IQR)	25.5 (6.4)	22.8 (4.1)	26 (6.6)	0.0706	0.83 (0.67, 1.01)
Platelet Count (x10^3/µl) median (IQR)	341.5 (239.5)	380 (254)	336 (236)	0.8814	1.00 (0.996, 1.01)
Absolute Reticulocyte Count (x10^9) n=49, median (IQR)	251.3 (234.1)	318.05(136.1)	248.4 (230.8)	0.4249	1.00 (0.996, 1.01)
AST (SGOT U/L) n=28, median (IQR)	36.5 (35)	47 (35)	34 (36)	0.9839	1.00 (0.98, 1.02)
Bilirubin (mg/dl) n=28, median (IQR)	1.75 (1.85)	2.2 (2.8)	1.7 (1.4)	0.8207	1.01 (0.89, 1.15)
Hb F (%), n=38, median (IQR)	13.5 (13.8)	7.2 (12.9)	15.0 (14.2)	0.1154	0.92 (0.82, 1.03)
Blood Culture n=42, (Negative) n (%)	34 (80.95)	7 (100)	2 (4.44)	1.0000	
Respiratory Rate n=51, median (IQR)	36 (15)	46 (16)	27 (77.14)	0.3124	
Respiratory Viral Panel, n=42, (Positive) n (%)	14 (29.17)	1(14.29)	13 (31.71)	0.6823	

*p<0.05, **p<0.01, ***p<0.001

Logistic regression modelling the odds of severe ACS, with the firth's bias correction was used to test for association with laboratory markers during acute ACS events in both Severe ACS and none severe ACS. An alpha of 0.05 suggests a significance level (alpha) of 0.05 (two-tailed), and therefore, a p-value less than 0.05 indicates an association

Table IV: Association of severe ACS with laboratory characteristics of the study population at follow-up

Study Population	**All Patients (n=33)**	**Severe ACS (n=6)**	**Non-Severe ACS (n=27)**	**p value (LRT)**	OR ($CI._{95}$)
WBC Count (10^3/µl), median (IQR)	8.2 (4.26)	7.7 (5.35)	8.37 (4.5)	0.7052	1.05 (0.82, 1.32)
Hb (g/dl), median (IQR)	9.5 (1.5)	9.2 (1.2)	9.6 (1.9)	0.1726	0.60 (0.27, 1.35)
Hct (%), median (IQR)	28.5 (3.3)	27.55 (4.2)	29.1 (3.5)	0.3056	0.88 (0.68, 1.14)
Platelet Count ($x10^3$/µl) median (IQR)	346 (266)	445 (126)	330 (294)	0.3206	1.00 (0.998 1.01)
Absolute Reticulocyte Count ($x10^9$), median (IQR)	317.4 (172.2)	372.9 (161.9)	299.8 (216.4)	0.4554	1.00 (0.995, 1.01)
AST (SGOT U/L) n=7, median (IQR)	33 (12)	33 (0)	33.5 (12)	0.9385	1.00 (0.88, 1.15)
Bilirubin (mg/dl) n=7, median (IQR)	2.5 (1.9)	2.5 (0)	2.45 (1.9)	0.9109	1.08 (0.23, 5.07)

*p<0.05, **p<0.01, ***p<0.001

Logistic regression modelling the odds of severe ACS, with the firth's bias correction was used to test for association with laboratory markers at follow up clinic visits in both Severe ACS and none severe ACS. An alpha of 0.05 suggests a significance level (alpha) of 0.05 (two-tailed), and therefore, a p-value less than 0.05 indicates an association

Table V: Association of severe ACS with heme and biomarkers of heme degradation during ACS

Biomarkers	**All Patients (n=52)**	**Severe ACS (n=7)**	**Non-Severe ACS (n=45)**	**p value (LRT)**	OR (CI_{95})
TPH (µM) n=50, median (IQR)	52.49 (28.01)	91.51 (71.43)	48.83 (19.53)	0.1805	1.01 (0.994, 1.03)
PFH (µM) n=50, median (IQR)	1.82 (0.24)	1.82 (0.1)	1.82 (0.37)	0.1644	0.05 (<0.001, 5.48)
HO-1 (ng/ml) n=42, median (IQR)	34.16 (48.10)	47.68 (9.48)	31.62 (49.21)	0.2306	1.02 (0.988, 1.05)
Hemopexin (mg/ml) n=48, median (IQR)	0.56 (0.6)	0.21 (0.43)	0.65 (0.62)	**0.0185***	**0.035 (0.001, 0.97)**
Bilirubin (mg/dl) n=50, median (IQR)	2.23 (1.47)	2.80 (6.29)	2.18 (1.40)	0.3562	1.05 (0.92,1.21)

*p<0.05, **p<0.01, ***p<0.001

Logistic regression modelling the odds of severe ACS, with the firth's bias correction was used to test for association with each individual heme biomarker (TPH, PFH, HO-A, Hpx and bilirubin) in both Severe ACS and none severe ACS groups during acute ACS events. An alpha of 0.05 suggests a significance level (alpha) of 0.05 (two-tailed), and therefore, a p-value less than 0.05 indicates an association

Table VI: Association of severe ACS with heme and biomarkers of heme degradation at follow up

Biomarkers	**All Patients (n=33)**	**Severe ACS (n=6)**	**Non-Severe ACS (n=27)**	**p value (LRT)**	OR (CI_{95})
TPH (µM) n=25, median (IQR)	72.22 (31.18)	93.13 (38.61)	70.52 (31.18)	0.1900	1.03 (0.98, 1.09)
PFH (µM) n=25, median (IQR)	1.95 (0.37)	1.97 (0.31)	1.95 (0.37)	0.9159	1.27 (0.01, 128.7)
HO-1 (ng/ml) n=21, median (IQR)	30.04 (28.65)	57.74 (60.02)	27.99 (18.73)	**0.0352****	1.03 (0.99, 1.08)
Hemopexin (mg/ml) n=24, median (IQR)	0.28 (0.58)	0.08 (0.16)	0.36 (0.61)	0.1642	0.061 (<0.001, 6.01)
Bilirubin (mg/dl) n=25, median (IQR)	3.16 (2.05)	3.58 (1.08)	2.93 (2.73)	0.9835	1.01 (0.51, 1.97)

*p<0.05, **p<0.01, ***p<0.001

Logistic regression modelling the odds of severe ACS, with the firth's bias correction was used to test for association with each individual heme biomarker (TPH, PFH, HO-A, Hpx and bilirubin) in both Severe ACS and none severe ACS groups at follow up clinic visits. An alpha of 0.05 suggests a significance level (alpha) of 0.05 (two-tailed), and therefore, a p-value less than 0.05 indicates an association

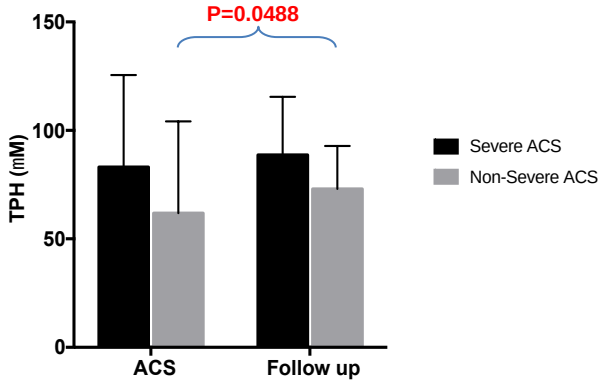
Table VII: Association between plasma concentration of TPH, PFH, HO-1, and Hpx during ACS compared with follow up

Biomarkers	**ACS**	**Follow-up**	**Wald p value**
Hb (g/dl) n=34, median (IQR)	8.5 (2.5)	9.5 (1.5)	**<0.0001**
TPH (µM) n=25, median (IQR)	52.29 (28.01)	72.22 (31.18)	**0.0488**
PFH (µM) n=25, median (IQR)	1.82 (0.24)	1.95 (0.37)	0.2867
HO-1 (ng/ml) n=18 median (IQR)	34.16 (48.1)	30.03 (28.65)	0.0652
Hemopexin (mg/ml) n=24, median (IQR)	0.56 (0.6)	0.28 (0.58)	**0.0009**
Bilirubin (mg/dl) n=25, median (IQR)	2.23 (1.47)	3.16(2.05)	0.1309

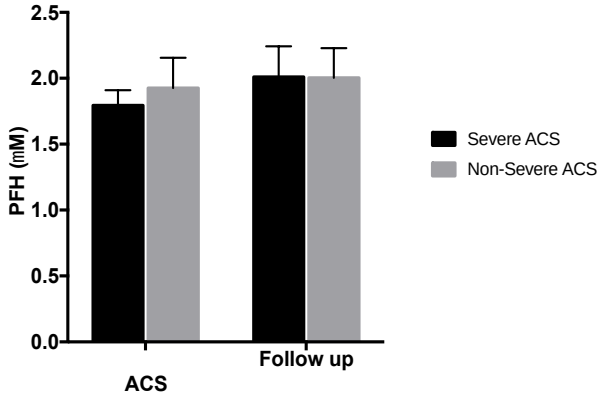
*p<0.05, **p<0.01, ***p<0.001

To determine the difference between plasma levels of individual heme degradation biomarker (TPH, PFH HO-, Hpx and bilirubin) during ACS and their corresponding plasma levels at follow up, we compared paired biomarkers using the Wilcoxon signed-rank test

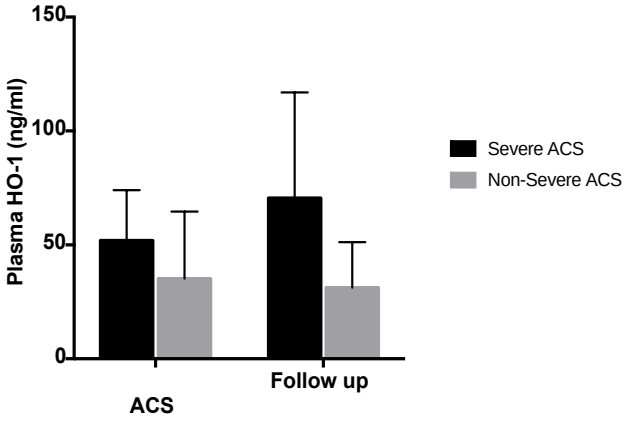
Figure 1: Heme and Biomarkers of Heme Degradation during ACS and at Follow up



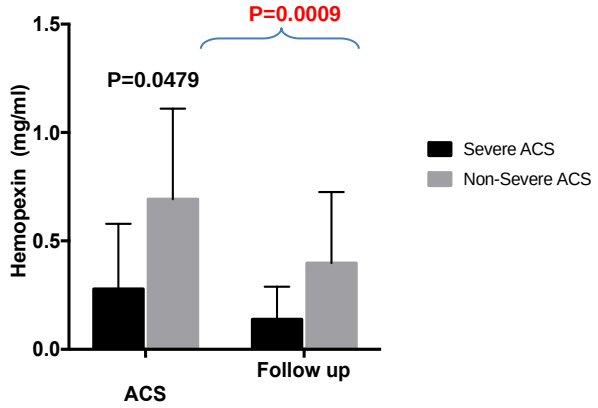
Total Plasma Heme during ACS and at Follow Up



Plasma Free Heme during ACS and at Follow Up



Plasma HO-1 during ACS and at Follow Up



Plasma Hemopexin during ACS and at Follow Up

Table VII: Correlation between heme degradation biomarkers (TPH, PFH, HO-1and Hpx), during ACS and at Follow-up

	Biomarker #1	**Biomarker #2**	**N**	**Spearman**	**P values**	**Pearson**	**P values**
ACS	TPH	Bilirubin	52	0.7739	<0.0001	0.94	<0.0001
		Hb	51	-0.4951	0.0002	-0.3469	0.0126
		Hb-Fup	33	-0.4038	0.0198	-0.4027	0.0201
	PFH	HO-1	44	-0.4169	0.0049	-0.4426	0.0026
		Hemopexin	50	0.3077	0.0300	0.3383	0.0163
		HO-1-fup	21	-0.5794	0.0059	-0.4365	0.0479
	HO-1	Hemopexin	44	-0.445	0.0025	-0.3729	0.0127
		Hb	43	-0.3661	0.0158	-0.3646	0.0162
		Hb-fup	18	0.5978	0.0020	0.5130	0.0295
	Hemopexin	Bilirubin	50	-0.3512	0.0124	-0.236	0.099
	Bilirubin	Hb	51	-0.4913	0.0003	-0.2596	0.0658
		Hb-fup	33	-0.4863	0.0041	-0.3493	0.0463
Follow-Up	TPH	Bilirubin	27	0.771	<0.0001	0.6985	<0.0001
		Hb	21	-0.5932	0.0046	-0.5822	0.0056
	Bilirubin	Hb	27	-0.5301	0.0135	-0.2838	0.1514

Table VIII: Association between individual biomarkers of heme and heme degradation (TPH, PFH, HO-1and Hpx) and severe ACS in children with SCD.

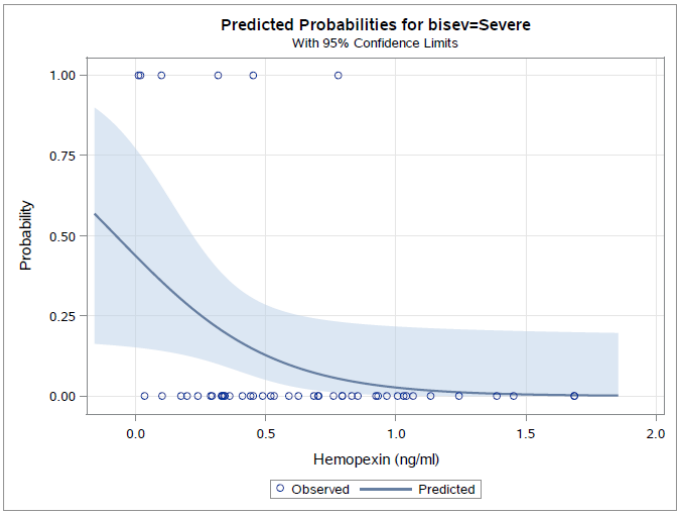
Variables	**Units**	**P values (LRT)**	**OR**	**95% CI**	**SE**
PFH (µM)	0.3	0.1644	0.409	0.064, 1.371	2.3885
Hemopexin (mg/ml)	**0.3**	**0.0185***	**0.367**	**0.1, 0.87**	**2.135**
Hb (g/dl)	**1**	**0.0312***	**0.559**	**0.273, 0.955**	**0.3061**
Δ Hb (g/dl)	**0.1**	**0.0172***	**1.154**	**1.021, 1.383**	**0.7213**
Δ HO-1 (ng/ml)	0.1	0.2284	1.002	0.999, 1.006	0.0207
Δ TPH (µM)	0.1	0.5915	1	0.998, 1.002	0.0096
Δ Hb + Hemopexin£	**0.3**	**∞0.0042****	**0.307**	**0.056, 0.918**	**2.1350**

*p<0.05, **p<0.01, ***p<0.001

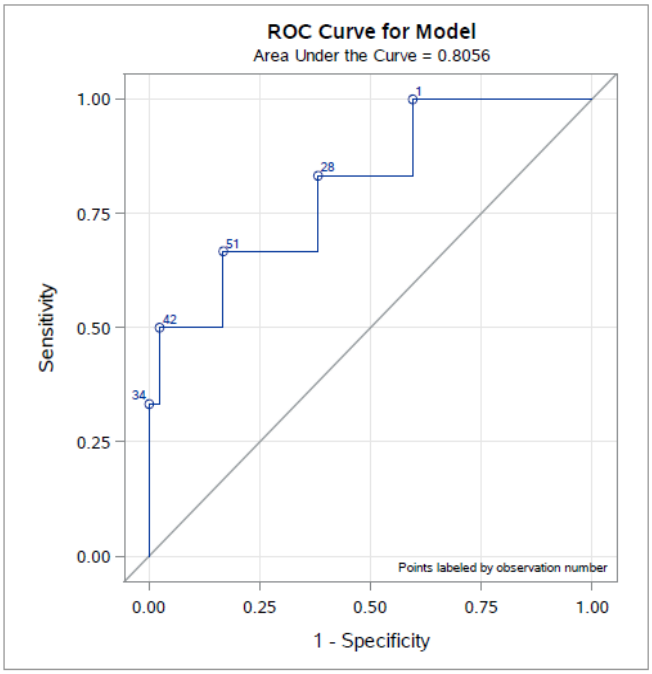
Δ Change
∞ OR for Hpx
£ Tests the null hypothesis that both of the estimates are not zero

Final Model: logit P (Severe ACS=1) = $\beta 0 + \beta 1 \Delta Hb + \beta 2 Hemopexin$

Figure 2: Model Diagnostics using the Hosmer-Lemeshow goodness of fit test and the Area Under the ROC (Receiver Operator Characteristic) Curve



Hosmer and Lemeshow Goodness-of-Fit Test		
Chi-Square	DF	Pr > ChiSq
5.1087	8	0.7459



Association of Predicted Probabilities and Observed Responses			
Percent Concordant	80.6	Somers' D	0.611
Percent Discordant	19.4	Gamma	0.611
Percent Tied	0.0	Tau-a	0.137
Pairs	252	c	0.806