HEMOGLOBINOPATHIES IN CHILDREN WITHIN A MALARIA HOLOENDEMIC REGION OF WESTERN KENYA

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B.F.A., Fashion Institute of Technology, 2002

Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2007

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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Plasmodium falciparum malaria is one of the predominant causes of morbidity and mortality in children under five years of age in sub-Saharan Africa. In malaria endemic regions, the intensity of transmission and the age at which malaria is first acquired are important in conditioning disease outcomes. In addition, inter-individual variability in disease severity among age-matched children (aged <3 yrs) with similar levels of parasite exposure is largely determined by genetic variability. Historical exposure to malaria in endemic populations has exerted tremendous selective pressure on the human genome, particularly in the host-immune response genes that mediate susceptibility and clinical outcomes. Hemoglobinopathies, such as the alpha thalassemia 3.7 kb deletion ($-\alpha^{3.7}$) and sickle-cell trait (HbAS) also confer protection against severe malaria through a mechanism(s) that are yet to be fully elucidated. As such, this study examined the role of $-\alpha^{3.7}$ and HbAS in protection against severe malaria anemia (SMA) in children (n=468; aged 3-36 months) residing in a holoendemic *P. falciparum* transmission region of western Kenya. These investigations demonstrated that successful genotyping of the $-\alpha^{3.7}$ required high-quality genomic DNA from large volumes of whole blood that was unavailable for most of the small, underweight-for-age, severely anemic children in which DNA was isolated from dried blood spots. Results presented here further demonstrated that the HbAS genotype was significantly associated with a reduced burden of both low (<10%; P=0.03) and high (≥10%;

P<0.001) pigment-containing monocytes (PCM). In addition, hemoglobin (Hb; P=0.05) and red blood cell (RBC; P=0.04) counts were significantly higher in the HbAS group relative to children with the HbAA genotype. The HbAS genotype was also significantly associated with protection against SMA using both the World Health Organization (i.e., <5.0 g/dL; P=0.04) and modified definitions of SMA (i.e., <6.0 g/dL; P=0.02). Taken together, results presented here suggest that the HbAS genotype confers protection against SMA by reducing the natural acquisition of malarial pigment (hemozoin) in monocytes. This study has significant public health importance by demonstrating that one of the mechanisms by which HbAS provides protection against SMA is through reducing the overall burden of hemozoin in monocytes.

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PREFACE

I would like to thank my thesis advisor, Dr. D.J. Perkins, for the opportunity to work on this project, and for his guidance. Additionally, I would like to thank Dr. Gordon Awandare and Greg Davenport, for their effort, “helpful” discussions, and overall assistance, none of this would have been possible without the two of you. Finally I would like to thank my committee members Dr. Bob Ferrell and Dr. Jeremy Martinson for all the time you spent helping me, even when it went above and beyond your role as a committee member.

I have been extremely lucky to have family and friends who have supported me throughout my two years here in IDM, without them, I have no idea where I would be! Special thanks to Lance Presser and Kelly Ogrodnik, you were and are my family here in Pittsburgh and I swear I would not have made it through without the two of you.

1.0 INTRODUCTION

1.1 MALARIA BACKGROUND

Malaria is the leading cause of childhood mortality, with 90% of this mortality occurring in Sub-Saharan Africa (Figure 1) (WHO 2000; McCombie 2002). Endemic to tropical and subtropical regions of the world, malaria causes 300-600 million clinical episodes and mortality in one to three million children under the age of five each year. (WHO 2000; Stevenson and Riley 2004; Struik and Riley 2004; Kwiatkowski 2005; Snow *et al.* 2005). The disease results from the transmission of the protozoan parasite(s), *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium vivax,* to the human host during the bite of an infected female *Anopheles* mosquito. *Plasmodium falciparum* is the most pathogenic species of protozoan**,** accounting for the majority of morbidity and mortality (Stevenson and Riley 2004; Struik and Riley 2004; Good *et al.* 2005). Children under the age of five are the most vulnerable to malaria morbidity and mortality due to their immune-naïve status (Perkins *et al.* 1999; Breman *et al.* 2001; Williams *et al.* 2005b). However, until the age of approximately six months children are protected from malaria by maternal antibodies (Crawley 2004). As maternal protection wanes, susceptibility to clinical disease and mortality increases greatly (Crawley 2004). The clinical manifestations associated with mortality in children with malaria include severe anemia (SMA), cerebral malaria (CM), hypoglycemia, hypoxia, and respiratory distress (Clark and

Cowden 2003). However, CM is uncommon in *P. falciparum* holoendemic transmission areas (Clark and Cowden 2003; Ong'echa *et al.* 2006). In rural settings, by the time many of the children reach the hospital they are exceedingly ill and have been medicated at home. In Kenya, the course of treatment typically involves chemotherapy with artemether-lumefantrine, (artemisin-based combination therapy; ACT), as drug resistance strains of *P. falciparum* have rendered chloroquine (CQ) and sulphadoxine/pyrimethamine (S/P) treatments ineffective (Amin *et al.* 2007). By adulthood, residents in holoendemic regions acquire partial immunity from frequent bouts of clinical malaria during childhood, which helps them manage future malaria complications such as SMA (Bloland *et al.* 1999b; Struik and Riley 2004). These adults co-exist with a parasite load with or without mild clinical presentation of disease (fever) for the duration of their residency in a malaria endemic region (Petersen *et al.* 1991).

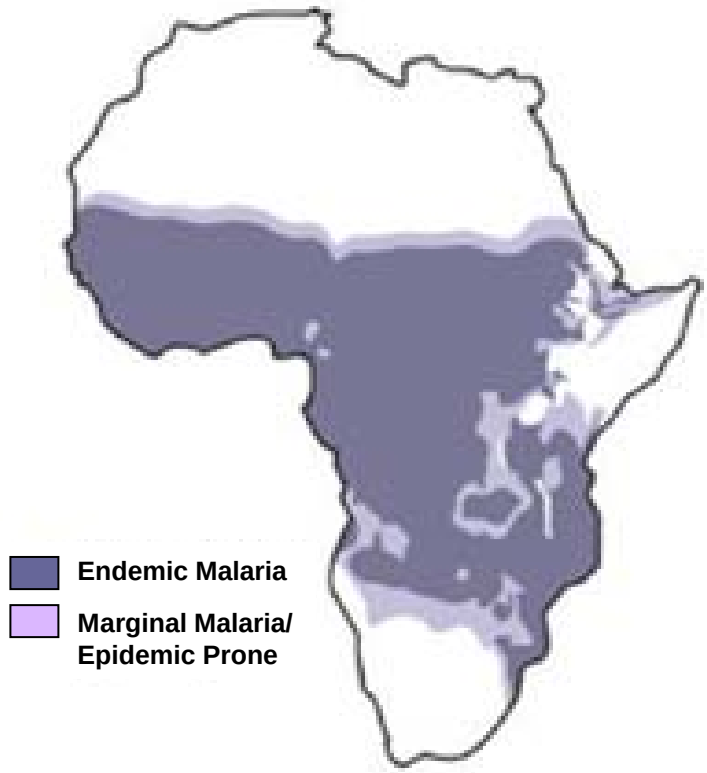

Figure 1: ***Map of Endemic Malaria within Africa***

The distribution of endemic malaria, a consistent transmission region, and marginal malaria, an unstable transmission region affected by low ambient temperatures within Africa (Shanks *et al.* 2005). Reprinted with permission from Macmillian Publishers Ltd: Nature Genetics (Wellems and Fairhurst 2005).

1.2 MALARIA PATHOGENESIS

1.2.1 Parasite Lifecycle

Infection of the human host occurs when a female *Anopheles* mosquito injects sporozoites during a blood meal into the dermis of the host (Miller *et al.* 2002)(Figure 2). Sporozoites migrate to the liver and invade hepatocytes utilizing the thrombospondin domain and the thrombospondin-related adhesive protein, which binds heparin sulphate proteoglycans on

hepatocytes (Miller *et al.* 2002; Jones and Good 2006). During the liver stage, the host is asymptomatic. Over a period of about one week, sporozoites develop by asexual mitosis into exoerythrocytic schizonts containing approximately 30,000 merozoites, which subsequently rupture the infected hepatocyte and infect red blood cells (RBC) (Jones and Good 2006), marking the onset of a 48-hour intraerythrocytic cycle within the newly parasitized RBC (pRBC). The merozoite in the newly invaded RBC develop asexually into the ring stage trophozoite, followed by a mature trophozoite, and finally, the schizont (Good *et al.* 2005) (Figure 2). During the occupation of the RBC, the parasite catabolizes hemoglobin using globin as a source of amino acids. The parasite renders the heme into an insoluble biomineralized product called hemozoin (Hz). The mature schizont gives rise to approximately 16 daughter merozoites, which are released upon the rupture of the pRBC (Good *et al.* 2005). This release marks the start of clinical disease presentation with fever (>37.5^{o}C), chills, headache, fatigue, seizures, and coma, primarily due to dysregulation of the cytokine response (Good *et al.* 2005). The life cycle of the parasite is complete when another female *Anopheles* mosquito ingests a gametocyte, a sexual stage parasite formed from merozoites, during a blood meal. Within the mosquito gut where the temperature is lower, male and female gametocytes emerge from the ingested pRBC and fuse in sexual replication to produce a zygote (Good *et al.* 2005). The zygote imbeds in the mid-gut of the mosquito and forms an oocyte that contains immature sporozoites (Figure 2). These sporozoites travel to the mosquitoes' salivary glands where they continue to develop prior to injection into the human host to start the parasitic life cycle anew (Good *et al.* 2005).

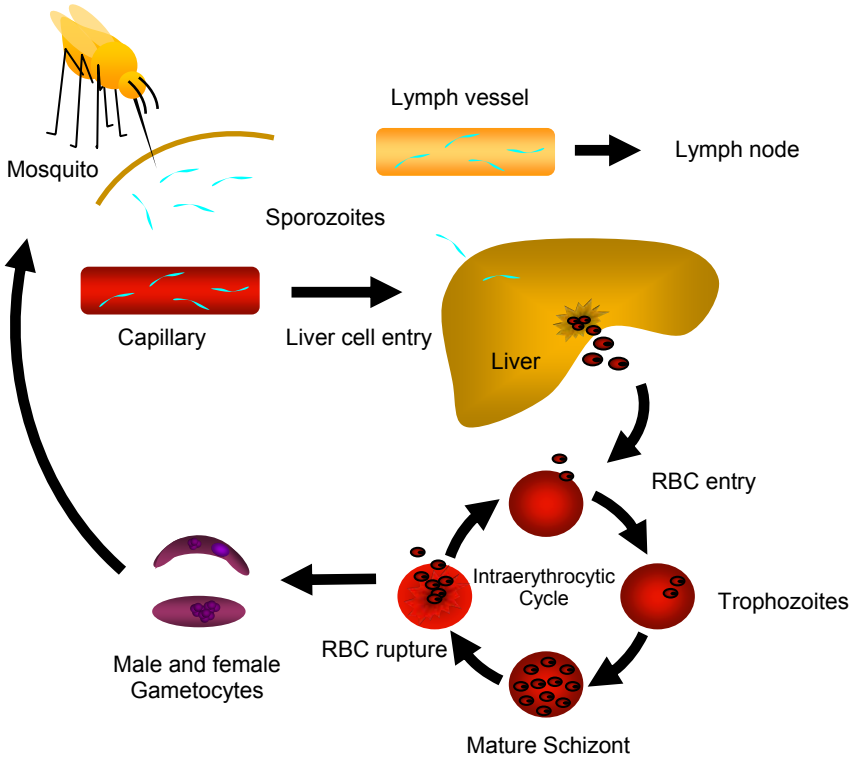

Figure 2: ***Life Cycle of Plasmodium falciparum in Humans***

The female *Anopheline* mosquito injects sporozoites into the human hosts' dermis where they migrate to the liver and invade hepatocytes. The invading sporozoites undergo 5-7 days of asexual replication to produce merozoites which rupture the hepatocyte. Merozoites migrate and invade the RBC initiating the intraerythrocytic cycle. During this 48 hour cycle, merozoites develop into trophozoites and schizonts. Schizogony gives rise to daughter merozoites that cause the rupture of the RBC. Many of these daughter merozoites will invade new RBC, but some differentiate into male and female gametocytes. Gametocytes are taken up by a new female *Anopheles* mosquito and migrate to the mid-gut to fuse in sexual reproduction forming a zygote containing immature sporozoites. These sporozoites will migrate to the mosquito's salivary glands for final development and inoculation into a new host. Adapted from Jones *et al*., 2006.

1.2.2 Hemozoin

During the intraerythrocytic cycle of infection, malaria pigment, (hemozoin, Hz), is synthesized from heme molecules (hematin, ferriprotoporphyrin IX) (Figure 3). Hz is formed and visible microscopically within the digestive vacuoles of late trophozoites and early schizonts and released as a by-product of parasitic proteolysis of host erythrocyte hemoglobin (Lawrence and Olson 1986; Sullivan *et al.* 1996; Egan 2002; Sullivan 2002). The parasite is unable to cleave the porphyrin ring therefore it must detoxify the heme by converting it to the insoluble Hz molecule (Figure 3). Upon pRBC rupture, free Hz is released into the blood stream where it is responsible for the induction of both pro- and anti-inflammatory cytokines and ingested by monocytes/macrophages and neutrophils (Schwarzer *et al.* 1998; Schwarzer *et al.* 2001; Lyke *et al.* 2003). However, monocytes/macrophages are unable to degrade ingested Hz and accumulation of this parasitic product leads to an inability to further ingest senescent pRBC, free Hz, and dysregulation in a number of cellular immunological functions (Ndungu *et al.* 2005). Previous studies have demonstrated that the presence of circulating Hz is an important marker of malaria severity both in adults and children (Nguyen *et al.* 1995; Amodu *et al.* 1998; Luty *et al.* 2000; Lyke *et al.* 2003) and is associated with increased mortality (Lyke *et al.* 2003).

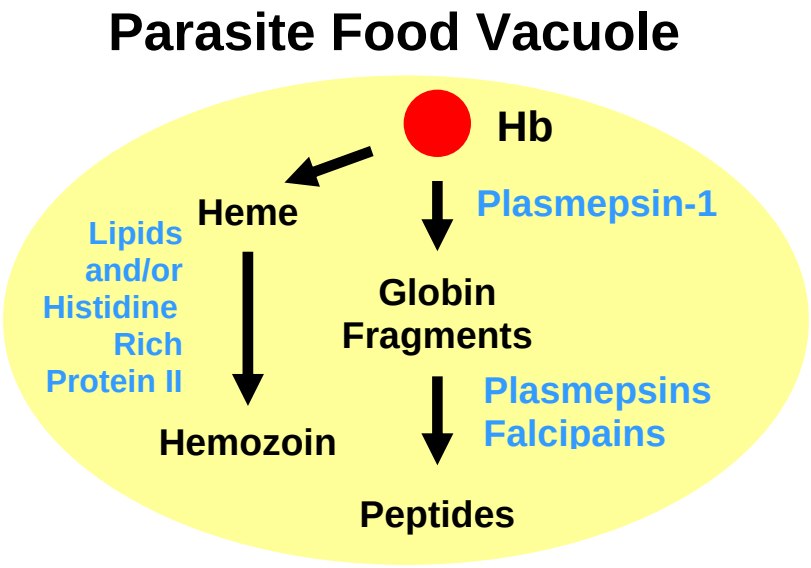

Figure 3: ***Diagram of Hemozoin Formation***

The parasite breaks down Hb as a source of amino acids creating heme as a by-product. Heme is toxic to the parasite and is converted to an insoluble biomineralized product called hemozoin. The mechanism(s) responsible for converting heme into hemozoin have yet to be elucidated, but are believed to involve phospholipids (Tripathi *et al.* 2002) and/or the histidine rich protein II, which is present in the RBC and secreted by the parasite (Sullivan *et al.* 1996; Egan 2002).

1.2.3 Immune Response

Clinical disease occurs during the intraerythrocytic cycle when pRBC rupture releasing merozoites and Hz into the blood leading to a cytokine response that can quickly become dysregulated (Struik and Riley 2004). When pRBC are phagocytized by scavenger monocytes/macrophages and neutrophils malaria pigment/Hz is ingested during this cellular process. The phagocytosis of pRBC, free Hz, and dead pigment-containing monocytes/macrophages aids greatly to the hosts' parasite management, clearance, and initial

immune response. However, the ingestion of Hz can lead to monocyte/macrophage dysfunction, reducing the ability to repeat phagocytosis, generate oxidative burst upon stimulation, and activate protein kinase C (Ndungu *et al.* 2005). It is the induction of cytokines from scavenger monocytes/macrophages, which are intended to protect the host, that have been implicated in the severity of disease. Activated monocytes/macrophages bind to pRBC through CD36 and subsequent phagocytosis results in the secretion of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine that has potent anti-parasitic properties (Othoro *et al.* 1999). However when present systemically in high amounts, TNF-α has been implicated in severe disease (Othoro *et al.* 1999). Nitric oxide (NO), also secreted from monocytes/macrophages, has anti-parasitic properties by increasing parasite clearance through oxidative damage to the pRBC (Ekvall 2003). However, long-term expression of NO has been shown to suppress erythropoiesis and induce apoptosis of CD34+ hematopoietic stem cells, thereby contributing to SMA (Kremsner *et al.* 1996; Reykdal *et al.* 1999). Interleukin-12 (IL-12) secreted from monocytes/macrophages plays a vital role in the regulation of immunological cells through its ability to up-regulate the secretion of TNF-α and interferon-γ (IFN-γ) (Luty *et al.* 2000; Perkins *et al.* 2000; Watford *et al.* 2003). Furthermore, IL-12 activates naïve T-cells to differentiate into T-helper 1 cells (T_H1) that further activate B cells to secrete IgG antibodies, which in turn opsonize pRBC (Struik and Riley 2004). T_H1 cells secrete IFN-γ and interleukin-2 (IL-2) thereby activating NK cells to secrete IFN-γ. IFN-γ is released early in the immune response and has been shown to be a strong modulator of the pro-inflammatory response (Stevenson and Riley 2004).Due to the ability of IFN-γ to up-regulate the secretion of TNF-α and interleukin-1 (IL-1), it has been linked to sev ere disease when present systemically in high amounts (Artavanis-Tsakonas *et al.* 2003). Furthermore, IFN-γ is implicated in the activation of naïve T cells, the

maturation of dendritic cells (DC), and the activation of monocytes/macrophages (Figure 4) (Stevenson and Riley 2004).

Increased ingestion of Hz by monocytes/macrophages leads to decreased IL-12 and increased TNF-α production (Keller *et al.* 2006). Furthermore, low plasma levels of IL-10 are insufficient to effectively down-regulate TNF-α production, which may lead to the suppression of erythropoiesis, a contributor to SMA (Ho *et al.* 1995; Othoro *et al.* 1999; Perkins *et al.* 2000; Good *et al.* 2005). Low IL-12 levels have also been implicated in severe disease due to the inability of the host to mount an effective immune response to clear and manage the infection (Luty *et al.* 2000; Perkins *et al.* 2000; Dodoo *et al.* 2002). Therefore, the balance between pro- and anti-inflammatory mediators determines if the response will be detrimental or protective (Kurtzhals *et al.* 1999; Othoro *et al.* 1999).

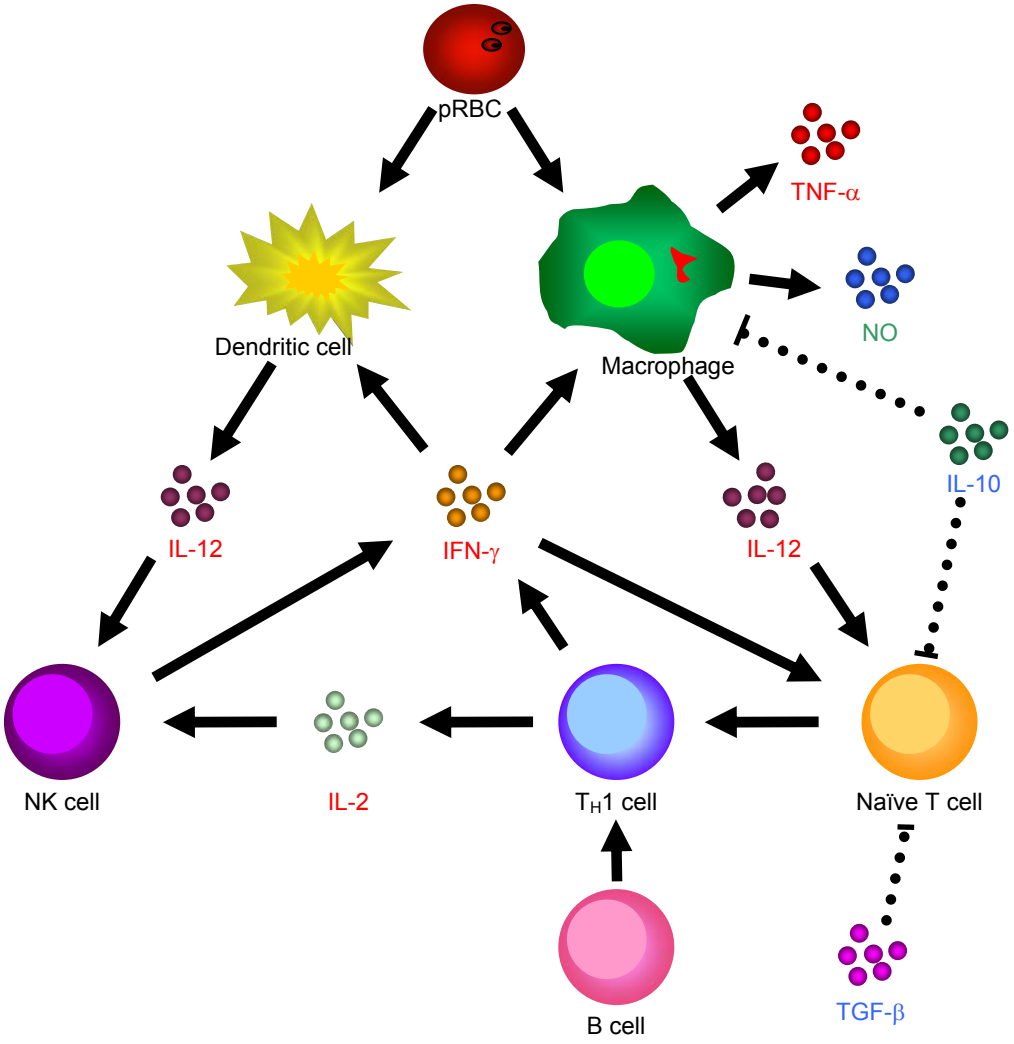

Figure 4: ***Model of the Cytokine Response to Malaria***

Immunity against malaria is regulated by pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines are represented in red text, anti-inflammatory cytokines in blue text, and effector molecules in green text. When monocytes/macrophages ingest pRBC or free Hz a cytokine cascade is initiated through the secretion of IL-12. IL-12, together with B cells, promote the differentiation of naïve T cells into T_H1 cells and the secretion of IFN-γ. Both IL-12 and IFN-γ stimulate monocytes/macrophages to secrete TNF-α. T_H1 cells secrete IL-2 to activate NK cells, which are also activated through DC-secreted IL-12, to secrete IFN-γ. The release of anti-inflammatory cytokines such as IL-10 and transforming growth factor -β (TGF-β) causes the down-regulation of IL-12, IFN-γ, and TNF-α thereby preventing elevated pro-inflammatory levels, which can lead to severe disease. Adapted from Stevenson and Riley, 2004.

1.2.4 Severe Malaria Anemia (SMA)

The pathophysiology of severe malaria anemia (SMA) is complex and multifactorial. SMA is the leading cause of mortality in children under the age of five with malaria (WHO 2000). In acute childhood malaria, suppression of bone marrow responsivness and the increased phagocytosis of pRBC and non-parasitized RBC (n-pRBC) play a major role in SMA pathogenesis (Price *et al.* 2001; Ekvall 2003). Although pregnant women are at risk for developing SMA, it is estimated that they comprise only ~10% of SMA cases in sub-Saharan Africa, and the ensuing anemia is more likely due to malnutrition, hookworm infection, and HIV complication (Ekvall 2003).

The destruction of pRBC in SMA occurs via rupture and phagocytosis of these RBC by monocytes/macrophages and neutrophils. However, destruction of RBC alone does not adequately account for the profound decrease in Hb observed in children with SMA. Previous results show that monocytes/macrophages clear n-pRBC and this process accounts for more than 90% of the loss in the hosts' RBC (Price *et al.* 2001; Ekvall 2003). Susceptibility of n-pRBC to increased phagocytosis is multifactorial and results from several mechanisms i.e., circulating monocytes/macrophages release oxygen and nitrogen radicals that can damage both the pRBC and n-pRBC membranes, leading to enhanced clearance of the overall RBC population (Greve *et al.* 1999; Perkins *et al.* 2000; Griffiths *et al.* 2001). Furthermore, oxidative stress causes the RBC membranes to undergo a conformational change, which leads to an increase in IgG binding to the band 3 protein on the RBC surface resulting in increased phagocytosis (Ekvall 2003; Turrini *et al.* 2003). Additionally, reduction in RBC deformability, characterized by membrane rigidity, and exposure of phosphatidylserine on the outer surface of the cell has been linked to disease severity (Dondorp *et al.* 2003; Lang *et al.* 2004). Reduced deformability is also implicated in the

removal of RBC in the spleen, where RBC can become entangled within splenic cords and subsequently removed (Ekvall 2003). Complement can also act synergistically with immunoglobulins for both enhanced induction of phagocytosis as well as the direct lysis of normal RBC (Ekvall 2003). n-pRBC are usually protected from complement activation, however the absence of CR1 and CD55 regulatory proteins, which protects the RBC from complement-mediated lysis, leads to increased phagocytosis and possibly SMA (Waitumbi *et al.* 2000).

Children with SMA characteristically exhibit bone marrow suppression and dyserythropoiesis. Normal erythropoiesis occurs when the erythroid progenitor, primitive erythroid burst-forming unit (pBRU-E), matures, loses the ability to divide and migrate, and becomes the erythroid colony–forming unit (CFU-E) (Roberts *et al.* 2005). During bone marrow dysfunction in malaria, there is a decreased responsiveness of erythroid progenitor cells and potential impairment in erythropoietin production; a potential consequence of increased inflammatory cytokine production (Ekvall 2003). Pro-inflammatory cytokines such as TNF-α and IFN-γ, and anti-inflammatory cytokines including IL-10, have been implicated in promoting dyserythropoiesis (Ekvall 2003). Elevated levels of TNF-α and IL-10 are found in children with severe malaria, with a low IL-10 to TNF-α ratio associated with childhood SMA (Kurtzhals *et al.* 1998; Othoro *et al.* 1999; Luty *et al.* 2000; Zamai *et al.* 2000; Ekvall 2003). Normally, IL-10 down-regulates the production of TNF-α, but due to decreased secretion of IL-10 the normally beneficial TNF-α levels become excessively elevated and contribute to SMA (Roberts *et al.* 2005).

1.3 HEMOGLOBIN SYNTHESIS

1.3.1 Heme Synthesis

Hemoglobin synthesis is the concerted effort between heme, a prosthetic group that mediates the reversible binding of oxygen (O_2) to Hb, and globin.

Heme synthesis is a step-wise process that involves enzymes in the mitochondria and cytosol of the cell. Within the mitochondria, condensation of succinyl coenzyme A (CoA) and glycine forms α-amino-β-ketoadipate, which become decarboxylated to form Δ-aminolevulinate (ALA) (Baggott and Dennis 1995). ALA is then transported to the cytosol where two ALA molecules undergo dehydration to release two molecules of water forming four molecules of porphobilinogen, the first pyrrole. The porphobilinogen molecules undergo three step-wise reactions before becoming coporphyringen III. Catalyzation of porphobilinogen by uroporphyrinogen I synthase results in the loss of amino groups (Baggott and Dennis 1995). Porphobilinogen then becomes a linear tetrapyrrole with alternating acetic and propionic acid groups, which slowly cyclize to create uroporphyrinogen I. The second reaction transforms the tetrapyrrole to cyclic uroporphyrinogen III through the enzymatic activity of uroporphyrinogen III cosynthase. The cyclic uroporphyrinogen III acetic acid groups are then decarboxylated by uroporphyrinogen decarboxylase to form methyl groups that become coproporphyrinogen III (Baggott and Dennis 1995). Coproporphyrinogen III within the mitochondria utilizes the enzyme coproporphyrinogen III oxidase to convert the propionic acid groups on ring I and III to vinyl groups, creating the product protoporphyrinogen IX. Protoporphyrinogen IX oxidase then converts the methylene bridges between the pyrrole rings of protoporphyrinogen IX to methyl bridges allowing for resonance around the entire ring structure and enhanced stabilization.

Lastly, synthesis of heme occurs when ferrochelatase adds iron (Fe^{2+}), aided by ascorbic acid and cysteine as reducing agents, to protoporphyrinogen IX to form heme (Baggott and Dennis 1995).

1.3.2 Globin Synthesis

There are two separate globin chains with their respective heme molecules coming together to form Hb. The alpha globin chain expresses the 141 residue alpha genes that are encoded on chromosome 16 (Figure 5). Chromosome 16 has two alpha globin genes, referred to as alpha 1 and alpha 2, that are identical and aligned one after the other. Each cell has two sets of chromosomes resulting in four alpha genes per cell. Each alpha gene produces approximately one quarter of the required alpha globin per Hb molecule (Bridges 2003).

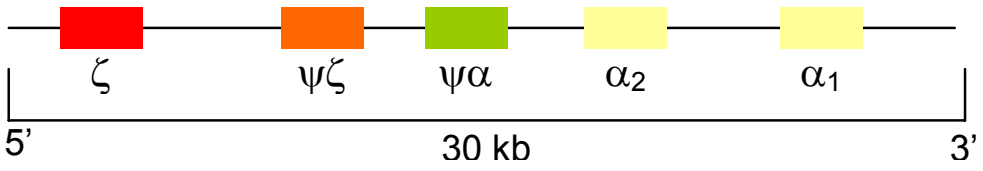

Figure 5: ***Schematic of the Alpha Chain of Hb***

The Hb alpha chain is 30 kb long and encoded on chromosome 16. Each chromosome 16 has two alpha genes, α_1 and α_2, which are identical, as well as a zeta (ζ) gene and two pseudogenes: pseudo-zeta (ψζ) and pseudo-alpha (ψα). Each of the four alpha genes produce exactly one quarter of the needed alpha globin required per Hb molecule.

The beta chain is 146 residues and is encoded on chromosome 11 (Figure 6). The beta globin genes are sequentially expressed from the 5' to 3' end during various developmental time points. The beta chain expresses epsilon (ε) during the first 12 weeks of embryogenesis then

moves to gamma (γ), delta (δ), and/or beta (β). On chromosome 11, there are two copies of the gamma gene, while the remaining genes are singularly present (Bridges 2003).

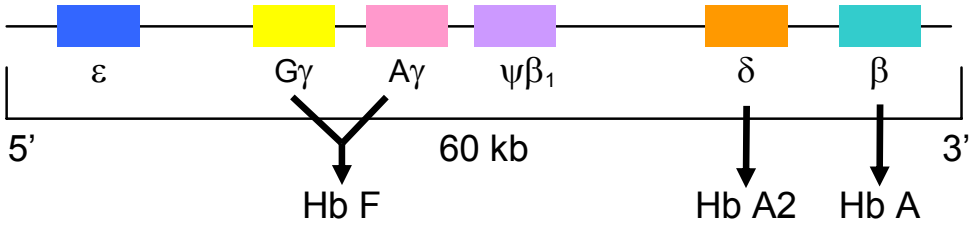

Figure 6: ***Schematic of the Beta Chain of Hb***

The beta chain within the Hb molecule is 60 kb long and encoded on chromosome 11. Expression of the beta genes begin during the first 12 weeks of embryogenesis with epsilon (ε) which then moves sequentially to gamma (γ), delta (δ), and/or beta (β). Both gamma genes comprise fetal hemoglobin (HbF), which is the predominate Hb until ~18 weeks of age. The delta A2 Hb constitutes approximately 3% of adult Hb, although it is not commonly produced.

During the first 12 weeks of embryogenesis Hb Gower 1 is expressed with the zeta gene of the alpha globin gene cluster (there is no alpha gene being expressed at this time) coupled with the epsilon gene as the non-alpha (Bridges 2003). The Gower 2 Hb is formed shortly after replacing the short-lived zeta gene with an alpha gene. Hb Portland is formed when the epsilon gene is replaced with the gamma genes on the non-alpha chain and the zeta gene expressed on the alpha chain (Bridges 2003). The combination of two alpha chains and two gamma chains form fetal Hb, which is the predominate Hb until approximately 18 weeks post-birth when adult Hb is formed from two alpha and two beta chains. However, it has been noted that the delta gene, located between gamma and beta, can be expressed in children and adults producing

hemoglobin A2. Hb A2 is comprised of two alpha and two delta chains, however, the delta globin normally comprises less then 3% of A2 Hb (Bridges 2003; Campbell and Farrell 2006).

1.3.3 Oxygen Transport

Oxygenation of Hb is favored within the alveoli of the lungs due to an acidic environment with high O_2 pressure (100 Torr) and low amounts of H^+ and CO_2. As Hb enters the lungs, it releases a H^+ molecule that is picked up in the capillary beds and exchanged for O_2 (Campbell and Farrell 2006). One Hb molecule is able to strongly bind four molecules of O_2 aided by the high O_2 pressure resulting in 100% saturation. The H^+ expelled from local tissues during metabolic activity binds to the bicarbonate ion (HCO^-_3) creating carbonic acid (H_2CO_3), which is then transported to the lungs. The secession of CO_2 from H_2CO_3 occurs immediately upon H_2CO_3 formation and then is subsequently expelled during exhalation (Campbell and Farrell 2006).

As Hb-containing RBC move into the capillaries O_2 pressure drops to around 20 Torr, this would typically yield an Hb saturation of approximately 50%. This drop in pressure is a physiological mechanism promoting Hb to release O_2 (Campbell and Farrell 2006). Within the capillary bed there is a reduced pH (~6.35) due to the production of CO_2, which rapidly forms H_2CO_3. Since the pH in blood is typically 7.4, approximately 90% of the dissolved CO_2 will take the form of bicarbonate ions and release H^+ to maintain the pH level within blood. Hb then releases the O_2 molecules that bind to the free H^+ that is carried back to the lungs to bind with HCO_3^- to start the process over again (Campbell and Farrell 2006).

1.4 α - THALASSEMIA

α-thalassemia was first identified in 1925 when Detroit pediatrician Dr. Thomas Cooley described a syndrome he observed in Italian children characterized by extreme anemia, splenomegaly, and bone deformities (Wintrobe and Lee 1999). Prior to genetic screening, individuals with α-thalassemia were classified based on the severity of their clinical presentation. Patients presenting with severe clinical disease were considered thalassemic major. Those with mild-to-moderate anemia were classified as thalassemic intermedia (Wintrobe and Lee 1999; Bridges 2003).

1.4.1 Molecular Basis of α-Thalassemia

The α-thalassemia hemoglobinopathy occurs when one or more of the α genes become deleted or non-functional on chromosome 16 p13.3 (Wintrobe and Lee 1999; Bridges 2003). The α globin genes are prone to deletions due to their tandem duplicate α gene clusters (Wintrobe and Lee 1999). These deletions lead to unbalanced pairing of α and β chains and depending on how many deletions are present, these deletions can be life-threatening (Wintrobe and Lee 1999; Bridges 2003). There are two α-thalassemia variants designated α^{o} and α^{+}. The α^{o} variant is the deletion of both alpha globin genes, where the α^{+} variant results in the deletion of only one of the two alpha globin genes (Wintrobe and Lee 1999).

1.4.2 Genetic Variants of α-Thalassemia

α-thalassemia is the most common genetic disorder worldwide with geographically specific variants. The highest frequencies of thalassemia are in Southeast Asia, Mediterranean, Middle East, and Africa (Figure 7)(Wintrobe and Lee 1999). In Saudi Arabia, greater than 50% of the population has the clinically silent form of thalassemia with the common deletional variant $-\alpha^{4.2}$. The $-\alpha^{4.2}$ genotype involves the deletion of a 4.2 kb segment of DNA from the 3' Ψα gene to the 3' α_2-gene (Wintrobe and Lee 1999). The α^o variant is prevalent in Mediterranean populations, affects both α globin genes, and includes 3 different deletions. The ($--^{MED}$) deletion occurs on the 5' end of the Ψζ gene and extends through both α globin genes (Wintrobe and Lee 1999). The ($-(\alpha)^{20.5}$) deletes the Ψζ, Ψα, α_2-gene, and the first 56 codons of the α_1-gene, while the smaller deletion variant ($-(\alpha)^{5.2}$) removes the α_2-gene and part of the α_1-gene. In Southeast Asia, the α^o variant is also common, but with the ($--^{SEA}$) deletion. This deletion extends from the 3' end of the ζ gene through the α_1-gene. In Africa, the $-\alpha^{3.7}$ deletion is the most common, causing a 3.7 kb fragment of DNA to be deleted, affecting only one of the alpha globin genes (Figure 7)(Wintrobe and Lee 1999).

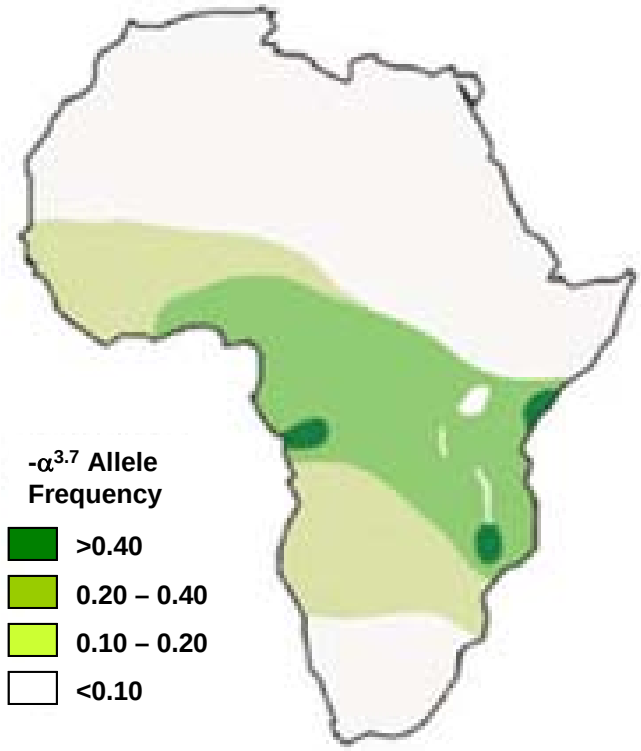

Figure 7: ***Allele Frequency of -α$^{3.7}$ within Africa***

Allelic frequency of the -α$^{3.7}$ genotype in Africa. Reprinted with permission from Macmillian Publishers Ltd: Nature Genetics (Wellems and Fairhurst 2005).

1.4.3 Pathophysiology of α-Thalassemia

There are four α-thalassemia syndromes currently recognized; silent carrier, α-thalassemia minor, HbH disease, and hydrops fetalis with Hb Bart's (Wintrobe and Lee 1999). However, depending on the specific variant of thalassemia, some syndromes may be rarer than others. Historically, testing for α-thalassemia required cord blood measurement to determine the amount of Hb Bart's, characterized by gamma chains that aggregate in groups of four causing an

abnormal Hb variant. However, PCR reactions have recently been optimized to genotype and diagnose α-thalassemic individuals (Wintrobe and Lee 1999; Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b; Bridges 2003).

1.4.3.1 Silent Carrier (αα / α-)

The silent carrier has the loss of only one functional α globin gene causing a slight reduction in protein production with no clinical or hematological abnormalities (Bridges 2003). Diagnosis of the silent carrier is most easily made at birth by PCR or by measuring the percentage of Hb Bart's (2-10%) in cord blood. The severity of thalassemia is directly proportional to the percentage of Hb Bart's (Wintrobe and Lee 1999). This method is highly effective in Indian, Asian, and Malaysian populations, whereas in the African, African-American, and Caribbean populations this methodology is less reliable. Within the African-American and Caribbean populations cord blood can only accurately predict 12% and 10%, respectively, of the expected silent carrier cases (Wintrobe and Lee 1999). In adults, one reliable method of detection is through gene mapping by measuring the α:β globin mRNA ratios, which can distinguish the number of functional α genes. (Wintrobe and Lee 1999).

1.4.3.2 α-Thalassemia Minor (αα / - -) / (α- / α-)

α-thalassemia minor is the most commonly detected form of thalassemia in Asian, Mediterranean, and African populations and may manifest in the heterozygous (αα / - -) or homozygous (α- / α-) form (Wintrobe and Lee 1999). Although fairly benign, the loss of two α globin genes does cause minor anemia that can be detected during routine blood testing (Bridges 2003). Individuals appear functionally normal but have a microcytic, hypochromic, and anisopoikilocytotic RBC morphology, characterized as small RBC with a pink coloration and an

irregular shape (Wintrobe and Lee 1999). The diagnosis of thalassemia minor is easily assessed at birth by measuring the percentage of Hb Bart's (10-20%) in cord blood and by determining the mean corpuscular volume (MCV; the measurement of the average RBC size and volume), and mean corpuscular hemoglobin (MCH; the measurement of Hb mass within a RBC) (Wintrobe and Lee 1999).

1.4.3.3 HbH Disease (α- / - -)

The loss of three α globin genes results in a serious disorder called HbH disease that is prevalent in South East Asia, parts of the Middle East, and Mediterranean. However, HbH disease is rarely found in African populations (Wintrobe and Lee 1999). HbH occurs when the β chains associate in groups of four due to the lack of α chains (Bridges 2003). Because of this anomaly, individuals experience severe anemia that often requires blood transfusions for survival. Additionally, HbH does not properly carry oxygen, making it functionally useless to the RBC resulting in membrane damage that leads to increased cell death. Affected individuals also typically exhibit bone abnormalities, i.e. bone flairs in the cheek and forehead region leading to facial disfigurement (Wintrobe and Lee 1999; Bridges 2003). In addition, the spleen will become enlarged and the individual will present as small and malnourished, even though they receive appropriate caloric intake (Bridges 2003). The malnourished presentation occurs because energy from consumed foods is utilized for the bone hyper-erythropoiesis, an accelerated process that mimics high-energy aerobic activity (Bridges, 2003 #201).

Detection of HbH can be achieved with 3 to 4 drops of blood added to 0.5 mL of 1% brilliant cresyl blue and incubated at room temperature for 20 minutes, which causes the precipitation of HbH (Wintrobe and Lee 1999). Another method is using Hb electrophoresis to measure the amount of Hb Bart's that is present, usually 20-40% of the individuals total Hb. The

concentration of Hb Bart's will decrease during the first months of life and will be replaced by HbH (Wintrobe and Lee 1999).

1.4.3.4 Hydrops Fetalis with Hb Bart's (- - / - -)

Hydrops fetalis is the most severe form of α-thalassemia with the loss of all four functional α globin genes and is incompatible with life. Hydrops fetalis is observed in South East Asia, occasionally in the Mediterranean, and rarely in African populations (Wintrobe and Lee 1999). The rarity of hydrops in African and Mediterranean populations is due to the geographic distribution of the α^o and α^+ haplotypes. Haplotypes α^o (- -) and α^+ (α-) occur in equal frequency in Asia, whereas the α^o (- -) haplotype is rare in the Mediterranean, and even more rare in Africa. Therefore, the Asian population has a (α- / α-) or (- - / αα) genotype for minor thalassemia, and the (α- / α-) genotype is present in Mediterranean and African populations (Wintrobe and Lee 1999).

The development of hydrops fetalis varies, with some fetuses developing normally until the third trimester, while others become severely hydropic with numerous abnormalities in the second trimester (Chui 2005). The infant is commonly delivered stillborn or with significant cardio-respiratory distress. Death usually occurs within an hour of birth, but there have been documented cases of survival after an immediate serial exchange transfusion followed by a red cell transfusion (Chui 2005).

Hematologically, the hydrops infant has Hb in the range of 4-10 g/dL, with a peripheral blood smear characterized by anisopoikilocytosis, severe hypochromia, and nucleated red blood cells (Chui 2005). When Hb electrophoresis is performed, HbF and HbA are absent, but there is a large, fast migrating band representing Hb Bart's, and a smaller band representing HbH (Chui

2005). However, there has been some promise with detecting fetal erythrocytes in maternal circulation early in pregnancy by immunofluorescence. Earlier detection offers the possibility of an *in utero* transfusion (Chui 2005).

1.4.4 $-\alpha^{3.7}$ and Falciparum Malaria

The relationship between $-\alpha^{3.7}$ and falciparum malaria is unclear and no mechanism(s) has been found to elucidate its protection. Interestingly, the genetic frequency of $\alpha^{3.7}$ mirrors the endemicity of both falciparum and vivax malaria (Williams *et al.* 2005c; Urban *et al.* 2006; Wambua *et al.* 2006b). Documentation from Africa to New Guinea has demonstrated frequencies as high as 70% in the population (Oppenheimer *et al.* 1987). On the islands of Vanuatu and New Guinea, protection was limited to severe malaria episodes only in homozygotes (α- / α-). Additionally, blood smears from homozygotous individuals had higher parasite counts than heterozygous and wild type individuals (Oppenheimer *et al.* 1987). However, another study from Vanuatu documented an increase in uncomplicated malaria in both heterozygous and homozygous α-thalassemia children (Williams *et al.* 1996).

A recent study in Kilifi, Kenya, found that $-\alpha^{3.7}$ heterozygotes and homozygotes had a 40% and 60% reduced risk of death, respectively, when presenting with severe malaria at the hospital; however, there was no effect of $-\alpha^{3.7}$ on parasite density or prevalence of symptomless carriage (Williams *et al.* 2005c). Further research in Kilifi confirmed that protection is afforded to both the heterozygous and homozygous individuals with the 3.7 kb deletion with no effect on parasitemia, suggesting that parasite density is not altered in $-\alpha^{3.7}$ individuals (Wambua *et al.* 2006a; Wambua *et al.* 2006b).

Studies from Ghana show that protection from severe falciparum malaria was only observed in heterozygotous individuals (Mockenhaupt *et al.* 2004). However, it is important to note that seasonal transmission of malaria and the low frequency of the $-\alpha^{3.7}$ genotype in Ghana, may result in $-\alpha^{3.7}$ protection not being as prominent (Mockenhaupt *et al.* 2004).

Several studies have been carried out to elucidate the mechanism(s) of protection in $-\alpha^{3.7}$. One study demonstrated that $-\alpha^{3.7}$ caused a reduced parasite invasion rate and/or a reduction in parasite growth within thalassemic RBC (Pattanapanyasat *et al.* 1999). Additionally, thalassemic RBC age more quickly due to oxidative stress leading to membrane damage and decreased parasite invasion (Yuthavong *et al.* 1988; Pattanapanyasat *et al.* 1999; Destro-Bisol *et al.* 1999b; Cheng *et al.* 2005; Senok *et al.* 2006). However, physiological studies showed that thalassemic RBC have no detectable protection against parasite load when compared to those from wild type individuals, suggesting that thalassemics have similar parasite invasion rates (Mockenhaupt *et al.* 2004; Williams *et al.* 2005c; Wambua *et al.* 2006b). Parasite growth may be reduced in RBC due to oxidative stress induced by the $-\alpha^{3.7}$ genotype. Oxidative stress increases when the parasite breaks-down cellular Hb leading to membrane damage and increased phagocytosis (Luzzi *et al.* 1991a; Luzzi *et al.* 1991b; Pattanapanyasat *et al.* 1999; Destro-Bisol 1999a; Destro-Bisol *et al.* 1999b; Cheng *et al.* 2005). Furthermore, surface antigens on the RBC undergo conformational changes when the parasite enters the cell. Previous studies documented increased phagocytosis by macrophages in thalassemic individuals, possibly due to conformational changes of surface antigens (Yuthavong *et al.* 1988). Additional studies also demonstrated a marked increase in the binding of IgG to the pRBC caused by a conformational change in band 3 protein on the RBC membrane (Luzzi *et al.* 1991a; Luzzi *et al.* 1991b).

1.5 SICKLE-CELL HEMOGLOBIN (HBS)

Sickle-cell disease was one of the first human genetic variants associated with a specific molecular defect (Pauling *et al.* 1949). The first formally documented case occurred in 1910 when Chicago cardiologist Dr. James Herrick recorded observations made during a physical exam of a 20-year-old West Indian student (Embury 1994).

1.5.1 Molecular Basis of Sickle-Cell Hemoglobin

The sickle-cell genotype is due to a mutation in the beta globin subunit located on the short arm of chromosome 11. A point mutation substituting thymine for adenine at the sixth codon of the β gene, (GAG → GTG), leads to valine incorporation, rather than glutamine. This single point mutation causes substantial changes in the molecular formation of Hb, resulting in deoxygenation, and polymerization (Embury 1986).

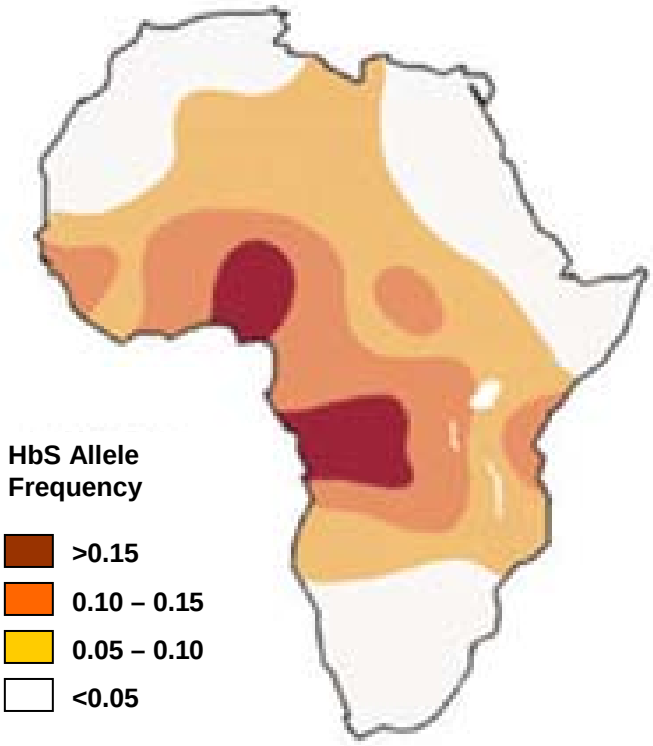

Figure 8: ***Allele Frequency of HbS within Africa***

Allele frequency of HbS in Africa. Reprinted with permission from Macmillian Publishers Ltd: Nature Genetics (Wellems and Fairhurst 2005).

1.5.2 Allele Frequency of Sickle-Cell Hemoglobin

The greatest prevalence of HbS occurs in Africa (Figure 8), with lower frequencies in the Middle East, Asia, parts of India, and Mediterranean basin region. Studies from these various locations revealed that the sickle gene arose from three independent mutations within Africa (Pagnier *et al.* 1984; Serjeant *et al.* 1994). The most common haplotype developed in Benin/Central West Africa, while the second and third haplotypes developed within the Senegal/African west coast and Central African Republic (Pagnier *et al.* 1984; Serjeant *et al.* 1994). All three haplotypes are prevalent among African-American and Jamaican populations who frequently have HbS. The HbS variant present in the eastern Middle East and central India

population is not associated with any of the three African haplotypes and contains a different DNA structure, indicating the fourth haplotype developed independently from the three African haplotypes (Serjeant *et al.* 1994). The Benin and Senegal haplotypes are prevalent among North Africans, Greeks, and Italians, suggesting that this haplotype may have been distributed through trade routes, intermarriage, and slave trade (Mears *et al.* 1981; Antonarakis *et al.* 1984).

1.5.3 Pathophysiology of Sickle-Cell Disease (SCD)

1.5.3.1 Molecular Basis of Sickling/Polymerization

Sickling of HbS-containing RBC results from Hb polymerization, poor solubility, and deoxygenation (Embury 1986; Embury 1994). The deoxygenated HbS polymer is a helical structure with fourteen Hb tetramers in each layer forming a central core of four strands and an outer sheath of ten additional strands. Only two of the β6 valine residues participate in the intermolecular bonding. The presence of this polymer is responsible for the reversible properties of the sickle RBC. Upon oxygenation, these polymers "melt" and the sickle RBC loses the characteristic sickle shape (Embury 1986; Embury 1994). Polymerization of the HbS RBC occurs in several stages. Normally, the RBC spends one to two seconds in arterial circulation, one second in microcirculation, and then requires about fifteen seconds to return to the lungs. If there is a delay greater then fifteen seconds during this process, the cell can return to the lungs to be reoxygenated before polymerization occurs (Embury 1986; Embury 1994). However in HbS RBC, if the delay time is between one and fifteen seconds, gelation (formation of a semi-solid crystalline HbS structure) will occur while the cell is in venous circulation. Sickling of the RBC within large veins does not cause vaso-occulsion, but the RBC membrane will be damaged, resulting in loss of water and a shorter delay in subsequent cycles through circulation (Embury

1986; Embury 1994). If the delay time is less than one second, gelation can occur while the RBC are in one of the narrow vessels of the microcirculation. This results in significant problems since the RBC are no longer deformable and may not be able to circulate though the narrow vessel, therefore, causing vaso-occulsion by being permanently or temporarily lodged (Embury 1986; Embury 1994; Wintrobe and Lee 1999). Additionally, sickled RBC may never fully regain their normal morphological shape upon reoxgenation in the lungs. This incomplete depolymerization can occur due to the total Hb concentration being at, or greater, than the solubility of arterial pressure, leading to a slower depolymerization rate. Therefore, when RBC enters the microcirculation it will polymerize faster than it did during the first circulating cycle since it has not fully depolymerized (Embury 1986; Embury 1994; Wintrobe and Lee 1999).

1.5.3.2 Cellular Hydration of Sickled RBC

During deoxygenation, sickled RBC lose the intracellular, monovalent cation, potassium (K^+). A decrease in potassium results in the loss of water from the cell, leading to increased concentrations of Hb. Even the small Hb concentration increases can have serious effects on the delay time of the HbS RBC, leading to increased sickling. In addition, there is an intracellular increase in calcium (Ca^{2+}) during the sickling process that is due to the disruption of the lipid bilayer from the RBC cytoskeleton, thereby, increasing the permeability of the RBC to Ca^{2+} (Embury 1986; Embury 1994).

In irreversibly sickled cells (ISC), the RBC is permanently in the crescent sickle shape, even in the presence of 100% O_2. These cells are characterized by a low MCV and high MCHC, properties of dehydrated RBC (Embury 1986; Embury 1994). ISC are usually formed shortly after release from the marrow and are quickly removed from the circulation. ISC contain substantially lower HbF than reversibly sickled cells and may be the primary cause of ISC

(Embury 1986; Embury 1994). ISC have a reduced K^+ concentration that exceeds the Na^+ gain, and an increase in Ca^{2+} concentration (Embury 1986; Embury 1994; Wintrobe and Lee 1999).

1.5.4 Treatment of SCD

Access and availability to treatment for SCD varies greatly depending on the patient's location. Within Africa resources can be plentiful or scarce depending on location. There is infrequent post-natal screening and many times the diagnosis of SCD occurs during the child's first crisis and treatment availability can be limited (Diallo and Tchernia 2002). More than half of the children with SCD in Africa will die before the age of five (Diallo and Tchernia 2002; WHO April 24, 2006). Many areas lack the resources to import needed drugs as well as the proper medical equipment for monitoring and follow-up care, and even then improperly treated children due to misleading information is likely (Diallo and Tchernia 2002). Many areas have access to chemotherapeutics such as penicillin, although not usually given prophylactically and hydroxyurea may be found in larger city hospitals and the occasional research clinic (Diallo and Tchernia 2002). Hydroxyurea targets the cell cycle and inhibits DNA synthesis of HbS while increasing the synthesis of HbF, thereby reducing the amount of HbS within cells and reducing the chance of sickling (Embury 1994; Wintrobe and Lee 1999; De Franceschi and Corrocher 2004). Blood transfusions are common, but pose a considerable health threat as the blood is not always properly screened prior to transfusion (Diallo and Tchernia 2002). Finally, in 2006 the WHO declared SCD a major health issue by outlining health care infrastructure to be implemented in all countries i.e.: surveillance, research, early detection, management programs for families (WHO April 24, 2006).

1.6 SICKLE-CELL TRAIT (HBAS)

1.6.1 Background of Sickle-Cell Trait

Sickle-cell trait is the heterozygous form of SCD where the individual has one HbA (normal) and one HbS (sickle-cell) gene. There is a 60/40 ratio between HbA and HbS, and although this ratio may change slightly, HbA is always the dominant globin form (Wintrobe and Lee 1999). Sickle-cell trait is prevalent in Africa where allele frequencies have reached 0.07 in coastal Kenya (Urban *et al.* 2006) and 0.26 in the western Kenya lowlands (Moormann *et al.* 2003). HbAS is also frequent in parts of the Mediterranean, India, and Asia. More interesting is the geographic prevalence of HbAS in relation to the distribution of falciparum malaria. The aforementioned countries are endemic areas of falciparum malaria and the higher the frequency of HbAS within the population is directly proportional to the malaria transmission rate (Moormann *et al.* 2003).

HbAS is associated with little or no clinical illness and the only consistent hematological abnormality is a mildly reduced MCV and MCH (Serjeant 1992). Life expectancy and overall mortality rate for an HbAS individual is the same as it would be for an HbAA individual (Serjeant 1992). There has been documentation, although rare, of sudden death occurring in soldiers or athletes participating in physically strenuous activity. However, these cases lack vital information as to the HbA/HbS ratio or the presence of any undetected underlying conditions (Embury 1994; Wintrobe and Lee 1999).

1.6.2 Sickle-Cell Trait and Falciparum Malaria

The first connection between malaria and sickle-cell trait occurred during 1946 and 1947 in Northern Rhodesia where Dr. E. A. Beet noted in two separate communities that malaria parasites occurred less frequently in blood films from individuals with HbAS (Embury 1994). Soon following in 1954 Dr. A. C. Allison, a British physician, observed that the HbS gene was highest in the areas of greatest malaria transmission, HbAS individuals developed less malaria with milder severity than those without HbAS, and parasite density was greatly reduced in HbAS individuals (Serjeant 1992; Embury 1994). This sparked a debate, as many researchers could not replicate Dr. Allison's results, or had contradictory findings. However, Dr. Allison is credited with having been the first investigator to link HbAS with a protective effect against malaria.

Sickle-cell trait affords carriers a 60%-90% protection against acute uncomplicated malaria, severe malaria, and malaria mortality (Allison 1954c; Raper 1955; Friedman 1978; Marsh *et al.* 1989; Greenwood *et al.* 1991; Hill *et al.* 1991; Aluoch 1997; Ntoumi *et al.* 1997a; Lell *et al.* 1999; Stirnadel *et al.* 1999; Aidoo *et al.* 2002). However, the mechanism(s) of protection is poorly understood, although several studies have been carried out to elucidate the protective pathway(s). One such study demonstrated a reduction in intracellular parasite growth due to the hypoxic environment of the sickle trait RBC. It is believed that the invading parasite's growth and development can be inhibited by this oxygen deprivation resulting in a break-down of the parasite's ability to carry out metabolic functions (Friedman 1978). Additionally, enhanced phagocytosis of the parasitized sickle trait RBC by monocytes has been hypothesized to modulate disease severity. When the hypoxic HbAS RBC is invaded by a parasite, oxidative stress is two-fold; one from the host's genetics and a second from the parasite's break-down of cellular Hb, causing the RBC to sickle (Ayi *et al.* 2004). This phenotypic change of the sickle

trait RBC causes an aggregation of band 3 protein, located on the RBC membrane, which then binds free iron and hemichromes. Band 3 protein aggregation causes the increased binding of IgG to the band 3 cluster on the RBC surface that leads to increased phagocytosis (Ayi *et al.* 2004). Furthermore, higher levels of antibodies to malarial antigens in peripheral blood mononuclear cells (PBMC) from children with HbAS, as compared to HbAA, were found in the Sudan (Bayoumi *et al.* 1990; Abu-Zeid *et al.* 1992b) and in The Gambia (Marsh *et al.* 1989). In Nigeria, HbAS children had higher plasma concentrations of IgG, IgM, and IgA than wild type children (Odegbemi and Williams 1995). Finally, HbAS children had higher levels of IgG3 and IgG2 in plasma samples whereas only IgG3 was detected in children with HbAA. This increase in IgG levels was associated with enhanced pRBC clearance (Ntoumi *et al.* 2002).

1.7 CO-INHERITANCE OF -$\alpha^{3.7}$ AND HBAS AND THE EFFECT ON FALCIPARUM MALARIA

Recent studies were conducted to investigate the co-inheritance of -$\alpha^{3.7}$ and HbAS and their subsequent protection from falciparum malaria (Williams *et al.* 2005c; Urban *et al.* 2006; Wambua *et al.* 2006a). In Kilifi, Kenya, recent reports documented that -$\alpha^{3.7}$ and HbAS co-inherited nullified each gene's protective qualities, leaving these children as susceptible to falciparum malaria as wild type children (Williams *et al.* 2005c; Urban *et al.* 2006). However, the available population with -$\alpha^{3.7}$ and HbAS was small in both studies (n=37 within the Williams study; and n=24 within the Urban study), therefore, these results may not be an accurate representation of the effects of co-inheritance of these hemoglobinopathies on falciparum malaria. In the same study population, Wambua *et al*., investigated the hematological

characteristics of co-inheritance, and noted that the HbAS genotype ameliorated some of the $-\alpha^{3.7}$ hematological abnormalities by reducing microcytosis, and hypochromia. Furthermore, the co-inheritance of both disorders resulted in the loss of malarial protection, possibly due to the interaction of the altered α and β chains (Wambua *et al.* 2006a). The α chains have a high affinity for normal β chains, which is greatly enhanced in thalassemia due to the lack of normal α chains, that can reduce the cellular HbS concentration and ameliorate protection. In summary, co-inheritance of $-\alpha^{3.7}$ and HbAS and the subsequent effect on falciparum malaria is not well understood and requires further validation.

2.0 HYPOTHESES AND OBJECTIVES

The strongest known selective pressure upon the human genome is malaria – a disease endemic to tropical and sub-tropical regions of the world where it kills at least two million children annually (WHO 2000; Stevenson and Riley 2004; Struik and Riley 2004; Kwiatkowski 2005). *Plasmodium falciparum* is the major cause of malaria-related morbidity and mortality, particularly in children less than five years of age within malaria endemic regions (WHO 2000; Breman *et al.* 2001; Rowe *et al.* 2006). Host defense mechanisms against *P. falciparum* and the molecular determinates of pathogenesis are complex and not fully understood. Human genetic factors, such as erythrocytic polymorphisms, $-\alpha^{3.7}$ and HbAS, can influence the disease severity and outcomes (Allison 1954c; Migot-Nabias *et al.* 2000; Mockenhaupt *et al.* 2004; Williams *et al.* 2005a; Williams *et al.* 2005b; Williams *et al.* 2005c; Williams *et al.* 2005c; Verra *et al.* 2006; Wambua *et al.* 2006a; Williams 2006a; Wambua *et al.* 2006b; WHO April 24, 2006).

These polymorphisms are believed to have arisen from evolutionary selection on the human genome in endemic malarial populations (Kwiatkowski 2005). However the mechanism(s) of protection that affords $-\alpha^{3.7}$ and HbAS individuals against malaria is unknown. Therefore, the role of $-\alpha^{3.7}$ and HbAS, in the protection of *P. falciparum* malaria morbidity and mortality was investigated.

2.1 SPECIFIC AIM 1

To determine if the -$\alpha^{3.7}$ polymorphism is associated with protection against severe malarial anemia in children residing in a holoendemic P. falciparum region.

Hypothesis: ***Children with the -$\alpha^{3.7}$ genotype will have reduced prevalence of severe malarial anemia and fatal malaria.***

To investigate the role of the α-thalassemia 3.7 kb deletion in children with falciparum malaria, finger-prick blood was drawn from children residing in a holoendemic *P. falciparum* transmission region of western Kenya. Genomic DNA was isolated from blood spots obtained from each participant (n=48), followed by α-thalassemia genotyping utilizing four different protocols.

2.2 SPECIFIC AIM 2

To determine if sickle-cell trait (HbAS) is associated with protection against severe malarial anemia in children residing in a holoendemic P. falciparum region by reducing the overall burden of parasitemia.

Hypothesis: ***Carriage of HbAS protects against severe malaria by reducing parasitemia, which results in decreased severe malarial anemia.***

To investigate the role of HbAS in children with falciparum malaria, finger-prick blood was obtained from children residing in a holoendemic *P. falciparum* transmission region of western Kenya. Children with a positive blood smear for *P. falciparum* and negative for HIV-1 and bacterial infections were included in the study. Study participants (n=467) were genotyped by Hb electrophoresis to determine HbAS status and placed into appropriate genotypic groups (HbAA=399; HbAS=68). To identify individuals with hematological abnormalities, complete blood counts were performed, along with parasite density and absolute counts of pigment containing monocytes (PCM) and neutrophils (PCN).

3.0 MATERIALS AND METHODS

3.1 STUDY LOCATION AND PARTICIPANTS

This study was conducted as part of an on-going prospective study of SMA in children (n=822, aged 3-36 months) at Siaya District Hospital in western Kenya. This area is holoendemic for *P. falciparum* infections with perennial transmission (Bloland *et al.* 1999a). Malaria anemia (MA; Hb <8.0 g/dL) and hyperparasitemia (≥50,000 parasites/μL) are the most common presentations of malaria, with cerebral malaria rarely occurring (Ong'echa *et al.* 2006). Residents of this area receive 100-300 infective mosquito bites per annum (Bloland *et al.* 1999a) and there is little seasonal variation in parasite prevalence and density in the region (Beier *et al.* 1994). The population in this area is socially and culturally homogeneous, with greater than 99% of the study participants being Luo (Bloland *et al.* 1999a). All blood samples were obtained prior to treatment with antimalarials and/or antipyretics. Routine clinical evaluations and laboratory measures were used to evaluate the patients. Children with malaria were given antimalarials and the appropriate supportive therapy according to Kenya Ministry of Health guidelines.

Informed consent was obtained from the parents/guardians of participating children. The study was approved by the ethics committees of the University of Pittsburgh Investigational Review Board and the Kenya Medical Research Ethical Review Board.

3.2 α-THALASSEMIA 3.7 KB GENOTYPE STUDY DESIGN

Genomic DNA from Tahitian subjects that were heterozygous or homozygous for the α-thalassemia 3.7 kb deletion were used as positive controls and supplied by Dr. Jeremy Martinson of the University of Pittsburgh, Graduate School of Public Health, Department of Infectious Diseases and Microbiology.

3.2.1 Chong Protocol for -$\alpha^{3.7}$ Genotyping

In 2000, a protocol for the PCR amplification of α-thalassemia was published from a group at John Hopkins University (Chong *et al.* 2000a; Chong *et al.* 2000b). Consistent with this protocol, a total reaction volume of 50 μL was carried out with 100 ng of genomic DNA, 1 mol/L Betaine (Sigma, St. Louis, MO; Cat#B-300), 0.2 mmol/L of each dNTP (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 2.5 U of Platinum® *Taq* (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1.5 mmol/L of $MgCl_2$ (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1X PCR buffer (Invitrogen™, Gaithersburg, MD; Cat#55082-5), and 200 nM per primer. Primer sequences were identical to those in the published manuscript (Table 1) (Chong *et al.* 2000a; Chong *et al.* 2000b). Amplification was performed in a PTC-100 thermacycler (MJ Research, Waltham, MA) with an initial heat inactivation step of 5 minutes at 95°C, followed by 30 cycles of denaturation at 97°C for 45 seconds, annealing at 60°C for 1 minute 15 seconds, extension at 72°C for 2 minutes 30 seconds, and a final extension at 72°C for 5 minutes. A total volume of 7-15 μL of PCR product and 5 μL of 1 kb ladder (Invitrogen™, Gaithersburg, MD; Cat#10488-085) was electrophoresed on an ethidium bromide stained 1% agarose gel (Invitrogen™,

Gaithersburg, MD; Cat#15510-027) in 1X Tris-Base-EDTA (TBE) for 1 hour and visualized on an ultraviolet transilluminator (Eagle Eye, Stratagene, La Jolla, CA).

3.2.2 Liu Protocol for -$\alpha^{3.7}$ Genotyping

Another paper was published in 2000 with a protocol for the PCR amplification of α-thalassemia from a group at Oxford University (Liu *et al.* 2000). Consistent with this protocol, a total reaction volume of 25 μL was carried out with 100 ng of genomic DNA, 0.75 mol/L of Betaine (Sigma, St. Louis, MO; Cat#B-300), 5% DMSO (Sigma, St. Louis, MO; Cat#D2650), 200 mmol/L of dNTPs (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1.25 U of Platinum® *Taq* (Invitrogen™, Gaithersburg, MD; Cat#10966-034), 1.5 mmol/L $MgCl_2$ (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1X PCR buffer (Invitrogen™, Gaithersburg, MD; Cat#10966-034), 300 nM forward primer, and 100 nM reverse 1 and 2 primers. Primer sequences were were identical to those in the published manuscript (Table 1) (Liu *et al.* 2000). Amplification was performed in a PTC-100 thermacycler (MJ Research, Waltham, MA) with an initial heat inactivation step of 15 minutes at 95°C, followed by 35 cycles of denaturation at 98°C for 1 minute, annealing at 65°C for 1 minute, extension at 78°C for 2 minutes 30 seconds, and a final extension of 72°C for 10 minutes. A total volume of 7-15 μL of PCR product and 5 μL of 1 kb ladder (Invitrogen™, Gaithersburg, MD; Cat#10488-085) was electrophoresed on an ethidium bromide stained 1% agarose gel (Invitrogen™, Gaithersburg, MD; Cat#15510-027) in 1X TBE for 1 hour and visualized on an ultraviolet transilluminator (Eagle Eye, Stratagene, La Jolla, CA).

3.2.3 GC-Rich PCR System for -$\alpha^{3.7}$ Genotyping

The GC-Rich PCR System is a propriety kit from Roche which enables the amplification of GC heavy regions by preventing DNA secondary structures. A total volume of 35 µL of master mix 1 containing 100 ng of DNA, 200 µM of each dNTP (Invitrogen™, Gaithersburg, MD; Cat#55082-5), varying concentration of 0-2 M of GC-Rich Resolution Solution (Roche Diagnostics, Mannheim, Germany; Cat#12657421), and 200 nM of each Chong and Liu primer was prepared (Table 1) (Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b). Master mix 2 had a total volume of 15 µL and was comprised of 1X GC-Rich reaction buffer (Roche Diagnostics, Mannheim, Germany; Cat#12657421), and 2 U of GC-Rich enzyme mix (Roche Diagnostics, Mannheim, Germany; Cat#12657421). For each reaction, a total volume of 50 µL (35 µL of master mix 1 and 15 µL of master mix 2 combined on ice and mixed well) was placed in the PTC-100 thermacycler (MJ Research, Waltham, MA) immediately. The amplification protocol was as follows: an initial heat inactivation step of 3 minutes at 95^{o}C, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 65^{o}C for 30 seconds, extension at 72^{o}C for 45 seconds with the addition of 5 seconds to the extension step per cycle, and a final extension of 72^{o}C for 7 minutes. A total volume of 7-15 µL of PCR product and 5 µL of 1 kb ladder (Invitrogen™, Gaithersburg, MD; Cat#10488-085) was electrophoresed on an ethidium bromide stained 1% agarose gel (Invitrogen™, Gaithersburg, MD; Cat#15510-027) in 1X TBE for 1 hour and visualized on an ultraviolet transilluminator (Eagle Eye, Stratagene, La Jolla, CA).

3.2.4 PCR Enhancer System for -$\alpha^{3.7}$ Genotyping

The PCR Enhancer System is a propriety kit from Invitrogen™ which enables the amplification of GC rich regions by preventing DNA secondary structures. A total reaction volume of 25 μL containing 100 ng of genomic DNA, 800 μM of dNTPs (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1.25 U of Platinum® *Taq* (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1.5 mM of $MgSO_4$ (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1X PCR buffer (Invitrogen™, Gaithersburg, MD; Cat#55082-5), 1.5X Enhancer Solution (Invitrogen™, Gaithersburg, MD; Cat#55082-5), Chong and Liu forward, reverse 1, and reverse 2 deletional primers at 600 nM, 200 nM, and 200 nM, respectively (Table 1) (Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b). Amplification was performed in a PTC-100 thermacycler (MJ Research, Waltham, MA) with an initial heat inactivation step of 15 minutes at 95^{o}C, followed by 35 cycles of denaturation at 95^{o}C for 1 minute, annealing at 65^{o}C for 1 minute, extension at 72^{o}C for 2 minutes 30 seconds, and a final extension of 72^{o}C for 10 minutes. A total volume of 7-15 μL of PCR product and 5 μL of 1 kb ladder (Invitrogen™, Gaithersburg, MD; Cat#10488-085) was electrophoresed on an ethidium bromide stained 1% agarose gel (Invitrogen™, Gaithersburg, MD; Cat#15510-027) in 1X TBE for 1 hour and visualized on an ultraviolet transilluminator (Eagle Eye, Stratagene, La Jolla, CA).

3.3 SICKLE-CELL STUDY DESIGN

Samples for this study were obtained from children (n=467) from the Siaya District Hospital in western Kenya. Since previous studies carried out in the same study population

demonstrated that HIV-1 status is an important determinant of malarial anemia severity (Otieno *et al.* 2006), all children were tested for HIV-1 seropositivity and HIV-1 infection. Children with a positive HIV-1 PCR test were subsequently removed from analyses. Pre- and post-HIV test counseling was provided for the parents/legal guardians of all participants. Additionally, bacterial cultures were performed on all children. Children positive for bacteremia were also excluded from analyses as previous studies have demonstrated that bacteremia can aggravate SMA (Brent *et al.* 2006). Informed consent was obtained from the parent/guardians of participating children and study section was approved as previously described in section 3.1.

3.3.1 Clinical Definitions

Fever was defined by axillary temperature >37.5^{0}C. Although the World Health Organization (WHO) defines SMA as Hb<5.0 g/dL (WHO 2000), childhood SMA in western Kenya is more appropriately defined as Hb<6.0 g/dL. This modified definition of SMA is based on over 14,000 repeated Hb measurements in children from birth until four years of age in an age- and geographically matched reference population in western Kenya (McElroy *et al.* 1999), High density parasitemia (HDP) was defined as ≥10,000 parasites/μL of blood and was based on standard definitions (Aidoo *et al.* 2002; Hobbs *et al.* 2002; Awandare *et al.* 2006; Ong'echa *et al.* 2006; Otieno *et al.* 2006).

3.3.2 Laboratory Procedures

Malaria diagnosis was performed using finger-prick blood on thin and thick blood smears stained with 3% Giemsa. Asexual malaria trophozoites were counted against 300 leukocytes

based on absolute counts of white blood cells/μL of blood, while parasite density was estimated as follows: parasites/μL = WBC count/μL x trophozoites/300. A total of 30 monocytes and 100 neutrophils were examined per slide and the number of pigment-containing monocytes (PCM) or pigment-containing neutrophils (PCN) were expressed as a percentage of the total number of monocytes and neutrophils (Nguyen *et al.* 1995; Lyke *et al.* 2003). Hb levels were determined using a HemoCue system (HemoCue AB, Angelholm, Sweden). Complete blood counts were performed by an automated hematology analyzer (Beckman Coulter® AcT diff2™, Beckman-Coulter Corporation, Miami, USA), while bacteremia was determined using the Wampole ISOLATOR 1.5-ml microbial system (Inverness Medical). Reticulocyte counts were determined from thin blood films stained with new methylene blue. Reticulocyte index (RI) was calculated as reticulocyte count (%) x (hematocrit/0.36) (Were *et al.* 2006). Reticulocyte production indices (RPI) was calculated as RI/maturation factor (MF), where MF=b + (m)(x), b=1, m=0.05, and x=(average normal population hematocrit – patient's hematocrit) (Were *et al.* 2006). The standard hematocrit (Hct) used to calculate RPI in western adults is 45%. However, the standard Hct in our calculations was 36%, since this value was age- and geographically matched by calculating the average Hct value in a cohort of non-anemic (Hb >11.0 g/dL), aparasitemic children in western Kenya (n=107) (Were *et al.* 2006). HIV-1 status was determined using two serological antibody tests (i.e., Unigold™ and Determine™) and HIV-1 DNA PCR analysis according to our previously published methods (Otieno *et al.* 2006). Trimethoprim-sulfamethoxazole was administered to all children that were positive for one or both serological tests.

3.3.3 Hemoglobin Electrophoresis

Determination of Hb variants was performed using an alkaline cellulose acetate electrophoresis. Bands representing different variants were visualized after staining with Ponceau S (Helena BioSciences, Beaumont, TX, USA) and results compared with those of the control. Titan III® cellulose acetate plates and the AFSC control were used according to the manufacturer's instructions (Helena BioSciences, Beaumont, TX, USA), while red blood cell hemolysate was prepared from whole blood.

3.3.4 Statistical Analysis

Statistical analysis of data was performed using the software Minitab v. 14.0 (Minitab Inc., State College, PA) and SPSS v. 15.0 (SPSS Inc., Chicago, IL). Proportional data were compared using χ^2-test, geomeans between the two groups were compared using Paired *t* test, and Mann-Whitney U tests were used to compare medians between the two groups. Statistical significance was set at $P \leq 0.05$. Logistical regression controlling for the confounding effect of age and gender was used to examine the association of different genotypes with susceptibility to SMA and HDP.

4.0 RESULTS

4.1 SPECIFIC AIM 1: TO DETERMINE IF THE $-\alpha^{3.7}$ POLYMORPHISM IS ASSOCIATED WITH PROTECTION AGAINST SEVERE MALARIAL ANEMIA IN CHILDREN RESIDING IN A HOLOENDEMIC *P. FALCIPARUM* REGION

4.1.1 $-\alpha^{3.7}$ Genotyping

Table 2 illustrates the different PCR fragments that are expected for each genotype per protocol primer sequence (Table 1). Thirteen randomly selected Kenyan genomic DNA samples were amplified utilizing the Chong protocol and electrophoresed on a 1% agarose gel (Chong *et al.* 2000a). Only twelve of the thirteen samples demonstrated banding at 1800 bp (Figure 9), which is indicative of the normal band for this primer set (Table 2). Additionally, one Kenyan sample failed to produce any visually detectable band(s). There was an absence of the deletional band at 2000 bp in all twelve samples. Furthermore, nine Tahitian genomic DNA samples were amplified utilizing the same protocol (Figure 10). Only two of the nine samples yielded results demonstrating the normal band at 1800 bp.

In Figure 12, three Kenyan genomic DNA samples and one University of Pittsburgh PCR positive control DNA sample (Pittsburgh control) were amplified using the Liu protocol (Liu *et al.* 2000). The Pittsburgh control demonstrated banding at 2200 bp representing the normal

genotype for this primer sequence (Table 2). However, the three amplified Kenyan DNA samples did not yield any banding and/or smears (Figure 11).

These results demonstrate that the Chong protocol and primer set could amplify the normal band when used with the Kenyan DNA, but when attempting to amplify the Tahitian samples, only two of the nine samples yielded the normal band. Additionally, the Liu protocol and primer set did not yield any banding for the Kenyan samples; however the Pittsburgh control sample demonstrated the normal band at 2200 bp.

Table 1: *-$a^{3.7}$ Primer Sequences*

Primer Name	Primer Sequence
Chong Forward	CCC CTC GCC AAG TCC ACC C
Chong Reverse 1	AGA CCA GGA AGG GCC GGT G
Chong Reverse Deletional	AAA GCA CTC TAG GGT CCA GCG
Liu Forward	AAG TCC ACC CCT TCC TTC CTC ACC
Liu Reverse 1	ATG AGA GAA ATG TTC TGG CAC CTG CAC TTG
Liu Reverse Deletional	TCC ATC CCC TCC TCC CGC TGC CTT TTC

Primer Sequences based on published protocols (Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b).

Table 2: *-$a^{3.7}$ Genotype Expected Band Sizes*

Primer Set	Genotype αα / αα	Genotype αα / α-
Chong	1800 bp	1800 bp & 2000 bp
Liu	2200 bp	2200 bp & 2000 bp

Expected banding for each genotype according to published protocols (Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b).

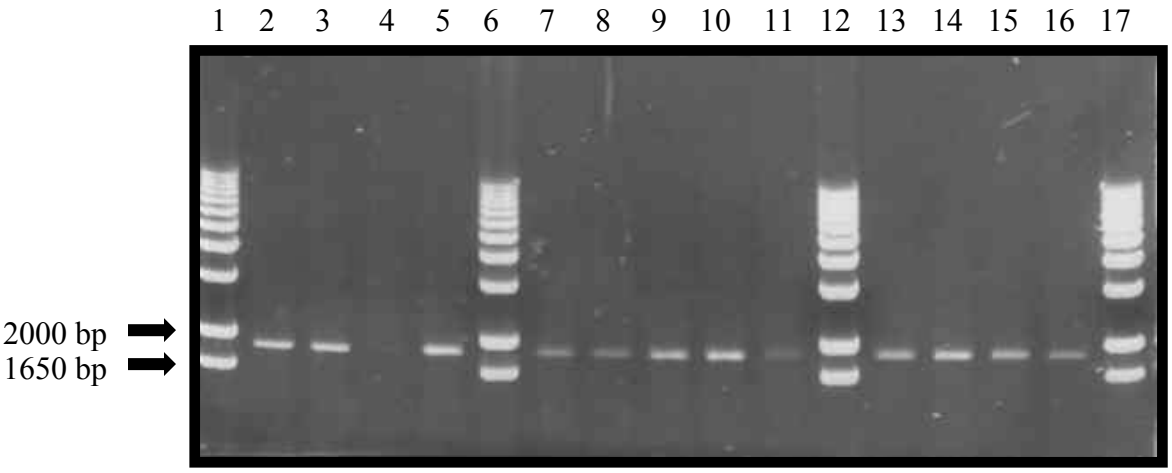

Figure 9: ***Agarose Gel of Kenyan Genomic DNA using the Chong Protocol***

Thirteen Kenyan genomic DNA samples were amplified utilizing the Chong protocol. The amplified product was then electrophoresed on a 1% agarose gel. Only twelve of the thirteen samples demonstrated banding at 1800 bp, which is indicative of the normal band for this primer set.

Gel Legend: Lane 1, 1 kb Ladder; Lane 2, Kenyan Genomic DNA #35; Lane 3, Kenyan Genomic DNA #36; Lane 4, Kenyan Genomic DNA #37; Lane 5, Kenyan Genomic DNA #39; Lane 6, 1 kb Ladder; Lane 7, Kenyan Genomic DNA #40; Lane 8, Kenyan Genomic DNA #41; Lane 9, Kenyan Genomic DNA #43; Lane 10, Kenyan Genomic DNA #44; Lane 11, Kenyan Genomic DNA #45; Lane 12, 1 kb Ladder; Lane 13, Kenyan Genomic DNA #46; Lane 14, Kenyan Genomic DNA #48; Lane 15, Kenyan Genomic DNA #50; Lane 16, Kenyan Genomic DNA #51; Lane 17, 1 kb Ladder.

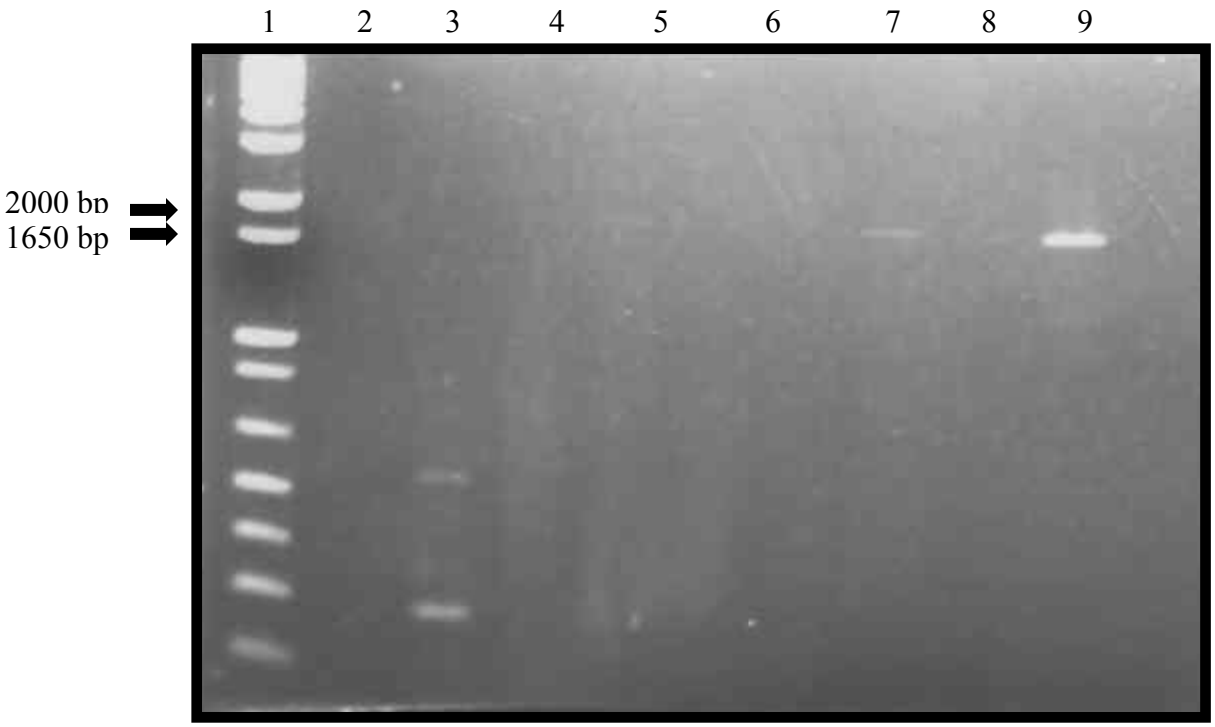

Figure 10: ***Agarose Gel of Tahitian Genomic DNA using the Chong Protocol***

Nine Tahitian genomic DNA samples were amplified utilizing the Chong protocol. The amplified product was then electrophoresed on a 1% agarose gel. Only two of the nine samples yielded results demonstrating the normal band at 1800 bp.

Gel Legend: Lane 1, 1 kb Ladder; Lane 2, Tahitian Homozygous Genomic DNA #460; Lane 3, Tahitian Heterozygous Genomic DNA #408; Lane 4, Tahitian Heterozygous Genomic DNA #412; Lane 5, Tahitian Heterozygous Genomic DNA #421; Lane 6, Tahitian Heterozygous Genomic DNA #433; Lane 7, Tahitian Heterozygous Genomic DNA #434; Lane 8, Tahitian Heterozygous Genomic DNA #435; Lane 9, Tahitian Heterozygous Genomic DNA #535.

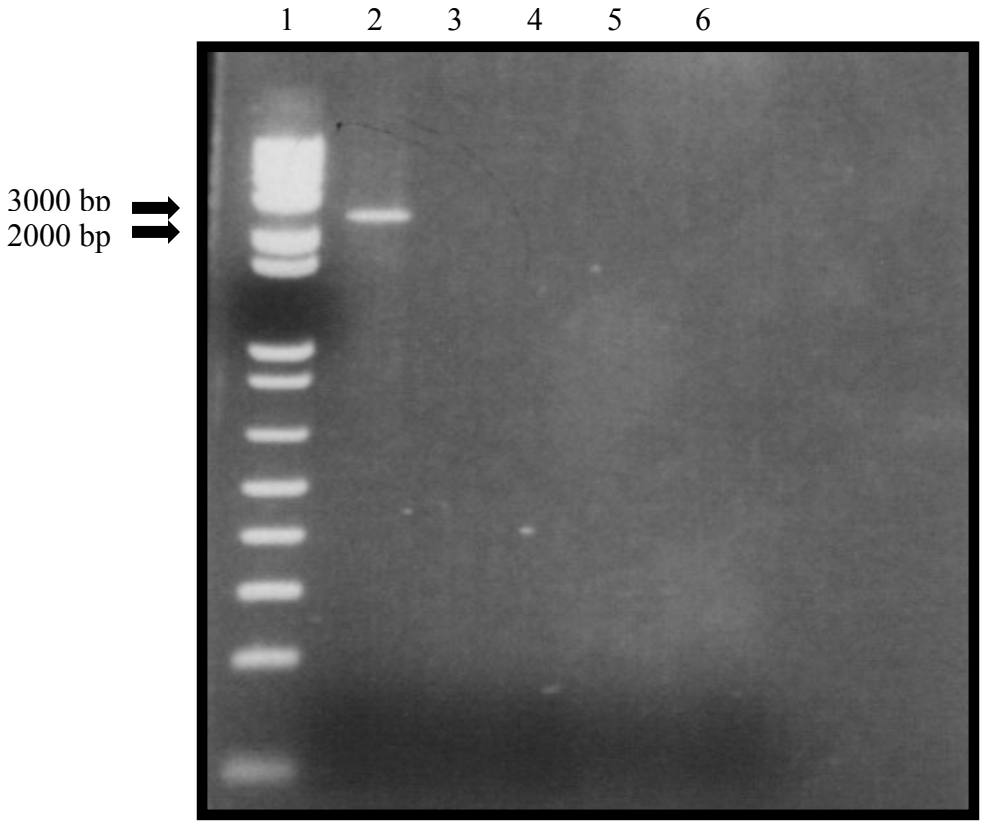

Figure 11: ***Agarose Gel of Kenyan Genomic DNA using the Liu Protocol***

Three Kenyan genomic DNA samples and one Pittsburgh control were amplified using the Liu protocol. The amplified products were then electrophoresed on a 1% agarose gel. The Pittsburgh control demonstrated banding at 2200 bp representing the normal genotype for this primer sequence. The three amplified Kenyan DNA samples did not yield any banding and/or smears.

Gel Legend: Lane 1, 1 kb Ladder; Lane 2, Pittsburgh Control; Lane 5, Negative Control; Lane 4, Kenyan Genomic DNA #10; Lane 5, Kenyan Genomic DNA #11; Lane 4, Kenyan Genomic DNA #12.

4.1.2 -$\alpha^{3.7}$ Specialty Kit Genotyping

The GC-Rich kit from Roche Diagnostics and PCR Enhancer System from Invitrogen™ are propriety kits designed to aid in the amplification of GC rich regions during PCR. Their priority solution is designed to linearize the GC regions preventing secondary structures and uniform amplification.

Using the Chong and Liu primer sequences (Table 1), Tahitian genomic DNA, Kenyan genomic, and Repli-G® samples (whole genome amplified DNA) were amplified. The Pittsburgh controls demonstrated consistent banding at 1800 bp for the normal band with the Chong primer sequence, while the Tahitian Repli-G® and Kenyan genomic DNA samples showed no visible banding. However, the Kenyan Repli-G® DNA samples showed bright and thick smears (Figure 12). Utilizing the Liu primer sequence, the Pittsburgh controls demonstrated normal banding at 2200 bp, while Tahitian genomic DNA homozygous sample #460 demonstrated a single band at approximately 2000 bp (Figure 13), hence showing the expected single deletion band. Additionally, three samples of Tahitian Repli-G® DNA were represented as smears with no distinct banding (Figure 13). Finally, the four heterozygous Tahitian genomic DNA samples did not produce clear banding, and only smears resulted (Figure 13).

In Figure 14, the Chong primer sequence was used with three Pittsburgh controls, which demonstrated the normal band at 1800 bp, while the four Tahitian genomic samples did not display any banding. In Figure 15, the Liu primer sequence was used with three Pittsburgh controls and four Kenyan genomic DNA samples. All three Pittsburgh controls displayed banding at 2200 bp representing the normal genotype. However, the four Kenyan genomic DNA samples did not exhibit any banding patterns or smears. Furthermore, in Figure 16 using the Liu

primer sequence, one Pittsburgh control demonstrated the normal genotype banding at 2200 bp but three Tahitian heterozygous genomic DNA samples did not exhibit any banding, although Tahitian sample #410 presented with a large smear.

Taken together, these results demonstrate that the specialty GC-rich PCR kits, GC-Rich, and PCR Enhancer have a high variability in the amplification of DNA samples. The GC-Rich kit was able to amplify the normal bands of two genomic Tahitian samples, two Pittsburgh controls, and the deletional band for a Tahitian homozygote. The PCR Enhancer kit amplified the normal band for the Pittsburgh controls with both primer sequences, but none of the Tahitian DNA samples, using the identical Tahitian samples between the two assays.

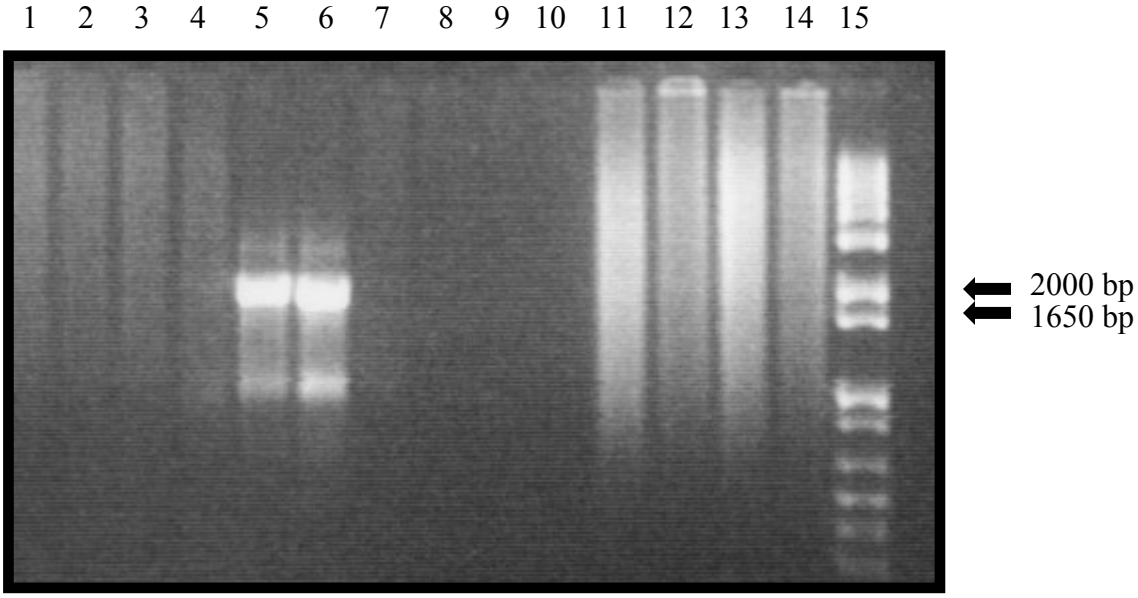

Figure 12: ***Agarose Gel of Tahitian Repli-G®, Kenyan Genomic and Repli-G® DNA using the GC-Rich PCR Kit and Chong Primer Sequence***

Two University of Pittsburgh controls, four Tahitian Repli-G®, four Kenyan Repli-G® and genomic DNA samples were utilized with the GC-Rich kit in conjunction with the Chong primer sequence. The amplified products were then electrophoresed on a 1% agarose gel. The Pittsburgh controls demonstrated banding at 1800 bp for the normal band while the Tahitian Repli-G® and Kenyan genomic DNA showed no visible banding and the Kenyan Repli-G® DNA showed bright and thick smears.

Gel Legend: Lane 1, Tahitian Homozygous Repli-G® #460; Lane 2, Tahitian Heterozygous Repli-G® #435; Lane 3, Tahitian Heterozygous Repli-G® #408; Lane 4, Tahitian Heterozygous Repli-G® #410; Lane 5, Pittsburgh Control #627; Lane 6, Pittsburgh Control #629; Lane 7, Kenyan Genomic DNA #40; Lane 8, Kenyan Genomic DNA #41; Lane 9, Kenyan Genomic DNA #43; Lane 10, Kenyan Genomic DNA #44; Lanes 11, Kenyan Repli-G® #40; Lanes 12, Kenyan Repli-G® #41; Lanes 13, Kenyan Repli-G® #43; Lanes 14, Kenyan Repli-G® #44; Lanes 15, 1 kb Ladder.

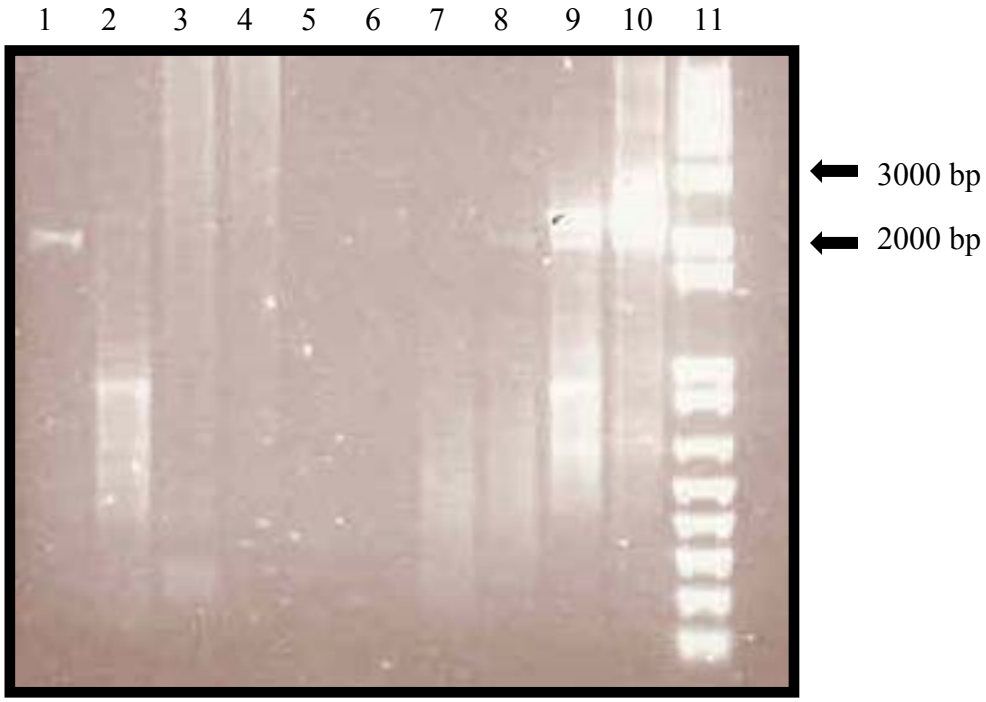

Figure 13: ***Agarose Gel of Tahitian Genomic and Repli-G® DNA using the GC-Rich PCR Kit and Liu Primer Sequence***

Two Pittsburgh controls, four Tahitian Repli-G® and genomic DNA samples were utilized with the GC-Rich kit in conjunction with the Liu primer sequence. The amplified products were then electrophoresed on a 1% agarose gel. The two Pittsburgh controls demonstrated normal banding at 2200 bp while one Tahitian homozygous sample demonstrated a single band at approximately 2000 bp. Three of the four Tahitian Repli-G® DNA samples presented as smears with no distinct banding.

Gel Legend: Lane 1, Tahitian Homozygous Genomic DNA #460; Lane 2, Tahitian Heterozygous Genomic DNA #435; Lane 3, Tahitian Heterozygous Genomic DNA #408; Lane 4, Tahitian Heterozygous Genomic DNA #410; Lane 5, Tahitian Homozygous Repli-G® #460; Lane 6, Tahitian Repli-G® #435; Lane 7, Tahitian Heterozygous Repli-G® #408; Lane 8, Tahitian Heterozygous Repli-G® #410; Lane 9, Pittsburgh Control #627; Lane 10, Pittsburgh Control #629; Lane 11, 1 kb Ladder.

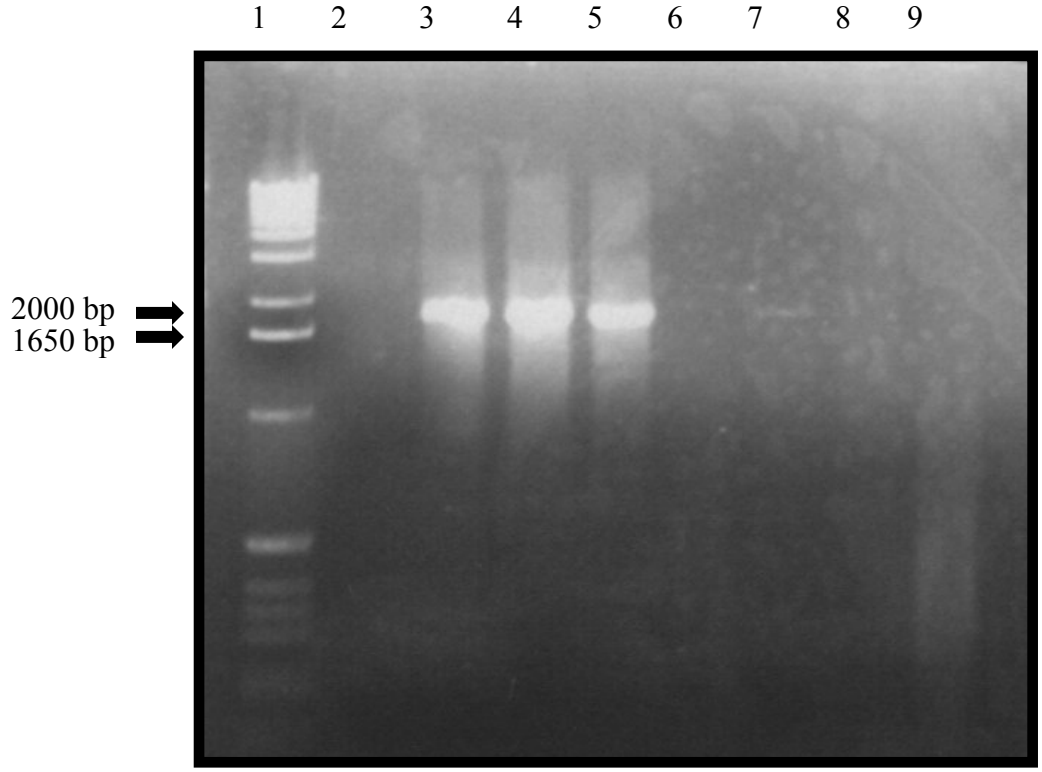

Figure 14: ***Agarose Gel of Tahitian Genomic DNA using the PCR Enhancer System and Chong Primer Sequence***

Three Pittsburgh controls and four Tahitian genomic DNA samples were amplified with the PCR Enhancer System in conjunction with the Chong primer sequence. The amplified products were then electrophoresed on a 1% agarose gel. The three Pittsburgh controls demonstrated the normal banding at 1800 bp while the four Tahitian genomic samples did not display any banding.

Gel Legend: Lane 1, 1 kb Ladder; Lane 2, Negative Control; Lane 3, Pittsburgh Control #631; Lane 4, Pittsburgh Control #627; Lane 5, Pittsburgh Control #629; Lane 6, Tahitian Homozygous Genomic DNA #460; Lane 7, Tahitian Heterozygous Genomic DNA #408; Lane 8, Tahitian Heterozygous Genomic DNA #412; Lane 9, Tahitian Heterozygous Genomic DNA #435.

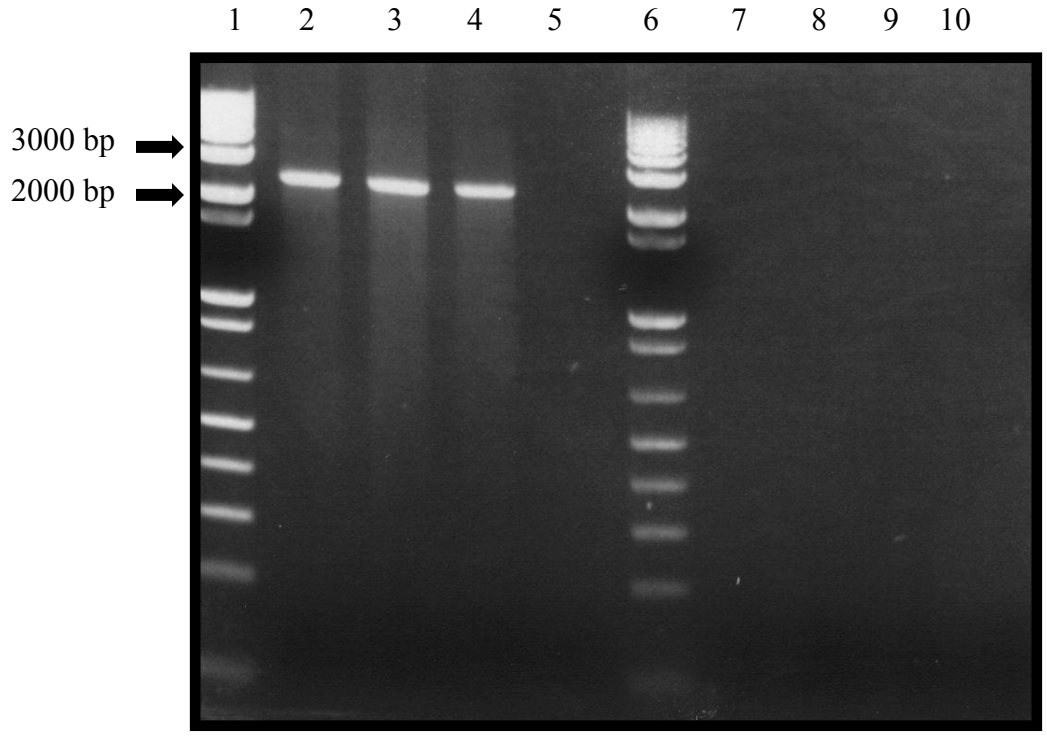

Figure 15: ***Agarose Gel of Kenyan Genomic DNA using the PCR Enhancer System and Liu Primer Sequence***

Three Pittsburgh controls and four Kenyan genomic DNA samples were amplified with the PCR Enhancer System in conjunction with the Liu primer sequence. The amplified products were then electrophoresed on a 1% agarose gel. All three Pittsburgh controls displayed banding at 2200 bp. The four Kenyan genomic DNA samples did not exhibit any banding or smears.

Gel Legend: Lane 1, 1 kb Ladder; Lane 2, Pittsburgh Control #627; Lane 3, Pittsburgh Control #629; Lane 4, Pittsburgh Control #631; Lane 5, Negative Control; Lane 6, 1 kb Ladder; Lane 7, Kenyan Genomic DNA #40; Lane 8, Kenyan Genomic DNA #41; Lane 9, Kenyan Genomic DNA #43; Lane 10, Kenyan Genomic DNA #44.

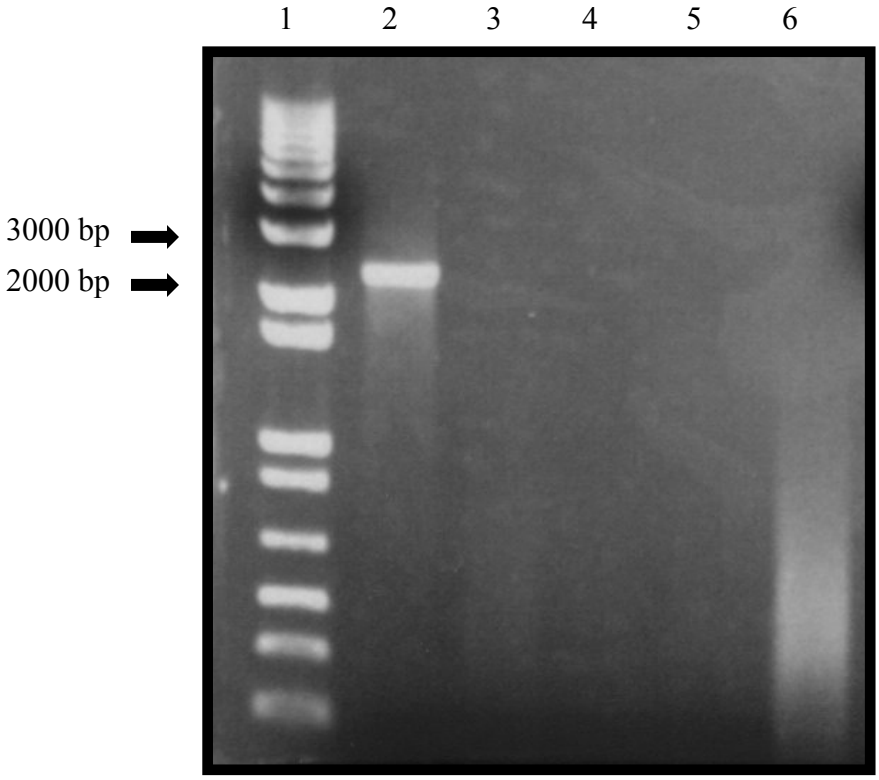

Figure 16: ***Agarose Gel of Tahitian Genomic DNA using the PCR Enhancer System and Liu Primer Sequence***

One Pittsburgh control and four Tahitian genomic DNA samples were amplified with the PCR Enhancer System in conjunction with the Liu primer sequence. The amplified products were then electrophoresed on a 1% agarose gel. The one Pittsburgh control demonstrated the normal genotype banding at 2200 bp. The three Tahitian genomic DNA samples did not exhibit any banding while Tahitian sample #410 presented with a large smear.

Gel Legend: Lane 1, 1 kb Ladder; Lane 2, Pittsburgh Control #627; Lane 3, Tahitian Homozygous Genomic DNA #460; Lane 4, Tahitian Heterozygous Genomic DNA #435; Lane 5, Tahitian Heterozygous Genomic DNA #408; Lane 6, Tahitian Heterozygous Genomic DNA #410.

4.2 SPECIFIC AIM 2: TO DETERMINE IF SICKLE-CELL TRAIT (HBAS) IS ASSOCIATED WITH PROTECTION AGAINST SEVERE MALARIAL ANEMIA IN CHILDREN RESIDING IN A HOLOENDEMIC *P. FALCIPARUM* REGION BY REDUCING THE OVERALL BURDEN OF PARASITEMIA

4.2.1 Demographic and Clinical Characteristics

Previous studies investigating the role of HbAS have demonstrated that after six months of age, maternal protection against malaria wanes and susceptibility to life threatening malarial infection increases (Lell *et al.* 1999; Aidoo *et al.* 2002; Williams *et al.* 2005a; Williams *et al.* 2005b). Additionally, children with HbAS from six months to five years demonstrate protective characteristics, therefore, making children of that age group ideal study participants (Lell *et al.* 1999; Aidoo *et al.* 2002; Williams *et al.* 2005a; Williams *et al.* 2005b). A total of 467 children were genotyped for the sickle-cell trait. When classified into different genotypic groups, 399 individuals were HbAA while 68 individuals were HbAS. Table 3 presents the clinical and demographic characteristics of the study participants. Mann-Whitney U test revealed no significant differences in age (P=0.969) and temperature (P=0.758; Table 3), and a proportionality test revealed no significant differences in the proportion of males vs. females (P=0.075). Since hypoglycemia (<2.2mmol/L) is a common complication of *P. falciparum* infection in endemic areas (Marsh *et al.* 1995; Planche *et al.* 2003; Planche *et al.* 2005), blood glucose levels were measured on all the enrolled children. However, blood glucose levels were comparable between HbAA and HbAS groups (P=0.599; Table 3).

Taken together, these results indicate that these parameters are not the predisposing determinants of malaria disease severity in children with different Hb variants.

Table 3: ***Demographic and Clinical Characteristics***

Characteristics	HbAA	HbAS	*P*
Number of subjects (n)	399	68	
Age (mos.)	10.0 (6.0 – 16.0)	10.00 (6.00 – 17.00)	0.969[a]
Gender			
Male (n, %)	201 (50.4)	42 (61.8)	0.075[b]
Female (n, %)	198 (49.6)	26 (38.2)	0.075[b]
Temperature (°C)	37.5 (36.7 – 38.4)	37.3 (36.8 – 38.3)	0.758[a]
Glucose (mmol/L)	5.0 (4.4 – 5.8)	5.2 (4.7 – 5.8)	0.599[a]

Data presented as medians (Q1-Q3) unless otherwise stated.

[a] Mann-Whitney U test

[b] χ^2 test

4.2.2 Treatment-Seeking Behavior

Elevated host body temperature (fever, >37.5°C) is a common clinical manifestation of malarial disease that leads to seeking of treatment at health facilities (Oakley *et al.* 2007). To determine if the history of malaria was comparable in the two groups, we investigated whether children with HbAS had a comparable fever history to children with HbAA. As such, children were stratified by their genetic group, and the history of fever was determined (Table 4). Children with the HbAS genotype had a similar history of fever as the HbAA children and there were no significant differences between the groups fever histories (no fever history: P=0.960, fever of 1-3 days: P=0.403, fever of 4-14 days: P=0.289, fever greater than 14 days: P=0.607; Table 4). Proportions of children having no fever history in both the HbAA and HbAS groups were also comparable (3.05% for HbAA vs. 2.94% for HbAS). Taken together, these results illustrate that both HbAS and HbAA children have a similar fever history and present to the hospital for treatment with comparable parameters. Thus, differences observed between the two groups is not likely due to one of the groups delaying presentation at the hospital, and therefore in a more advanced state of disease.

Table 4: ***Fever History as an Index of Disease Staging***

Fever History	HbAA (n=393)	HbAS (n=68)	*P*
None (n, %)	12 (3.05)	2 (2.94)	0.960
1 - 3 Days (n, %)	204 (51.91)	39 (57.35)	0.403
4 - 14 Days (n, %)	165 (41.98)	24 (35.29)	0.289
>14 Days (n, %)	12 (3.05)	3 (4.41)	0.607

Data presented as medians (Q1-Q3) unless otherwise stated.

Differences between the proportions were calculated by χ^2 test.

4.2.3 Parasite Burden

Previous studies carried out at a neighboring study site within western Kenya (Aidoo *et al.* 2002) and in the coastal regions of Kenya (Lell *et al.* 1999; Aidoo *et al.* 2002; Williams *et al.* 2005a; Williams *et al.* 2005b) showed that children with the HbAS, genotype have significantly reduced parasite burden in both mild and severe disease. To further investigate this observation, we examined parasite loads in HbAA and HbAS individuals. Results show that relative to the HbAA group, there were reduced peripheral parasitemia levels in the HbAS group; however, this relationship did not reach significance (P=0.202; Figure 17a). Furthermore, the geomean parasitemia in the HbAA group was non-significantly increased compared to the HbAS group (P=0.651; Figure 17b). Since high-density parasitemia (HDP; ≥10,000/μL) is a common clinical presentation in children from this holoendemic area (Ong'echa *et al.* 2006), we determined if the proportion of children with HDP differed among the groups. Figure 17c shows the proportion of children with HDP stratified according to sickle-cell status. A total of 294 children (74.43%) in the HbAA group and 44 children (70.59%) in the HbAS group had HDP (P=0.117; Figure 17c). These results suggest that, despite the lack of statistical significance, differences in the proportions of HDP between HbAA and HbAS groups represent a decreased parasite load in the HbAS group.

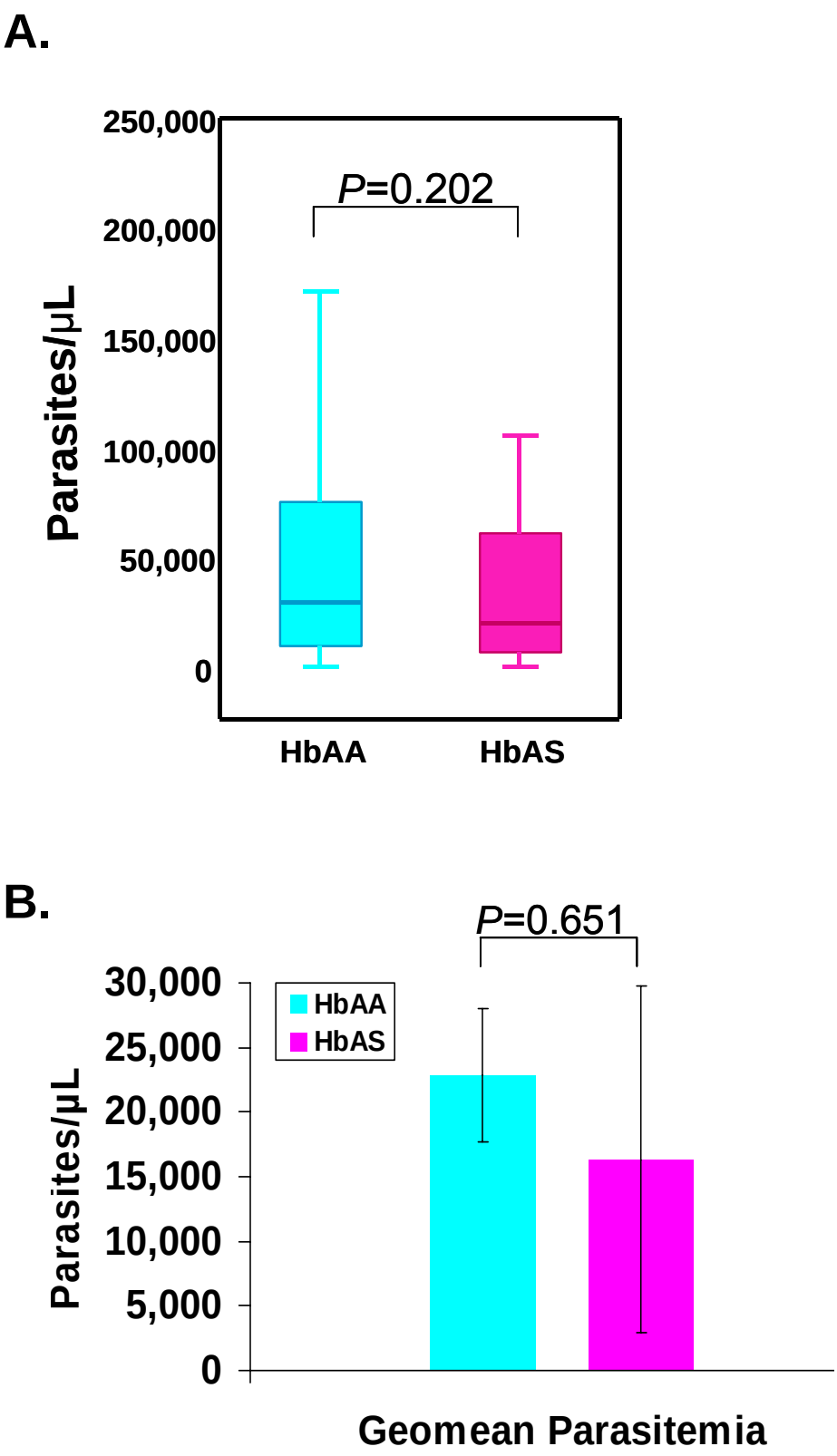

Figure 17: ***(A) Parasitemia and (B) Geomean Parasitemia According to Sickle-Cell Gene Status***

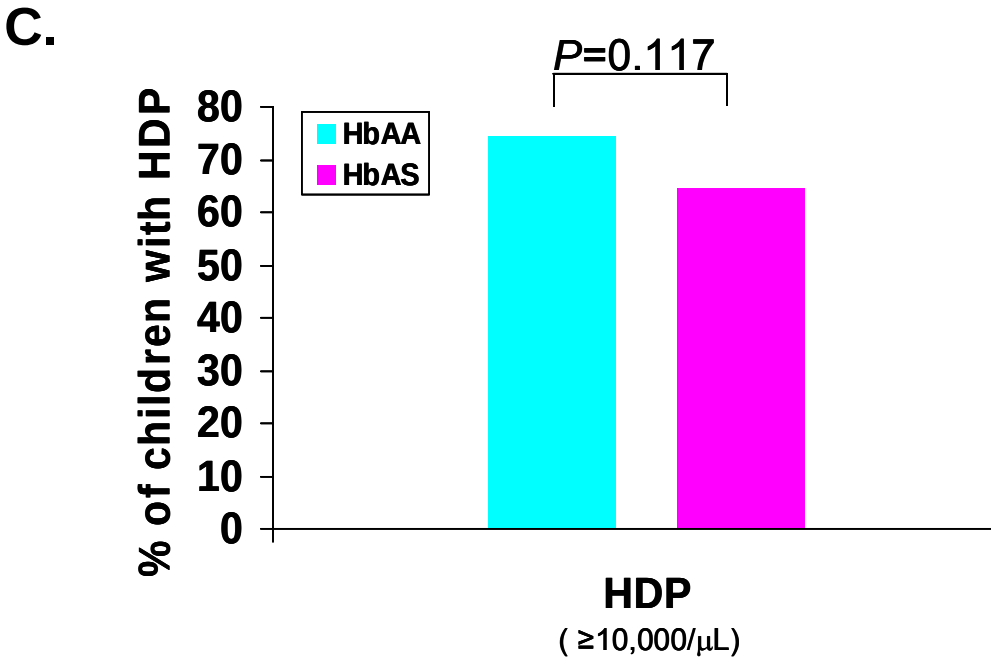

Figure 17: (C) ***Proportion of HDP According to Sickle-Cell Gene Status***

(**A)** Peripheral blood smears were prepared and stained with Giemsa reagent and examined for malaria parasites in children with HbAA (n=395) and HbAS (n=68) genotypes. Data is presented as a box plot where the box represents the interquartile range, the line through the box represents the median, whiskers indicate the 10^{th} and 90^{th} percentiles, and individual symbols are outliers. Differences in parasitemia between HbAA and HbAS genotypes were not found to be statistically significant (P=0.202, Mann-Whitney U test). (**B**) Geomean parasitemia was calculated for HbAA (n=395) and HbAS (n=68) genotypes. Bars represent ± SEM of the genotypes parasite load with differences in geomean parasitemia between HbAA and HbAS genotypes not found to be statistically significant (P=0.651, Paired t test). (**C**) Children who presented with HDP (≥10,000/µL) were stratified by sickle-cell gene status, HbAA (n=294) and HbAS (n=44), with bars representing the percentage of the genotype with HDP. Differences in HDP between HbAA and HbAS genotypes were not found to be statistically significant (P=0.117, χ^2test).

4.2.4 Malaria Pigment Characteristics

Previously, we and others have shown that the ingestion of malarial pigment by monocytes contributes to the impairment of the immune response (Huy *et al.* 2006; Awandare *et al.* 2007) and is an important marker of malaria morbidity and mortality (Nguyen *et al.* 1995; Amodu *et al.* 1998; Lyke *et al.* 2003). However, since the role of monocyte/macrophage-acquired Hz has not been documented in HbAS individuals, we examined pigment containing monocytes (PCM) and pigment containing neutrophils (PCN) in each of the genetic groups. There was a statistically significant difference in the absolute count of PCM between the two genetic groups (P=0.002; Figure 19a). The HbAA group presented with a higher absolute PCM count (mean [SEM], 17.92 [1.99]) than the HbAS group (11.54 [0.89]). This trend was reflected in the percent PCM where HbAA children had a significantly higher percentage of PCM (6.43% [1.73]) than their HbAS counterparts (4.75% [1.11]) (P=0.002; Figure 19b). However, for the absolute PCN counts, both genetic groups had comparable measurements (HbAA, 12.96 [3.07], HbAS, 6.1 [2.83]; P=0.790; Figure 20a). Furthermore, the genetic groups demonstrated a non-significant relationship of PCN (HbAA, 0.26% [0.05]; HbAS, 0.15% [0.07]; P=0.814; Figure 20b).

Since the HbAS genotype was significantly associated with PCM, we decided to investigate the role that HbAS plays in modulating the amount of PCM, thereby possibly reducing immune dysfunction. In order to test this, children were stratified by sickle-cell gene status, low pigment-containing monocytes (Low PCM; <10% PCM), and high pigment-containing monocytes (High PCM; ≥10% PCM). There were significant differences in proportions (P=0.031; Figure 21) of children having low PCM in HbAA (41.21%; n=164) relative to HbAS (29.41%, n=20). Furthermore, 11.31% (n=45) of HbAA children presented

with high PCM, while there was a significant reduction in the percentage of those having high PCM in the HbAS children, only 1.47% (n=1, P=0.001; Figure 21). Taken together, these results demonstrate that HbAS children have a reduced absolute PCM and percent PCM count suggesting a reduced ingestion of malarial pigment by monocytes that may lead to less immunological dysregulation and enhanced protection against severe malaria.

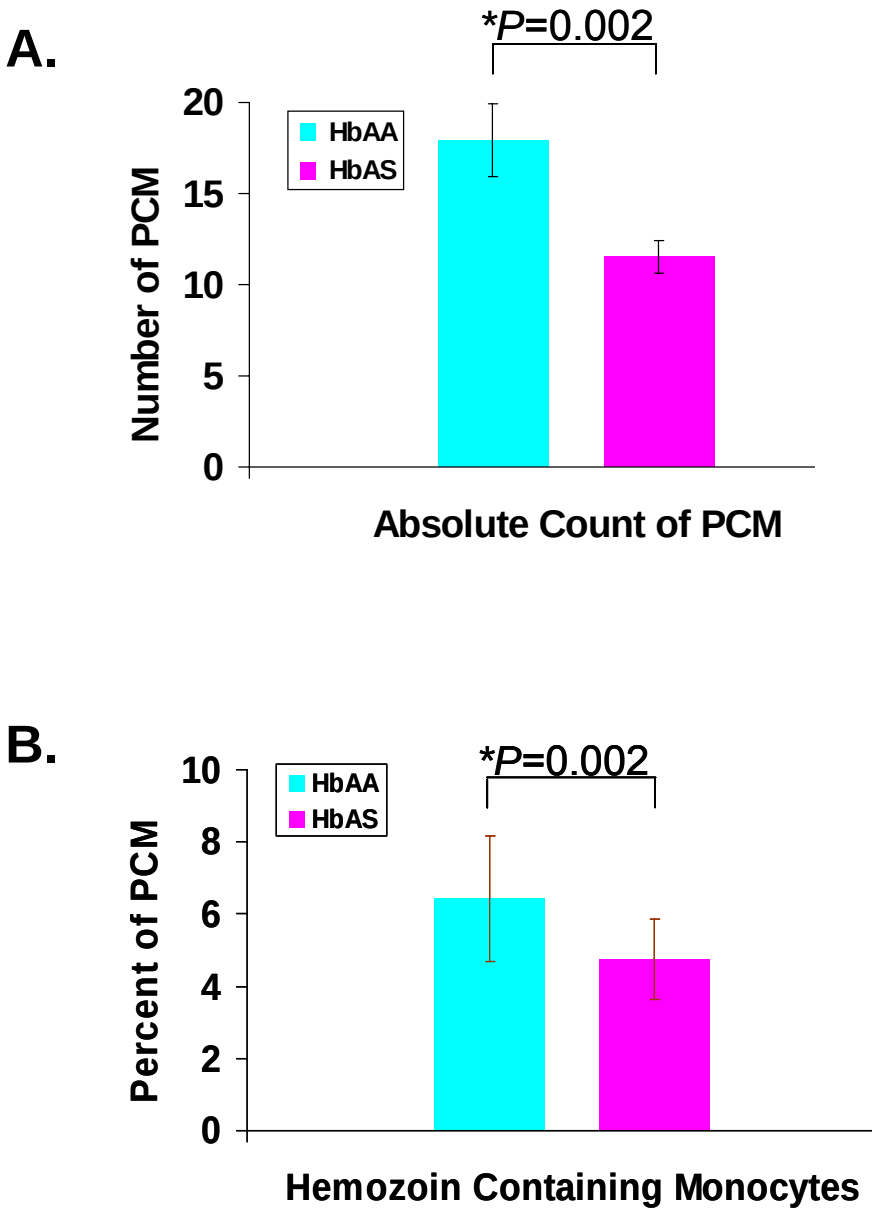

Figure 18: ***(A) Absolute Count and (B) Percent PCM According to Sickle-Cell Gene Status***

(**A**) The absolute count of PCM was calculated for HbAA (n=399) and HbAS (n=68) genotypes. Bars represent ± SEM of the absolute count of PCM. Differences in the absolute count of PCM between HbAA and HbAS genotypes were found to be statistically significant (P=0.002, Mann-Whitney U test). (**B**) The percent of PCM was calculated for HbAA (n=399) and HbAS (n=68) genotypes. Bars represent ± SEM of the percent of PCM with differences between HbAA and HbAS genotypes found to be statistically significant (P=0.002, Mann-Whitney U test).

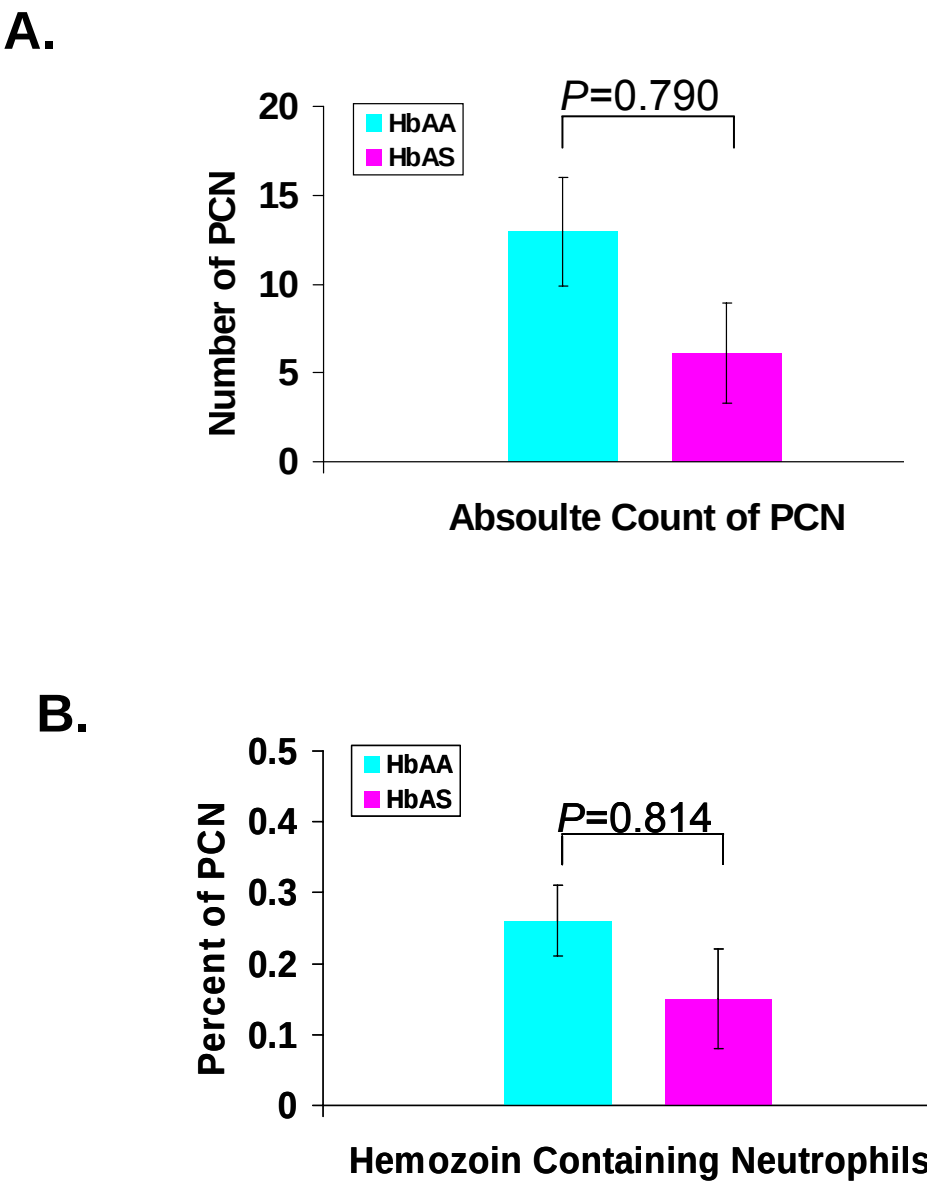

Figure 19: ***(A) Absolute Count and (B) Percent PCN According to Sickle-Cell Gene Status***

(**A**) The absolute count of PCN was calculated for HbAA (n=399) and HbAS (n=68) genotypes. Bars represent ± SEM of the absolute count of PCN. Differences in the absolute count of PCN between HbAA and HbAS genotypes were not found to be statistically significant (P=0.790, Mann-Whitney U test). (**B**) The percent of PCN was calculated for HbAA (n=399) and HbAS (n=68) genotypes. Bars represent ± SEM of the percent of PCN with differences between HbAA and HbAS genotypes not found to be statistically significant (P=0.814, Mann-Whitney U test).

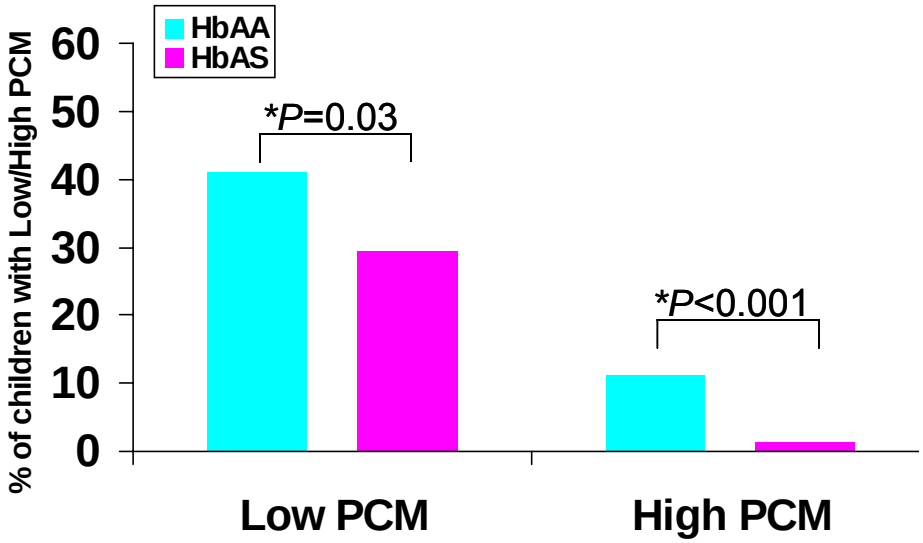

Figure 20: ***Low and High PCM According to Sickle-Cell Gene Status***

Children were stratified by low pigment containing monocytes (Low PCM; <10% PCM), high pigment containing monocytes (High PCM; ≥10% PCM), and sickle-cell gene status. Low PCM HbAA (n=164), Low PCM HbAS (n=20); High PCM HbAA (n=45), High PCM HbAS (n=1). Bars represent percent of children with low/high PCM. Differences in the low PCM between HbAA and HbAS genotypes were found to be statistically significant (P=0.03, χ^2test). Differences in the high PCM between HbAA and HbAS genotypes were found to be statistically significant (P<0.001, χ^2test).

4.2.5 Hematological Characteristics

Hematological measurements were obtained for all study participants to characterize the hematological profile of the two groups of children. The Hb concentrations were significantly reduced in the HbAA group, (median [Q1-Q3]), (6.40 g/dL [5.20 – 8.30 g/dL]) when compared to the HbAS group (7.10 g/dL [5.80 – 8.55 g/dL]; P=0.05; Figure 22a). Additionally, HbAA children had a significantly reduced RBC count, (3.08 x 10^6/μL [2.29 – 3.92 x 10^6/μL]) relative to the HbAS children 3.62 x 10^6/μL [2.88– 4.13 x 10^6/μL], P=0.004; Figure 22b). However, the MCV and the MCH in the HbAA group were significantly increased (MCV, 70.35 fL [64.80 – 76.60 fL]), (MCH, 22.60 pg [20.60 – 24.70 pg]) compared to the HbAS group (MCV, 65.70 fL [59.88 – 72.10 fL]; P<0.001; Table 5), (MCH, 21.05 pg [18.70 – 23.07 pg]; P<0.0001; Table 5). There were no significant differences between the genetic groups' median cell Hb concentration (MCHC; HbAA, (32.10 g/dL [30.70 – 33.10 g/dL]; HbAS (32.10 g/dL [30.32 – 33.20 g/dL]); P=0.816; Table 5). HbAA children had significantly reduced red cell distribution width (RDW; 21.30% [19.00 – 23.70%], compared to children in the HbAS genetic group (22.75% [19.50 - 26.22%]; P=0.026; Table 5). There were no significant differences between the genetic groups medians for absolute reticulocyte number (ARN; HbAA, 0.09 x 10^9/L [0.04 – 0.17 x 10^9/L]; HbAS, 0.09 x 10^9/L [0.05 – 0.16 x 10^9/L]; P=0.686; Table 5). The HbAS group had a similar proportion of children with a suppression of erythropoiesis [reticulocyte production indices (RPI) less than 2.0 (<2.0)] relative to the HbAA group (HbAA, 305 (76.4%); HbAS, 57 (83.8%); P=0.135; Table 5). Additionally, the HbAS group had a significantly lower proportion of children with an appropriate erythropoietic response [RPI greater than and including 3.0 (≥3.0)] when compared to the HbAA group (HbAA, 15 (3.75%); HbAS, 0 (0%); P=0.0001; Table 5).

To examine the relationship between HbAS and severe malaria disease we compared the proportion of enrolled children with SMA and non-SMA in the HbAA and HbAS groups. In this analyses, we used both the defined WHO criteria (Hb<5.0 g/dL (WHO 2000) and the modified definition of SMA in western Kenya (Hb<6.0 g/dL; (McElroy *et al.* 1999). When stratified according to the WHO definition of SMA, the HbAA group (n=89, 22.42%) had a significantly higher proportion of children with SMA relative to the HbAS group (n=9, 13.23%, P=0.046; Figure 23). The same pattern was maintained when the modified definition of SMA was used. The HbAA group had a significantly higher proportion of children with SMA (n=164, 41.41%) as compared to HbAS group (n=19, 27.94%, P=0.024; Figure 23).

These results illustrate that the presence of either AA or AS genotype resulted in identifiable hematological phenotypes as displayed by distinct red cell characteristics such as Hb, RBC counts, MCV, MCH, RDW, and RPI. In addition, children with HbAS genotype were protected against SMA whether the WHO or modified definitions of SMA was used.

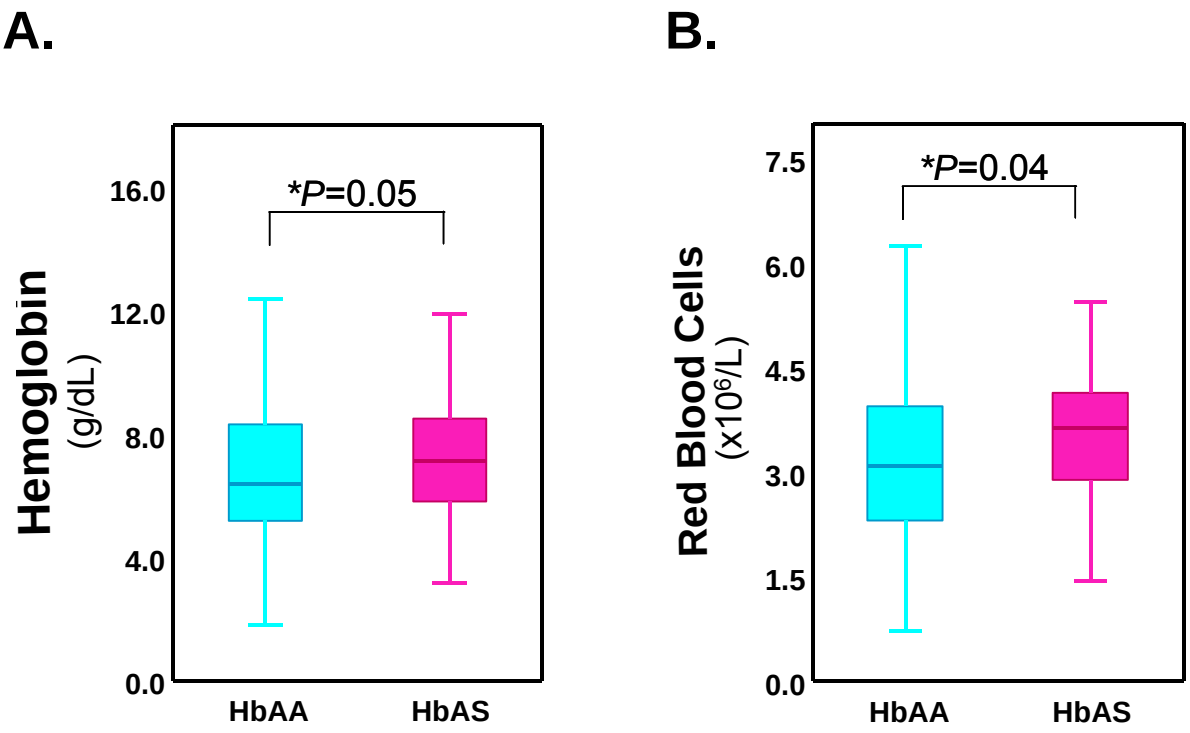

Figure 21: ***(A) Hemoglobin and (B) Red Blood Cell Measurements According to Sickle-Cell Status***

Data presented as box plots where the box represents interquartile range, the line through the box represents the median, whiskers indicate the 10^{th} and 90^{th} percentiles, and individual symbols are outliers. (**A**) Hb levels of parasitemic children with HbAA (n=399) and HbAS (n=68) genotypes were measured with the HemoCue system. Differences in Hb levels between HbAA and HbAS genotypes were statistically significant (P=0.05, Mann-Whitney U test). (**B**) RBC counts of parasitemic children with HbAA (n=399) and HbAS (n=68) genotypes were measured by a Beckman Coulter AcT diff2 counter. Differences in RBC counts between the HbAA and HbAS genotypes were statistically significant (P=0.04, Mann-Whitney U test).

Table 5: ***Red Blood Cell Indices According to Sickle-Cell Status***

Characteristics	HbAA (n=399)	HbAS (n=68)	*P*
Red blood Cell Indices			
Mean cell volume (fL)	70.35 (64.80 – 76.60)	65.70 (59.88-72.10)	0.00001[a]
Mean cell hemoglobin (pg)	22.60 (20.60 – 24.70)	21.05 (18.7–23.07)	0.0002 [a]
Mean cell hemoglobin concentration (g/dL)	32.10 (30.70 – 33.10)	32.10 (30.32-33.20)	0.816 [a]
Red cell distribution width (%)	21.30 (19.00 – 23.70)	22.75 (19.50-26.22)	0.026 [a]
Absolute reticulocyte number (x 10^9/L)	0.09 (0.04 – 0.17)	0.09 (0.05 – 0.16)	0.686 [a]
Reticulocyte Production Index <2.0, n (%)	305 (76.4)	57 (83.8)	0.135[b]
Reticulocyte Production Index ≥3.0, n (%)	15 (3.7)	0 (0)	0.0001 [b]

Hematological measurements were obtained using heel/finger-prick blood with a Beckman Coulter AcT diff2 counter.

Data presented as medians (Q1-Q3), unless other wise noted.

[a] Mann-Whitney U test

[b] χ^2 test

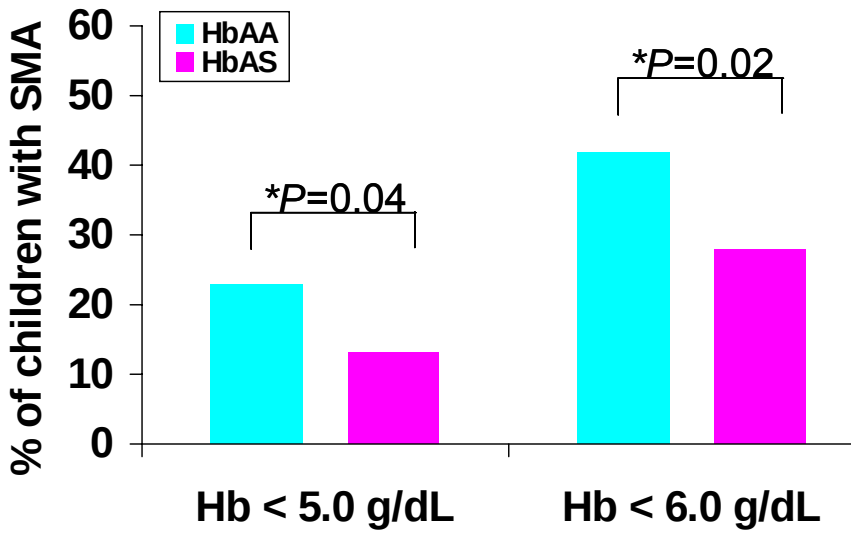

Figure 22: ***Proportion of Children with SMA According to Sickle-Cell Gene Status***

The proportion of children with SMA was investigated using the definition of SMA by the WHO as Hb<5.0 g/dL (WHO 2000), HbAA (n=89) and HbAS (n=9), and by the modified definition of Hb<6.0 g/dL (McElroy *et al.* 1999), HbAA (n=164) and HbAS (n=19), then stratified by their sickle-cell gene status. Bars represent percentage of children with SMA. Differences in SMA, Hb<5.0 g/dL and Hb<6.0 g/dL, between HbAA and HbAS genotypes were found to be statistically significant (P=0.04, and P=0.02; χ^2test).

4.2.6 Leukocyte and Platelet Indices

Leukocyte and platelet indices were obtained for all study participants to characterize any anomalies between the groups. When leukocyte and platelet indices were compared, results revealed that were no significant differences between the genetic groups for white blood cell counts (WBC; HbAA, 11.00 x 10^3/L [8.60 – 15.30 x 10^3/L]; HbAS, 12.20, x 10^3/L [9.12 – 14.97 x 10^3/L]; P=0.305), percent lymphocytes (LY; HbAA 50.70% [40.25 – 59.05%]; HbAS, 49.00% [39.58 – 59.78%]; P=0.733), percent monocytes (MO; HbAA, 9.00% [6.60 – 12.27%]; HbAS, 8.40% [5.80 – 11.32%]; P=0.287), and percent granulocytes (GR; HbAA, 39.65 [28.25 – 50.60%]; HbAS, 40.65% [30.03 – 52.15%]; P=0.505) (Table 6).

Similarly, there were no significant differences between the genetic groups for platelet counts (Plt; HbAA, 160.50 x 10^3/L [108.00 – 235.50 x 10^3/L]; HbAS, 159.00 x 10^3/L [106.50 – 271.50 x 10^3/L]; P=0.603), mean platelet volume (MPV; HbAA, 8.30 fL [7.50 – 13.90 fL]; HbAS, 8.60 fL [7.70 – 9.80 fL]; P=0.070), plateletcrit (Pct; HbAA, 0.14% [0.10 – 0.19%]; HbAS, 0.14% [0.10 – 0.23%]; P=0.382), and platelet distribution width (PDW; HbAA, 17.50% [16.70 – 18.20%]; HbAS, 17.55% [17.00 – 18.20%]; P=0.804) (Table 6).

These results showing comparable leukocyte and platelet indices indicate that these parameters are not significantly altered between the HbAA and HbAS genotypes.

Table 6: ***Leukocyte and Platelet Indices According to Sickle-Cell Gene Status***

Characteristics	HbAA (n=399)	HbAS (n=68)	*P*
Leukocyte Indices	**Median (Q1 – Q3)**	**Median (Q1 – Q3)**	
White blood cells (x 10^3/L)	11.00 (8.60 – 15.30)	12.20 (9.12 – 14.97)	0.305
Lymphocytes (%)	50.70 (40.25 – 59.05)	49.00 (39.58 – 59.78)	0.733
Monocytes (%)	9.00 (6.60 – 12.27)	8.40 (5.80 – 11.32)	0.287
Granulocytes (%)	39.65 (28.25 – 50.60)	40.65 (30.03 – 52.15)	0.505
Platelet Indices			
Platelet counts (x 10^3/L)	160.50 (108.00 – 236.50)	159.00 (106.50 – 271.50)	0.603
Mean platelet volume (fL)	8.30 (7.50 – 13.90)	8.60 (7.70 – 9.80)	0.070
Plateletcrit (%)	0.14 (0.10 – 0.19)	0.14 (0.10 – 0.23)	0.382
Platelet distribution width (%)	17.50 (16.70 – 18.20)	17.55 (17.00 – 18.20)	0.804

The leukocyte and platelets measurements were obtained using heel/finger-prick blood with a Beckman Coulter AcT diff2 counter.

Data presented as medians (Q1-Q3).

Differences between the two genetic groups medians were calculated using Mann-Whitney U test, and $p<0.05$ was considered statistically significant.

4.2.7 Association of HbAS with SMA, HDP, and PCM

The association of HbAS and malaria disease severity was determined by binary logistic regression analyses. SMA (defined by Hb<5.0 g/dL and Hb<6.0 g/dL), HDP, and high PCM were independent variables, while controlling the confounding effects of age and gender. Results reveal that relative to the HbAA group, children with the HbAS genotype had a 47% and 45% reduced risk of developing SMA, according to the WHO (Hb<5.0 g/dL; P=0.091) and modified definitions (Hb<6.0 g/dL; P=0.038; Table 7), respectively. Additionally, children with the HbAS genotype had a 37% (P=0.097) decreased risk of developing HDP than children with the HbAA genotype. Furthermore, HbAS children had a 58% (P=0.0001; Table 7) reduction in high PCM.

Taken together, these results illustrate that children with HbAS have a decreased susceptibility to SMA, independent of parasite burden. In addition, the HbAS individuals are protected against increased pigment burden relative to the HbAA group.

Table 7: ***Association of Sickle-Cell Trait with SMA, HDP, and PCM***

Characteristic	HbAA			HbAS		
	OR	**95% CI**	***P***	**OR**	**95% CI**	***P***
SMA (Hb<5.0 g/dL)	1.00	-	-	0.53	0.25 – 1.11	0.091
SMA (Hb<6.0 g/dL)	1.00	-	-	0.55	0.31 – 0.97	0.038
HDP (10,000/μL)	1.00	-	-	0.63	0.36 – 1.09	0.097
High PCM (≥10%)	1.00	-	-	0.42	0.26 – 0.68	0.0001

Abbreviations: SMA, severe malaria anemia; HDP, high-density parasitemia; PCM, pigment containing monocytes.

Data presented are results of a binary logistic regression analysis controlling for age and gender.

The association of sickle-cell trait with SMA, HDP and high PCM was examined in 68 parasitemic HbAS children, while the HbAA genotype was used as a reference for this analysis.

5.0 DISCUSSION

P. falciparum is the major cause of malaria-related morbidity and mortality, particularly in children less than five years of age residing in malaria endemic regions (Breman *et al.* 2001; Williams *et al.* 2005a). The host defense mechanisms that mediate protection against *P. falciparum* are complex and only partially defined. Genetic factors, such as the erythrocytic polymorphisms -$\alpha^{3.7}$ and HbAS appear to condition susceptibility to malaria and the consequent outcomes of infection (Allison 1954c; Williams *et al.* 1996; Migot-Nabias *et al.* 2000; Mockenhaupt *et al.* 2004; Williams *et al.* 2005a; Williams *et al.* 2005b; Williams *et al.* 2005c; Williams *et al.* 2005c; Verra *et al.* 2006; Wambua *et al.* 2006a; Williams 2006a; Wambua *et al.* 2006b; Williams 2006b). However, for more than fifty years, the mechanism(s) responsible for protection against falciparum malaria in individuals with -$\alpha^{3.7}$ and HbAS have remained elusive. The role of -$\alpha^{3.7}$ and HbAS in mediating protection against SMA was, therefore, investigated in children residing in a holoendemic *P. falciparum* transmission region of equatorial Africa.

To investigate the role of -$\alpha^{3.7}$ and HbAS in protection against SMA, we examined a cohort of children, aged 3-36 months, in a holoendemic *P. falciparum* transmission region of western Kenya: an area in which SMA is the most common clinical manifestation of severe malaria (McElroy *et al.* 1999; Ong'echa *et al.* 2006). In addition, all children were examined for HIV-1 infection and bacteremia, and those children found to be positive for either or both of these infections were excluded from the current study. This is a novel and important aspect of

the current study since previous investigations have not taken into account these co-infections. Since we and others have shown that HIV-1 and bacteremia influence hematological characteristics and progression of severe malaria (Crawley 2004; Brent *et al.* 2006; Otieno *et al.* 2006), the current investigations provide one of the first well controlled studies examining the relationship between $-\alpha^{3.7}$, HbAS, and SMA.

The $-\alpha^{3.7}$ genotype was previously detected by Southern blot analysis (Dozy *et al.* 1979), a technique that requires high quantity and quality of genomic DNA. However, in 2000, two separate papers were published outlining the detection of α-thalassemia variants in a single tube multiplex PCR reaction (Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b). This proved to be an advantageous method due to the short time required to generate reliable results, and by being more affordable and sensitive than Southern blotting. However, both of these protocols require high quality and large quantities of genomic DNA (100 ng). Since the amplification regions contained in the amplified sequence have a high GC content, DMSO and/or betaine are required to prevent the formation of secondary structure(s).

The availability of both high quality and quantity of genomic DNA was limited in our study since DNA was isolated from dried blood spots. This DNA collection was used since the amount of blood that can be drawn from severely anemic children is limited. In addition, DNA isolated from dried blood spots can contain residual hemolysate that can interfere with the PCR reaction. The starting material for the PCR-based reactions utilized to identify variation in the α-thalassemia gene appears to, therefore, have generated significant technical limitations.

The two primary protocols used for amplifying the α-thalassemia gene, published by Chong et al., and Liu et al., (Liu *et al.* 2000; Chong *et al.* 2000a; Chong *et al.* 2000b) consistently yielded amplification of the "normal" fragment for Pittsburgh controls; malaria

naïve donors from the University of Pittsburgh used as PCR positive controls. Additionally, with the Chong protocol, there was consistent amplification of the expected 1800 bp band using 100 ng of Kenyan genomic DNA and Pittsburgh control DNA. The conclusion of these results was that the PCR reactions worked well for detecting the normal band; but was never able to amplify the deletional band in Kenyan genomic DNA isolated from dried blood spots.

To more fully develop the PCR conditions, we utilized a positive control for $-\alpha^{3.7}$ represented by genomic DNA isolated from a Tahitian population. However, when using these positive controls for the $-\alpha^{3.7}$ in the Chong and Liu protocols, we observed inconsistent amplification of the normal bands at 1800 bp and 2200 bp, respectively, and never identified the deletional band at 2000 bp.

Further failure to amplify the deletional band with the positive control Tahitian genomic and the Kenyan genomic DNA samples led us to hypothesize that the high GC content could be interfering with the amplification. Although these protocols use DMSO and/or Betaine that stabilize and prevent secondary structures of GC rich regions, it is possible that these two reagents were not linearizing DNA to the extent required for proper amplification. Therefore, we attempted to optimize the PCR reactions using two commercially available PCR amplification kits, the GC-Rich kit from Roche Diagnostics and the PCR Enhancer System by Invitrogen™, both of which are capable of amplifying GC rich regions.

Utilizing the GC-Rich kit, and working in conjunction with the University of Pittsburgh, Graduate School of Public Health, Department of Human Genetics, we attempted the PCR amplifications with both the Chong and Liu primer sequence using Tahitian genomic DNA, Pittsburgh control genomic DNA, and Kenyan genomic DNA. Additionally, since we had a limited amount of Tahitian and Kenyan genomic DNA available, we performed whole genome

amplification on a number of the samples using the Repli-G® kit from Qiagen. Whole genome amplification is a PCR reaction requiring a limited amount of template genomic DNA (~10 ng), and through strand displacement, makes carbon copies of the starting material, yielding approximately 45 ng/μL in a reaction volume of 50 μL. With the Chong and Liu primer sequences, the Pittsburgh controls amplified the normal band consistently; the Kenyan genomic DNA did not yield any bands, and the Repli-G® DNA for both the Tahitian and Kenyan samples demonstrated smears when run out on an agarose gel. Interestingly, only one of the Tahitian genomic samples (a homozygous individual) demonstrated banding at 2000 bp with the Liu primer sequence; the correct position for the deletional band. Although this was a promising result, it could not be reproduced after many attempts with additional DNA samples.

Finally, the PCR Enhancer System was utilized with both the Chong and Liu primer sequences, along with the Tahitian genomic DNA, Pittsburgh control genomic DNA, and Kenyan genomic DNA (all at 100 ng). The Pittsburgh control normal band (at 1800 bp and 2200 bp) using the two primer sequences was amplified consistently with the majority of the samples. However, the Tahitian samples did not produce any banding with the Chong primer sequence, while the Liu primer sequence yielded one sample that produced a smear and additional Tahitian samples that produced neither banding patterns nor smears. Additional genomic positive controls for the $-\alpha^{3.7}$ will be required to appropriately optimize the PCR methodologies required for genotyping the Kenyan samples. Taken together, these results suggest that high quality and quantities of genomic DNA are required to utilize the current methodologies available for determining variation in the α-thalassemia gene.

The role of HbAS in protection against SMA was examined in a Kenyan cohort of children (3-36 months) that were age- and gender-matched. Since this was a cross-sectional

study, we wanted to determine if children in the HbAA or HbAS groups presented at hospital during the same clinical stage of disease. To accomplish this, we utilized the fever histories that were available for the two groups. The malarial fever typically occurs immediately after rupture of malaria-infected RBC through cytokine generation and the release of other fever-inducing mediators from host cells (Karunaweera *et al.* 1992; Kwiatkowski *et al.* 1993). Although fever is a generalized clinical response to most infectious pathogens, it serves as an indicator for caregivers to seek medical attention since fever is associated with malaria in most African countries (Lubanga *et al.* 1997; Olaleye *et al.* 1998; Amin *et al.* 2003; Guyatt and Snow 2004; Beausoleil 1984; Bruce-Chwatt 1987; Dicko *et al.* 2005). As such, our finding that children in the HbAA or HbAS groups presented at hospital within comparable time-frames, based on their fever history, indicates that the two groups were in the same phase of disease upon presentation at hospital. This data allowed us to further explore the relationship between HbAS and protection against malaria without the concern of having the case (HbAS) and control (HbAA) groups at differing continuums of infection.

Previous studies showed that children with the HbAS genotype had significantly reduced parasite density, median parasitemia, and HDP to children with the HbAA genotype (Allison 1954c; Carnevale 1981; Gendrel *et al.* 1991; Le Hesran *et al.* 1999; Aidoo *et al.* 2002; Williams *et al.* 2005a; Williams *et al.* 2005b). However, findings presented here demonstrate that parasite density was not significantly associated with carriage of HbAS. The reason for this apparent difference is presently unclear. It is possible that results presented here differed from previous investigations due to the fact that children in the previous studies likely included a significant number of children with additional co-infections, such as HIV-1 and bacteremia that can influence the course of a malaria infection. It is also important to note that the previous study in a

comparable area of falciparum endemicity was performed as a community-based longitudinal investigation in which both malaria morbidity and all-cause mortality was assessed in children from birth to five years of age (Aidoo *et al.* 2002). Thus, it is possible that the children investigated here in a cross-sectional study design that presented at hospital with their first episode of malaria may be clinically very distinct from those children residing in the community that are presumably less ill than those recruited in a hospital setting.

Our findings that children with the HbAS genotype have a similar parasite burden and HDP to the HbAA group prompted us to investigate if the hemozoin burden was comparable in the two groups. These studies showed for the first time that HbAA individuals have an increased number of PCM compared to HbAS individuals with acute malaria. During a malaria infection, monocytes and neutrophils ingest Hz (Nguyen *et al.* 1995; Lyke *et al.* 2003). Monocytes and neutrophils play a vital role in the defense against invading pathogens through responses such as phagocytosis, enhanced cytotoxicity, and increased cytokine production (Abdalla and Pasvol 2004). Although the role of Hz in malaria severity is not completely understood, the presence of Hz in circulating monocytes and neutrophils has been associated with increased morbidity and mortality in both children and adults (Nguyen *et al.* 1995; Lyke *et al.* 2003). Elevated PCM relative to PCN is an indicator of a chronic malarial infection, whereas higher predominance of PCN is indicative of a more acute malarial infection (Day *et al.* 1996; Awandare *et al.* 2007). This rationale is based on the fact that PCM have a longer half-life (days to weeks) than PCN (9 hours) due to the clearance kinetics of the two populations of white blood cells in circulation (Day *et al.* 1996; Awandare *et al.* 2007). During a malarial infection, Hz ingestion severely impairs the function of monocytes by creating a cellular milieu that can no longer generate oxidative burst, repeat phagocytose, and activate protein kinase C signaling pathways

(Schwarzer *et al.* 1998). Other studies have also shown that phagocytosis of opsonized Hz impairs the expression of major histocompatibility complex (MHC) class II antigens, CD54, and CD11c in human monocytes (Schwarzer *et al.* 1998). Recently, Hz was shown to activate leukocytes, including monocytes and neutrophils that leads to enhanced severity of severe malaria (Huy *et al.* 2006).

Based on differences in PCM between the two groups, it was of interest to stratify the Hz burden into high versus low for both monocytes and neutrophils. These results demonstrated that the HbAS group was significantly protected against both high and low PCM compared to the HbAA genotype. The precise mechanism(s) responsible for this observed difference remains to be determined. However, we hypothesize that there may be more efficient control of parasitemia in the early ring stage of parasitemia due to the more hypoxic nature of the RBC in the HbAS group. Although this mechanism may not significantly limit the overall parasite burden, it may limit efficient formation of Hz within pRBC in individuals with the HbAS genotype. Additional studies are required to verify this hypothesis.

The hematological data presented here unique in that individuals with HIV-1 and bacteremia were excluded from the analyses. To our knowledge, this represents the first investigation comparing hematological indices in children with HbAS and HbAA that took into account the important influence of these co-pathogens. This fact is illustrated by previous studies conducted by our group demonstrating that HIV-1 promotes enhanced SMA (Crawley 2004; Otieno *et al.* 2006). Additionally, bacteremia significantly enhances anemia severity in individuals with malaria (Brent *et al.* 2006). Taken together, the hematological data presented here more accurately reflect the true effect of carriage of HbAS on hematological outcomes relative to those with HbAA genotype.

Children with the HbAS genotype presented with a significantly higher median Hb and RBC count than children with the HbAA genotype. Both Hb and RBC counts are used as early indictors of anemia. An increase in Hb and RBC count has been attributed to limiting parasitemia that leads to reductions in anemia (Desai *et al.* 2005; Wambua *et al.* 2006a). However, the fact that Hb and RBC counts were significantly different in the two groups, despite non-significant differences in parasitemia does not support previous investigations. Additional results presented here demonstrate that the children with HbAS genotype had significantly reduced MCV and MCH compared to children with the HbAA genotype; this finding is consistent with previous studies carried out in both adults and children (Sheehan and Frenkel 1983; Mockenhaupt *et al.* 2000; Wambua *et al.* 2006a). Although the implications of reduced MCV and MCH in individuals with HbAS has not been definitively determined, MCV and MCH are additional hematological indices that can contribute to anemia (Davidson and Hamilton 1978; George-Gay and Parker 2003). For example, increased MCV and MCH are important markers of underlying dyserythropoiesis caused by deficiencies of B12 and/or folic acid (George-Gay and Parker 2003). HbAS individuals also presented with significantly higher RDW than those with the HbAA genotype. RDW is used to account for the amount of variation in RBC volume, and is typically increased in both iron deficiency and megaloblastic anemia's (Helleberg *et al.* 2005). Increased RDW has been previously observed in malaria, and this increase has been attributed to the red cells response to the parasite (Bunyaratvej *et al.* 1993). The clinical value associated with determining the RDW is due to its ability to reflect active erythropoiesis (Roberts and El Badawi 1985). In a previous study carried out in Ghana, RDW was used as a marker for the release of young erythrocytes and reticulocytes, an indication of appropriate bone marrow responsiveness (Kurtzhals *et al.* 1997). In a different study conducted in malaria-infected

children, the RDW increased in response to low Hb values and was used as an index of a positive erythropoietic response, while a reduced RDW was associated with bone marrow suppression (Helleberg *et al.* 2005). Therefore, the increased RDW in HbAS children may indicate increased erythropoiesis and the release of young RBC into circulation. However, this phenomenon was not supported by our measurement of the reticulocytes production indices (RPI) in the two groups. The RPI is a standard index that accounts for reticulocyte production in the bone marrow of anemic patients taking into account the degree of anemia (Were *et al.* 2006). An index of less then 2.0 implies suppression of erythropoiesis, while an index of greater than 3.0 is indicative of an appropriate erythropoietic response. The majority of children with HbAS had a RPI indices <2.0, a hallmark of suppression of erythropoiesis. Therefore, this decrease in RPI contradicts the RDW measurements that suggested increased bone marrow responsiveness, suggesting that protection against anemia in children with the HbAS may not be due to an enhanced erythropoietic response.

In agreement with previous findings in children with malaria, there was a greater proportion of children with SMA in the HbAA group compared to the HbAS group (Aidoo *et al.* 2002). Although the cause of anemia in tropical and sub-tropical areas is multifactorial, *P. falciparum* infection is a major contributory factor to its etiology in children living in holoendemic malaria areas (WHO 2000; Crawley 2004). Based on all of the findings presented here, we hypothesize that protection against SMA in the HbAS group is due to a reduced burden of Hz in monocytes. Previous results show that Hz can contribute to enhanced anemia through suppression of erythropoiesis (Casals-Pascual *et al.* 2006). Additional studies examining the erythropoietic response in longitudinal fashion will be required to determine if children with HbAS have more efficient production of RBC.

Taken together, results from this study demonstrate that although we were unsuccessful in genotyping -$\alpha^{3.7}$ in the Kenyan cohort, we now have a better understanding of the starting material required to successfully genotype this cohort of children in the future. For example, isolation of DNA from whole blood will be essential to yield both high quality and quantity genomic DNA that will be required for these investigations. Additionally, the acquisition of a positive -$\alpha^{3.7}$ sample from Kenyan descent will provide an accurate positive control for the PCR reactions. Results presented here also demonstrate that HbAS is protective against SMA when appropriately controlling for the confounding effects of common co-pathogens, such as HIV-1 and bacteremia that occur at high percentages in malaria endemic areas. In addition, these investigations showed that protection against SMA in children with the HbAS genotype is not significantly associated reduced parasite burden. This study demonstrates the novel finding that HbAS is associated with significantly reduced levels of PCM. The underlying mechanism(s) for this finding require additional investigations that may provide important insight into the pathogenesis of childhood SMA.

This study has important public health implications since malaria is one of the major causes of childhood morbidity and mortality around the globe (WHO 2000; Breman *et al.* 2001; Rowe *et al.* 2006). An improved understanding of how hemoglobinopathies protect against fatal malaria may provide important information required for the development of novel therapeutics in the future.

6.0 BIBLIOGRAPHY

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