# Effect of hemoglobins S and C on the *in vivo* expression and immune recognition of *Plasmodium falciparum* erythrocyte membrane protein 1 variants in Malian children

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# Statement of work performed

The work in this thesis was performed with great help from many people. Below is a description of my contributions and others' contributions in the body of work presented in this thesis.

The clinical study in chapter 2 was designed by Dr. Rick Fairhurst and carried out by Saibou Doumbia, Mory Doumbouya, and Drissa Konate. The statistical analysis of the data from this study was conducted by Dr. Michael Fay with direction from Dr. Rick Fairhurst, Dr. Tatiana Lopera, and me. The experiments detailed in chapters 3, 4, and 5 were conceived and designed by me under the direction of Dr. Rick Fairhurst, Professor Chris Newbold, and Professor Nicholas White. The cytoadherence comparisons in chapter 3 were performed by me with help from Seidina Diakite and Michael Krause. I performed the analysis in this chapter in consultation with Dr. Michael Fay, a statistician. For the work presented in chapter 4, I cloned and the Wellcome Trust *Sanger Institute* sequenced the expressed PfEMP1s from 50 Malian samples. I performed the clean-up and analysis of these sequences. The Northern blots were performed with help from Dr. Sue Kyes, who did all of the radioactive work. Dr. Louise Turner developed the Luminex assay described in chapter 5 and tested our samples using her assay. I performed the analysis of the data from this experiment in consultation with Dr. Kazutoyo Miura and Jeffrey Skinner. The thesis, in its entirety, was written solely by me with minor editing by Dr. Rick Fairhurst and Professor Nicholas White.

# Abstract

The enormous mortality burden exerted by *P. falciparum* malaria has evolutionarily selected for red blood cell (RBC) polymorphisms which confer protection against the severe manifestations of this disease. Although the epidemiological protection by these polymorphisms has been well-established for the past half-century, the mechanisms underlying this protection are still being uncovered. Recent studies implicate impaired cytoadherence to microvascular endothelial cells (MVECs) due to reduced surface levels and altered display of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as a mechanism of protection against severe malaria by sickle hemoglobin (Hb) S and HbC. Consequently, in this thesis, I have described three separate, but related investigations into whether hemoglobins S and C influence a parasite's cytoadherence binding phenotype (**Chapter 3**), the PfEMP1 variants that parasites express *in vivo* (**Chapter 4**), and the IgG recognition of PfEMP1 domains in Malian children (**Chapter 5**). We found that parasites from HbAS children show statistically insignificant increased binding to MVECs and that parasites did not express a restricted subset of *var* genes in HbAS and HbAC children. Compared to HbAA and HbAC children, HbAS children demonstrated a slower rate of acquisition of IgG responses to a repertoire of PfEMP1 domains. These findings suggest that, although hemoglobin type influences the binding phenotype of *P. falciparum* isolates and the acquisition of PfEMP1-specific IgG responses, other factors more likely determine the expressed *var* gene repertoire within parasites than hemoglobin type.

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# Abbreviations

| | |
|---|---|
| ACT | artemisinin combination therapy |
| AMA | apical merozoite antigen |
| Amp | ampicillin |
| ABE | assay buffer E |
| BBB | blood brain barrier |
| BCR | B cell receptors |
| BM | binding media |
| CI | confidence interval |
| CIDR | cysteine-rich interdomain region |
| CM | cerebral malaria |
| CO | carbon monoxide |
| CSA | chondroitin sulfate A |
| CQ | chloroquine |
| CR1 | complement receptor 1 |
| DBL | Duffy binding ligand |
| DDT | dichlorophenyltrichloroethane |
| DEPC | diethylpyrocarbonate |
| dNTPs | deoxynucleotide triphosphate |
| DV | digestive vacuole |
| EBA-175 | erythrocyte-binding antigen 175 |
| EIR | entomological inoculation rate |
| ELISA | enzyme-linked immunoabsorbant assay |
| ER | endoplasmic reticulum |
| FISH | fluorescent *in situ* hybridization |
| FPIX | ferriprotoporphyrin IX |
| G6PD | glucose-6-phosphate-dehydrogenase |
| gC1qR | globular head of the C1q receptor |
| GSH | glutathione |
| H3K9 | histone 3 lysine 9 |
| Hb | hemoglobin |
| HbF | fetal hemoglobin |
| HO-1 | heme oxgenase-1 |
| HPFH | hereditary persistence of fetal hemoglobin |
| HRP-2 | histidine rich protein 2 |
| ICAM-1 | intracellular adhesion molecule 1 |
| Ig | immunoglobulin |
| IL | interleukin |
| IRS | indoor residual spraying |
| ITN | insecticide-treated nets |
| KAHRP | knob-associated histitidine-rich protein |
| LB | Luria broth |
| MAHRP1 | membrane-associated histidine-rich protein 1 |
| MCHC | mean corpuscular hemoglobin concentration |

| | |
|---|---|
| MFI | mean fluorescence intensity |
| MSP | merozoite surface protein |
| MVEC | microvascular endothelial cell |
| NAD | nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NAI | naturally acquired immunity |
| NIAID | National Institute of Allergy and Infectious Diseases |
| NO | nitric oxide |
| nt | nucleotide |
| PAM | pregnancy associated malaria |
| PEXEL | protein export element |
| PfCRT | *Plasmodium falciparum* chloroquine resistance transporter |
| PfEMP1 | *P. falciparum* erythrocyte membrane protein 1 |
| PoLV | positions of limited variability |
| PV | parasitophorous vacuole |
| RBC | red blood cell |
| RD | respiratory distress |
| RR | relative risk |
| RDT | rapid diagnostic test |
| TLR | toll-like receptor |
| TNF | tissue necrosis factor |
| TVN | tubulovesicular network |
| UM | uncomplicated malaria |
| SBP1 | skeleton binding protein 1 |
| SCA | sickle cell anemia |
| SCC | saline sodium citrate |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel |
| Sir | silent information regulator |
| SM | severe malaria |
| SMA | severe malaria anemia |
| TBE | Tris-Borate-EDTA |
| UV | ultraviolet |
| VSA | variant surface antigen |
| VCAM-1 | vascular cell adhesion molecule 1 |
| WHO | World Health Organization |

# 1 Introduction – Literature Review

## 1.1 Malaria

Malaria, caused by the mosquito-borne hemoprotozoa of the genus *Plasmodium*, has been a major cause of morbidity and mortality globally for millennia. Despite major efforts to reduce the burden of malaria over the past century, there are an estimated 300-500 million clinical cases and 1-2 million deaths per year worldwide [1]. As a result of the parasite's dependence on both human and anopheline hosts, the intensity of transmission is climate-related. Most malaria cases occur in tropical regions of Africa, Asia, and South America with the majority of deaths occurring in children under 5 years old and in pregnant women in sub-Saharan Africa [2]. Most of these cases and nearly all of the fatalities are caused by *Plasmodium falciparum,* one of the five major *Plasmodium* species that cause malaria in humans [2].

### 1.1.1 Plasmodium falciparum lifecycle

While malaria has played a large role in shaping human history due to its widespread distribution globally and its debilitating nature, the cause of malaria was not accurately understood until the late 1800s. In fact, the name "malaria," derived from the Italian words meaning "bad air," reflects the widespread belief for centuries that malaria's characteristic periodic fever was a result of bad air emanating from swamps and marshes [3]. It was not until 1880 that *Plasmodium* was first observed in human blood by Laveran, a French military surgeon [4]. In 1897, another military surgeon, Ronald Ross, confirmed the transmission of the parasite from bird-to-bird by mosquitoes [5]. It is now widely understood that mosquitoes of the genus *Anopheles* transmit the *Plasmodium* species that cause human malaria: *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi* [6].

#### 1.1.1.1 Mosquito & liver stage

Similar to the other *Plasmodium* species, *P. falciparum* has two distinct phases of its lifecycle: sexual and asexual. The sexual phase occurs begins in the human host with the differentiation of asexual forms into macrogametocytes (female) and microgametocytes (male) inside parasitized red blood cells (RBCs). *Anopheles* mosquitoes ingest these haploid gametocytes as they take a blood meal on a *P. falciparum*-infected human. Within the mosquito midgut, fertilization occurs and the resulting zygotes mature into sporozoites, replicate, and migrate to the salivary glands over the next 10-14 days. When the mosquito feeds on another human, tens to hundreds of sporozoites are passed to the new host through the mosquito's saliva. Within minutes, the sporozoites deposited into the subcutaneous tissue of the host cross the endothelium lining the host's vasculature, enter the bloodstream, and travel to the liver where they pass through several hepatocytes before invasion [7]. Invasion is mediated by the binding of thrombospondin domains on the circumsporozoite protein and on thrombospondin-related adhesive protein of parasites to heparin sulfate proteoglycans on hepatocytes [8, 9]. Once inside the hepatocytes, the sporozoites undergo a 40,000-fold expansion within a parasite-derived vacuole where they differentiate into merozoites. After a symptomless period of 5-7 days, the merozoites egress from the hepatocytes. In addition to producing this initial wave of parasitemia within the peripheral blood, *P. vivax* and *P. ovale* are capable of persisting asymptomatically in the liver as hypnozoites for months to years before relapsing into the hepatic sinuses, entering the bloodstream, and rapidly invading RBCs.

#### 1.1.1.2 Invasion of RBCs

The ellipsoidal *P. falciparum* merozoites contain all of the components necessary for invasion of RBCs [10]. The apical organelles, which are involved in invasion, include rhoptries, micronemes, and dense granules, and are common to all members of the phylum

Apicomplexa. Once the merozoites egress from the hepatocytes, they invade RBCs by attaching, reorienting their apical end, forming an irreversible junction, and entering the RBC within a parasitophorous vacuole (PV), which enables the parasite to remain isolated from the RBC cytoplasm. The initial attachment of the merozoite to its host RBC is reversible and likely mediated by a number of identified merozoite surface proteins (MSPs), whose role in invasion has yet to be well-defined [11, 12]. The merozoite then undergoes apical reorientation, which aligns the apical pole of the merozoite in direct apposition from the attached RBC membrane and allows for closer interaction between the RBC and merozoite. This step is potentially mediated through a Type 1 integral membrane protein called apical merozoite antigen (AMA) 1 [13]. Once the merozoite has apically reoriented, a tight junction is formed between the merozoite and RBC [14, 15]. This junction is irreversible and marks the commitment of the parasite to invade that particular RBC. The parasite then uses the movement of the junction to penetrate the RBC.

#### 1.1.1.3 Intraerythrocytic cycle

The invasion of RBCs by merozoites marks the onset of the intraerythrocytic cycle, which is responsible for the symptoms of malaria. In the tertian malarias (*P. falciparum, P. vivax, and P. malariae*), this cycle is characterized by its synchronous 48-hour periodicity. As its name implies, the intraerythrocytic cycle occurs exclusively within host RBCs.

The *P. falciparum* intraerythrocytic cycle begins with the ring stage, named for the characteristic “ring” appearance of the parasite in Giemsa-stained blood films [16]. After about 24 hours, parasites mature into trophozoites, which are able to sequester in the host’s microvasculature due to the binding of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of infected RBCs to host receptors [17-19]. As a result, rings and sexual gametocytes are typically the only parasite forms observed in peripheral blood smears of a *P. falciparum*-infected individuals [20]. The parasite then mitotically replicates

its DNA multiple times in the schizont stage to produce 16-20 daughter merozoites [21]. Upon rupture, each merozoite may infect another RBC, resulting in an exponential expansion of parasite density.

Growth within the metabolically sluggish, enucleated RBCs presents a challenge for *P. falciparum* parasites. To more efficiently perform their specialized role as oxygen and carbon dioxide transporters, terminally-differentiated RBCs lack organelles, and thus the ability to synthesize and traffic proteins, [22] leading to their description as a "sack of hemoglobin." As a result, parasites need to extensively remodel their host RBC to promote their survival. Within the PV, parasites export an estimated 400 proteins to their host RBC [23-25] through the PV-derived tubulovesicular network (TVN) and Maurer's clefts. In addition to exporting proteins involved in PfEMP1 trafficking, parasites export proteins that decrease the deformability of the RBC membrane [26]. The presence of a pentameric protein export element (PEXEL) motif (RXLXE) designates a protein for export, although this motif does not appear to be absolutely required since some exported proteins lack the PEXEL motif [27]. Additionally, parasites take up hemoglobin (Hb) and digest it using proteases, which provides space in the RBC for the parasite to grow, amino acids for the parasite's development, and osmolytes for preventing premature lysis of the RBC [28-30]. The byproduct of Hb digestion, haematin, has redox and detergent-like properties. Consequently, the parasite sequesters haematin by crystallizing it into inert hemozoin within the digestive vacuole (DV) [31].

### 1.1.2 Clinical features of malaria

In malaria endemic areas, individuals are constantly being exposed to bites from *P. falciparum*-infected mosquitoes. The rate at which people are bitten by infected mosquitoes is defined as the entomological inoculation rate (EIR) [32, 33] and varies dramatically on a population level due to climate and on an individual level due to proximity

to larval habitats [34], differential attractiveness to mosquitoes [35], and public health measures such as the use of bed nets. Approximately half of these infectious bites will result in infection of the human host [36].

The spectrum of clinical outcomes from *P. falciparum* infection is wide-ranging and involves a progression from asymptomatic parasitemia to uncomplicated malaria to severe malaria to death. The outcome of a particular infection is influenced by a number of both human and parasite factors. Host factors that play a role in this progression include genetic polymorphisms, immunity, nutritional status, comorbidities (most notably HIV/AIDS), and timely treatment. The initial size of the sporozoite inoculum and parasite genetics also contribute to this multifactorial process.

#### 1.1.2.1 Asymptomatic parasitemia

In a given population, the proportion of *Plasmodium*-infected individuals is known as the parasite prevalence and is used to categorize the endemicity of a given region as follows: hypoendemic (<10%), mesoendemic (10-49%), hyperendemic (50-75%), and holoendemic (>75%) [37]. In the areas of most intense transmission, it is not uncommon for almost all individuals to be infected with *P. falciparum*. Approximately half of these individuals will not develop a malaria syndrome from a particular infection and consequently remain asymptomatically parasitemic [36]. These individuals are not considered to have clinical malaria. Incidental parasitemias may be found in children in endemic areas who present with febrile illnesses of other etiologies.

#### 1.1.2.2 Uncomplicated malaria

Individuals with uncomplicated malaria (UM) experience an undifferentiated febrile illness with the presence of peripheral blood parasitemia, normally detected microscopically in thick blood films. UM is often accompanied by chills, headache, body

aches, and malaise [38]. Gastrointestinal symptoms may also occur. Children who are too young to verbalize complaints such as headache may be irritable or feed poorly. Individuals with malaria may experience paroxysms [39], which are caused by the host's physiological response to the synchronous rupture of schizonts every 48 hours and are characterized by acute fever, shaking chills, and drenching sweats. The experience of paroxysms is most commonly associated with *P. vivax* malaria [40]. Most individuals with UM recover with timely administration of effective antimalarial drugs.

#### 1.1.2.3 Severe malaria

Only about 2% of symptomatic infections will lead to the severe, life-threatening manifestations of *P. falciparum* malaria, most notably cerebral malaria (CM), severe malarial anemia (SMA) [41], and respiratory distress (RD) [36]. Children may experience two or more of these syndromes at the same time, and the presence of multiple syndromes simultaneously predicts poor clinical outcome [42]. CM is characterized by impaired consciousness. The Glasgow and Blantyre coma scores are used to assess consciousness in adults and children, respectively, by evaluating ocular, motor, and verbal responsiveness. The Blantyre coma score is measured on a scale of 0 (unconscious) to 5 (normal) with a score of ≤2 generally used to diagnose CM in African children [38]. SMA is defined by the World Health Organization (WHO) [2] as a Hb level <5 g/dL and may be complicated by secondary jaundice or renal failure [38]. Hyperventilation and labored breathing are the major features of RD, which results from metabolic acidosis [38]. Other criteria of severe malaria (SM) include prostration, hypoglycemia, jaundice, renal insufficiency, hemoglobinuria, shock, cessation of eating and drinking, repetitive vomiting, and hyperpyrexia [43]. Individuals who meet these criteria are typically treated with parenteral antimalarial therapy and aggressively managed to prevent death. Patients with high parasite densities (>100,000 asexual forms per µL of whole blood in low transmission areas,

>250,000 per µL in high transmission areas) are clinically managed similarly to patients with SM even if they do not meet any of the clinical criteria [38].

Age and malaria endemicity affect the susceptibility to SM and certain manifestations of SM. For example, SMA is found most commonly in young (<10 years old) children with the prevalence decreasing with age. In contrast, renal insufficiency caused by malaria is almost exclusively seen in adults and only rarely seen in Africa where children experience the greatest risk of SM [44]. Paradoxically, individuals in high transmission areas appear less likely to progress to severe disease compared to those in areas of lower transmission areas [45]. Until recently, *P. falciparum* was the only *Plasmodium spp.* known to cause SM. However, recent reports suggest that some *P. vivax* malaria cases meet the WHO criteria for SM [46-49].

#### 1.1.2.4 Fatal malaria

Death occurs in only a small proportion of malaria cases although it is estimated that without treatment, most individuals with severe malaria would die [38]. Approximately 6% of SM cases in young children (<10 years old) result in death. However, this percentage increases in older children and adults as well as in malaria-naïve individuals [44, 50]. Acidosis, cerebral manifestations of malaria such as coma and convulsions, and elevated blood urea nitrogen have all been associated with risk of death from malaria [42, 51]. Additionally, underlying chronic illness independently predicts an increased risk of death for children with SM [42] suggesting that co-morbidities must be seriously considered both in clinical management of patients as well as design and interpretation of studies involving SM outcomes.

#### 1.1.2.5 Pregnancy-associated malaria

Pregnant women are particularly susceptible to one particular form of malaria, termed pregnancy-associated malaria (PAM). PAM poses significant risk to both mother and infant and occurs even in women from endemic areas who have already developed immunity to *P. falciparum* in their non-pregnant state. Mothers may experience maternal anemia and hypertension. Infants, in addition to being at risk of spontaneous abortion *in utero*, are likely to have a low birth weight and may have a modified risk of subsequently developing *P. falciparum* malaria due to *in utero* sensitization to parasite antigens [52, 53].

### 1.1.3 Pathogenesis of falciparum malaria

The pathogenesis of malaria is complex and involves multiple organ systems. Both host and parasite factors contribute significantly to the clinical outcome of malaria. Of these, two important factors discussed here are parasite multiplication rate and cytoadherence. The pathogenesis of two severe malaria syndromes, CM and SMA, are discussed as well.

#### 1.1.3.1 Parasite multiplication rate

One of the major factors in the progression from asymptomatic parasitemia to death is the parasite multiplication rate. High parasite multiplication rates are believed to yield high parasite densities, which tend to correlate with poor clinical outcomes [43]. On an individual level, however, this correlation is often not strong due to immunity and other host and parasite factors. Asymptomatic children in endemic areas may tolerate high parasite densities and conversely, malaria-naïve individuals may experience SM at very low parasite densities.

Nevertheless, high parasite densities impact malaria pathogenesis through several mechanisms. First, *P. falciparum* parasites metabolize approximately 30-75 times more plasma glucose than uninfected RBCs in the course of the intraerythrocytic cycle [28, 54,

55]. The significance of this is two-fold. First, parasite consumption of glucose along with impaired hepatic gluconeogenesis and fasting commonly experienced by children with SM contributes to hypoglycemia, which may lead to coma and seizures [55]. Also, a byproduct of this process, lactic acid, can lead to deep breathing states and ultimately, respiratory distress at high concentrations. Second, in the course of their development, parasites form reactive oxygen species. Nonparasitized RBCs and platelets that are oxidatively damaged by reactive oxygen species are then prematurely removed by the spleen, which may contribute to the development of anemia and thrombocytopenia. Lastly, schizont rupture of parasites results in the release of a number of proinflammatory factors, such as *P. falciparum* glycosylphosphatidylinositol (*Pf*GPI), which results in release of tissue necrosis factor (TNF) from macrophages, and uric acid (discussed in further detail in **1.1.3.4**), which may also play a role in the development of anemia through dyserythropoiesis [56, 57].

Parasite multiplication rate is determined by multiple factors. One such factor is the RBC selectivity of invading parasites. Most parasitized RBCs have been invaded by a single merozoite and display a single ring form, but others may harbor two or more parasites. In certain infections, the distribution of such multiply-infected cells differs from the distribution that would be expected from a random process. High parasitemias and SM have been independently associated with a lack of selectivity in RBC invasion [58]. This finding suggests that parasites that are not selective in invading RBCs are more likely to rapidly multiply to high parasite densities and to cause SM than those infecting only a subset of the host RBCs.

Another strategy used by parasite to multiply at high rates is rosetting. Rosetting, the binding of multiple uninfected RBCs to parasitized RBCs, has also been associated with SM and high parasitemias [59-61]. Rosetting has been shown to occur in infections of 3 plasmodial species: *P. falciparum, P. vivax,* and *P. ovale* [62, 63]. In *P. falciparum* infections,

it occurs during the mature intraerythrocytic stages of parasite development through the interaction between parasite protein PfEMP1 (further discussed in **1.1.3.2** and **1.3**) on the parasitized RBC surface and receptors, complement receptor 1 (CR1) and heparan sulfate, on the nonparasitized RBC. By bringing the parasitized RBC into close proximity to multiple nonparasitized RBCs, rosetting increases the likelihood that the merozoites released from the parasitized RBC are able to find and invade nonparasitized RBCs more efficiently.

#### 1.1.3.2 Cytoadherence

Cytoadherence, the binding of mature parasitized RBCs to human microvascular endothelium, is crucial to parasite survival within the human host. Italian scientists, Marchiafava and Bignami, first observed and reported cytoadherence in their pathological studies of malaria near the turn of the 20$^{th}$ century, noting that "malignancy coincides with an exceptionally abundant quantity of parasite forms, a quantity much more abundant – where the cases terminate fatally – in the blood of the viscera than in the blood of the finger" [64]. Today, it is understood that one important effect of cytoadherence is preventing the removal of *P. falciparum*-infected RBCs from the peripheral circulation by the spleen. The spleen acts as filter for abnormal or senescent RBCs by retaining any RBCs that are unable to undergo the deformation necessary to pass through the interendothelial slits in the venous sinuses [65]. Parasite maturation within RBCs leads to large reductions in the deformability of the normally-flexible RBC. Although RBCs containing early rings, which maintain their deformability, are able to pass through these slits, any circulating RBCs containing trophozoites, schizonts, and some mature rings are retained in the red pulp of the spleen and prevented from re-entering the peripheral circulation [66]. The circulation of mature blood-stage parasites may occur during high parasitemia infections in which large numbers of parasites have exhausted most binding sites within the peripheral circulation forcing any additional parasites to circulate through the spleen. The accumulation of

mature blood-stage parasites along with unparasitized RBCs, hemozoin, and macrophages within the spleen during an acute malaria episode explains the splenomegaly observed in some malaria patients [67].

The ability of *P. falciparum*-infected RBCs to avoid removal by the spleen is dependent on the exportation of a major virulence factor, PfEMP1, to the RBC surface during the mature intraerythrocytic stages . PfEMP1 mediates binding to host endothelial receptors and allows parasitized RBCs to sequester in the microvessels of many organs including brain, lung, kidney, heart, liver, and intestine as well as in dermal, adipose, and muscle tissue [68]. Inoculation of *P. falciparum* into spleen-intact monkeys by David *et al.* resulted in the cyclical disappearance then re-appearance of ring-stage parasitized RBCs demonstrating that parasites were undergoing 48-hour cycles of sequestration [66]. Interestingly, the maintenance of a parasite's ability to sequester requires an intact spleen [66, 69]. In the same series of experiments, parasites from splenectomized monkeys demonstrated reductions in their cytoadherence in an *in vitro* cellular binding assay [66]. Furthermore, *in vivo* parasitemias in these animals increased continuously in daily peripheral blood smears, and mature trophozoite- and schizont-stage parasites were seen in addition to ring-stage parasites. These results indicate that a mixture of developmental stages circulate in the bloodstream of splenectomized patients due to a loss of the cytoadherence phenotype and of the removal of any non-adherent trophozoites and schizonts, [70, 71].

The significance of cytoadherence on the pathogenesis of SM is also seen upon autopsy. Autopsies reveal massive sequestration in the organs of individuals who died of malaria [72, 73]. In the microvessels within the brains of CM patients, sequestered parasitized RBCs co-localize with host microvascular endothelial cell (MVEC) receptors intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1),

and E-selectin [72-74]. Of the receptors, CD36 is most abundantly and ubiquitously found in the body's microvasculature and almost all *P. falciparum* isolates from malaria patients bind to CD36 [75]. The expression levels of this and other receptors on host MVECs vary drastically by organ. The differential affinity of parasites for specific host receptors enables parasitized RBCs to sequester in an organ-specific manner and is believed to affect both the clinical presentation and severity of the infection although studies have yielded conflicting results. In particular, some studies have found that isolates from African UM patients preferentially bind to CD36 while parasites from CM patients demonstrate a phenotype of increased binding to ICAM-1 [75, 76]. Two studies from Southeast Asia have additionally suggested that increased binding to CD36 might be correlated to SM [77, 78] and another showed that CD36 deficiency might result in protection against SMA [79] implying that the correlation between binding phenotype and clinical severity might be dependent on genetic background and intensity of transmission. However, other studies have shown no relationship between binding with either CD36 or ICAM-1 and SM [75, 80]. The globular head of C1q receptor (gC1qR) has also been implicated as an important receptor in cytoadherence, platelet-mediated clumping, and SMA [81, 82]. However, the most dramatic example of the relationship between the parasite's ability to bind to a specific host receptor and clinical disease is in PAM, which is caused by the sequestration of parasites within the placental intervillous space [83]. Sequestration in PAM is mediated by a special subset of PfEMP1 variants that are unable to bind to CD36, but instead bind specifically to chondroitin sulfate A (CSA), which is only expressed in the placenta [84-86].

To achieve stable adherence to endothelium *in vivo*, parasites undergo a number of mechanical interactions with host receptors that parallel the steps that leukocytes undergo to adhere to vascular endothelium [87]. Under flow conditions in a blood vessel, a parasite first makes initial contact through tethering. The parasite then rolls along host endothelium while making contact with various receptors before achieving stable adherence. As a result,

PfEMP1 likely interacts with multiple receptors acting synergistically to promote cytoadherence to the lining of microvessels. The involvement of multiple receptors in this multistep process enhances the overall avidity of the interaction [88, 89]. It appears that ICAM-1, VCAM-1, thrombospondin, and P-selectin promote rolling adhesion in contrast to CD36, which promotes static adherence [87, 90-92]. The initial contact of PfEMP1 with CD36 appears to initiate actin recruitment and CD36 clustering through the phosphorylation of Src family kinases, which strengthens cytoadherence and may, in part, explain CD36's role in static adherence [93].

While cytoadherence clearly allows parasites to multiply to high densities, it also likely plays a role in the pathogenesis of malaria through the obstruction of microvessels and the activation of MVECs. Mechanical obstruction of microvessels results from the narrowing of the effective luminal vessel diameter due to the adherence of trophozoites and schizonts to the endothelium. Rosette formation and increased rigidity of parasitized RBCs also increase local resistance and decrease blood flow within microvessels [94-96]. Autopsies of individuals who succumbed to malaria have revealed that platelets, fibrin, nonparasitized RBCs, and leukocytes are found occluding microvessels in addition to parasitized RBCS [97]. The obstruction of microvessels causes impaired tissue perfusion and ischemia, which contribute to the inflammatory response.

Activation of MVECs results from the PfEMP1-mediated interaction between parasitized RBCs and MVECs and leads to downstream signaling within MVECs. These events result in the release of inflammatory cytokines, the upregulation of adhesion receptors, and the loss of microvascular integrity [98] all of which contribute to malaria's pathogenesis. Activation of MVECs during a malaria episode leads to a positive feedback cycle of concurrent inflammation and coagulation. Inflammation triggers activation of coagulation through the exposure of tissue factor (TF) on MVECs. Conversely, this pro-

coagulant state leads to increased inflammatory cytokine production and expression of receptors [98].

Evidence for the activation of MVECs during *P. falciparum* infection has come from *in vivo* and *in vitro* studies. Levels of soluble adhesion molecules such as ICAM-1, E-selectin, and VCAM-1 are elevated in patients with malaria compared to nonparasitized adults and are further increased in SM patients [99]. Results from immunohistochemical studies performed on tissue obtained from dermal and skeletal muscle biopsies of patients with malaria also show that endothelial cell expression of receptors ICAM-1, P-selectin, and CD36 is correlated with severity [73, 74]. Upregulation of receptors on the endothelium leads to further increases in cytoadherence as well as leukocyte binding [100]. Furthermore, the interaction between parasitized RBC and MVECs has been shown in *in vitro* experiments to expose functional TF on MVECs [101], which activates the coagulation pathway and promotes thrombocytopenia. Members of the coagulation pathway also upregulate cytokine production and thus stimulate further TF expression on MVEC.

In addition to PfEMP1, members of other multigene families including RIFINs and STEVORs are found on the surface of parasitized RBCs [102-105]. Although their function is still not well-understood, several studies have implicated a role for these other VSAs in cytoadherence [106] to CD31 [102] or to uninfected RBCs to form rosettes [103].

#### 1.1.3.3 Development of cerebral malaria

Of the severe, life-threatening manifestations of malaria, CM and SMA are the best-studied because of their impact on malaria mortality. CM develops from the consequences of massive sequestration within cerebral microvessels [107] although significant levels of sequestration occur in other organs during CM, most notably in the gastrointestinal system [108, 109]. Cerebral MVECs express high levels of ICAM-1 and low levels of CD36. Thus, the

binding of parasitized RBCs to ICAM-1, and perhaps to gC1qR as well [81], is particularly important in CM [110]. As described in **1.1.3.2**, cytoadherence of parasitized RBCs contributes to pathogenesis through obstruction and inflammation.

Other factors also play a role in obstruction of microvessels and inflammation in CM. First, rosette formation has been shown to occur at higher rates in CM [61, 111]. Furthermore, rosette-disrupting antibodies [111] and genetic polymorphisms which reduce rosetting (CR1 polymorphisms and blood group O) protect against CM [112]. Second, other blood elements such as leukocytes, fibrin, and platelets have been shown associating with the parasitized RBCs obstructing microvessels at autopsy [97]. Third, mononuclear leukocytes and platelets induce inflammation. Activation of monocytes by parasite antigens leads to release of proinflammatory cytokines such as TNF, which induces ICAM-1 expression on the cerebral endothelium and increases cytoadherence [73]. However, most studies examining serum levels of TNF in children with UM and SM have not found a significant difference between TNF levels in CM and UM cases [113-115], although a couple of studies have found lower TNF levels in SMA cases compared to UM cases [116, 117].

Autopsy studies of patients who have succumbed to CM have revealed the presence of macrophages containing malaria parasite pigment such as hemozoin, and edema within the brain, which sometimes causes frank herniation. Petechial hemorrhages in cerebral and cerebellar cortices, which are indicative of disruption of the blood brain barrier (BBB) in these patients, are also found upon autopsy of CM patients [118-126] and also contribute to the edema these patients experience. Additionally, the loss of endothelial-cell junctional proteins ZO-1, occludin, and vinculin particularly in cerebral vessels with sequestered parasitized RBCs, provide further evidence of BBB disruption in CM patients [127]. On clinical examination, many CM patients present with retinopathic findings such as retinal whitening, hemorrhages, vessel changes, and papilledema. These

indicators of retinopathy correlate well with autopsy findings, are predictive of clinical outcomes, and may be used as clinical tool for diagnosing and monitoring CM [128-133].

#### 1.1.3.4 Development of severe malaria anemia

SMA, which is clinically characterized by fatigue, malaise, and dyspnea, is driven by the removal of both parasitized and nonparasitized RBCs as well as suppression of erythropoiesis. The relative contribution of these two mechanisms to SMA development is likely determined by host immunity. In areas of low endemicity where most individuals lack immunity, acute removal of RBCs is more likely to rapidly drop Hb levels to <5 g/dL and result in SMA. In contrast, in areas of higher endemicity where individuals are chronically parasitized, Hb levels may gradually drop as removed RBCs are not sufficiently replaced by erythropoiesis. It is also important to note that an individual's baseline Hb level is an important factor in the development of SMA. Baseline Hb levels are affected by a number of environmental and genetic factors such as micronutrient deficiencies, inheritance of RBC polymorphisms such as HbS or HbC, or chronic infection with HIV or parasites.

Both parasitized and nonparasitized RBCs are removed from the peripheral circulation during an acute malaria episode. Parasitized RBCs are removed by one of a couple mechanisms. First, the remodeling of the RBC by *P. falciparum* causes the loss of RBC deformability as the parasite matures into trophozoites and schizonts [26] leading to the removal of any circulating mature forms from the peripheral circulation by the spleen [66]. Consequently, any circulating mature forms are retained by the spleen and removed from the peripheral circulation [66]. Second, parasitized RBCs are opsonized by immunoglobulin (Ig) G and complement, which leads to their phagocytosis by macrophages [134, 135]. However, the removal of parasitized RBCs does not account for the majority of RBC loss during a malaria episode. Multiple studies have suggested that up to 10 times more nonparasitized RBCs are destroyed in *P. falciparum* infection than parasitized RBCs

[136-139]. Therefore, removal of nonparasitized RBCs also plays an important role in the development of SMA. While the mechanisms of removal of nonparasitized RBCs is still not well-understood, potential mechanisms include the stimulation of macrophage phagocytosis by the imbalance of proinflammatory and anti-inflammatory cytokines, removal by the spleen due to reduced RBC deformability, and accelerated senescence due to oxidative stress.

SMA may also result from a diminished ability to replace RBCs removed over the course of infection. Patients with SMA may experience parenchymal damage in the bone marrow, dyserythropoiesis, and impaired erythropoiesis [140]. Erythropoietin appears to be appropriately elevated in these children according to their degree of anemia [141]. Instead, the reduced erythropoiesis may be due the effects of hemozoin on the regulation of inflammatory mediators, such as TNF, interferon (IFN)-γ, interleukin (IL)-10, and IL-12 [142, 143]. Dysregulation of such cytokines leads to a pro-inflammatory state, which may prevent a proper response of RBC precursors to erythropoietin. Therefore, the imbalance of pro- and anti-inflammatory cytokines may play a role in SMA through both removal of RBCs and failure to replace them.

### 1.1.4 Diagnosis, treatment, and prevention of malaria

Eradication of malaria remains a major goal for the WHO. Considerable resources have been dedicated to reducing malaria incidence, morbidity, and mortality through preventative measures and treatment over the last century. These efforts have succeeded in shrinking the geographical limits of malaria transmission particularly in temperate areas such as the United States, Greece, and Italy in the post-World War II era [144]. Accurate diagnosis, effective treatment, and successful vector control are necessary to control malaria in countries with high transmission and to eliminate malaria in countries with low endemicity. Significant efforts to develop a malaria vaccine have been unsuccessful thus far

although the recent vaccine RTS,S has shown some promise. Studies involving RTS,S will be discussed further in **1.1.4.4**. Reports of drug resistance to artemisinin, the crucial drug in the current first-line of treatment of artemisinin-based combination therapies (ACTs), have also threatened elimination efforts.

#### 1.1.4.1 Diagnosis

Diagnosis of malaria is typically made by microscopic evaluation of peripheral blood smears alongside clinical assessment. However, the parasite density demonstrated by such a smear does not reflect the full parasite load since mature forms of the parasite remain sequestered in deep organs. Large differences in parasitemias calculated from peripheral blood smears may be observed over a period of several hours as a result of sequestration and synchronous development of parasites [20]. In isolated, rural villages where either a microscope or an expert microscopist is unavailable, rapid diagnostic tests (RDTs) can be used in place of a peripheral blood smear [38]. These tests rely on detection of histidine rich protein 2 (HRP-2), a protein which is released from blood-stage parasites [145]. HRP-2 has also been used to estimate the total parasite biomass since the levels of HRP-2 correlate with the total number of circulating and sequestered parasites [146].

#### 1.1.4.2 Antimalarial drugs

Quinine was initially discovered in the powdered bark of the cinchona tree and used by the indigenous Quechuas in South America for treatment of ‘fever’ [147]. The Jesuits introduced the drug to Europe in the 1600s, and since then, much effort and many resources have been dedicated towards developing new and effective chemotherapeutics to treat *P. falciparum* malaria. Most chemotherapeutics used for treatment of malaria today work by interfering with the metabolism of folate (such as sulfadoxine and pyrimethamine) or with the metabolism of Hb (such as quinine and chloroquine).

In particular, chloroquine (CQ) [148] has played an especially important role in the history of malaria treatment and prophylaxis. Before the emergence of CQ-resistant strains of *P. falciparum* in the 1960s, CQ was used widely, most notably in the WHO's Global Eradication Program, due to its safety, affordability, and efficacy. CQ acts upon the parasite's ability to digest Hb and prevents its polymerization into hemozoin by interacting with toxic ferriprotoporphyrin IX (FPIX) [149]. Although the exact mechanism by which FPIX exerts its toxic effects is still being elucidated, the inability of the parasite to detoxify FPIX in the presence of CQ leads to the parasite's death. However, the widespread use of chloroquine in eradication efforts led to the development and spread of resistant strains globally. It is estimated that 80% of wild isolates are CQ-resistant, making it functionally useless in the clinical treatment of *P. falciparum* malaria [150].

Resistance to CQ is caused by a mutation in the gene which encodes for the chloroquine resistance transporter (PfCRT) [151]. This 10 domain transmembrane transport protein is located in the membrane of the DV and in its wildtype state prevents movement of CQ across the DV membrane thereby retaining the drug in the DV [11], CQ's putative locus of action [152]. The K76T mutation replaces a positively charged side chain with a neutral moiety, which allows transport of CQ from the DV to the cytosol resulting in the drug's functional neutralization [153]. Resistance to other antimalarials continues to be a major concern especially since a certain degree of cross-resistance exists between antimalarials.

Due to this high degree of resistance to many of the antimalarials previously used, the mainstay of falciparum malaria treatment today is ACTs. Artemisinin was first isolated in 1972 from *Artemisia annua*, an herb also known as "sweet wormwood," although the Chinese had used this herb for over 1000 years in the treatment of fever and chills. The mechanism of artemisinin's plasmodicidal action is intensely debated and not well-

understood. However, it is well-established that artemisinin is capable of acting rapidly to clear parasites from the peripheral circulation and to resolve symptoms of malaria [154]. However, as a result of their short half-life and increasing concerns about artemisinin resistance [155, 156] due to use of artemisinin monotherapy and counterfeit drugs [157, 158], a 3-day treatment course of artemisinin or one of its derivatives is combined with a longer-acting partner drug to ensure that all parasites are cleared [38]. Artemisinin resistance on the border of the Thai-Cambodian border was first reported in 2008 [159], and has since been confirmed by other studies [160, 161].

ACTs are also used for treatment of *P. vivax* in areas where CQ-resistant *P. vivax* has developed. However, neither ACTs nor CQ are sufficient to achieve radical cure for vivax malaria since both clear blood-stage, but not the liver-stage parasites. The only drug currently known to eliminate hypnozoites is primaquine [38], which is rarely given due to the high prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency within malarious areas [162]. Although G6PD deficiency has been shown to provide protection against malaria (see **1.2.3.1**), primaquine's oxidant properties cause hemolysis in individuals carrying this mutation [163].

#### 1.1.4.3 Vector control

Vector control also plays an important role in reducing the global burden of malaria through the use of such measures as indoor residual spraying (IRS) and insecticide-treated nets (ITNs). The outdoor spraying of long-acting insecticide dichlorodiphenyltrichloroethane (DDT) played a large role in the elimination of malaria transmission in many countries, including the United States. Despite concerns about the ecological impact and health effects of DDT, it is still used for IRS to reduce mosquito populations in countries that have not yet eliminated malaria [164-167]. The distribution and use of ITNs has been an effective method of reducing malaria incidence by not only providing a physical barrier between the

nocturnally-biting female *Anopheles* mosquito and the individual, but also by killing mosquitoes that come into contact with the nets [168]. In addition, environmental measures, including draining mosquito-breeding sites, have been used to reduce mosquito populations [169]. Lastly, genetically-modified mosquitoes that are resistant to parasite infection, have a reduced lifespan, or produce sterile offspring have been proposed as potential tools for vector control [170].

#### 1.1.4.4 Vaccine efforts

Research efforts to develop a safe, effective vaccine against falciparum malaria have failed to produce a licensed vaccine. However, several candidates in recent years have shown promise. One of these candidates, RTS,S, has generated much interest due to its relative success in successive clinical trials and is currently in phase III efficacy trials with 15,000 enrolled children over 11 sites in 7 African countries [148]. RTS,S is a subunit vaccine that induces antibody and CD4 T cell responses to circumsporozoite protein, which is expressed on the surface of sporozoites. When partnered with the AS01 or AS02 adjuvant system, RTS,S has shown 30-50% protection against malaria, which makes it the most effective candidate to date [171].

Other groups have taken a whole *P. falciparum* sporozoite vaccination approach. This approach is based on the finding that immunization by mosquito bite with attenuated whole sporozoites leads to sterile immunity [172]. Current research on whole sporozoite vaccines examines the efficacy of radiation-attenuated sporozoites, genetically-attenuated parasites, and infection-treatment immunization. In the radiation-attenuated sporozoite approach, sporozoites are able to infect hepatocytes, but are unable to complete liver stage development due to radiation damage [173, 174]. This approach has been most extensively studied of the whole sporozoite approaches in clinical trials, but results thus far have been disappointing due to low immunogenicity by intradermal or subcutaneous delivery [175].

Experiments performed in rodents and non-human primates suggest that an intravenous delivery route may provide greater immunogenicity [175].

### 1.1.5 Immunity to malaria

Both the innate and adaptive arms of the immune system play important roles in the host response to *P. falciparum* infection. The innate arm mediates the cytokine response to the erythrocytic stage of the parasite. Although cytokines are important in combating the parasite, they are also responsible for many of the disease manifestations associated with parasite infection. One such cytokine, TNF, is released from monocytes as a result of cytoadherence interactions with infected RBCs or recognition of parasite-derived factors by Toll-like receptors (TLRs) and the resulting downstream signaling. Although TNF helps control parasite densities, it also contributes to malaria pathogenesis through impaired erythropoiesis, increased expression of TF, and upregulation of adhesion receptors on cerebral MVECs, which then allows for sequestration of parasitized RBCs. Accordingly, children with SM and fatal outcomes have higher levels of TNF than those with uncomplicated malaria or asymptomatic parasitemia [72, 176, 177].

The humoral arm of the immune system is responsible for the age dependence of malaria protection in areas of high endemicity. In these areas, repeated exposure to *P. falciparum* malaria leads to acquisition of species-specific naturally acquired immunity (NAI). Our understanding of the development of NAI to *P. falciparum* malaria began with Robert Koch's comparison of the frequency and density of parasitemia in areas of high and low endemicity in 1900. He observed age-dependent protection against SM in the area of high endemicity, but not in the area of low endemicity causing him to conclude that heavy and uninterrupted exposure to malaria is necessary for development of immunity [178].

#### 1.1.5.1 Naturally acquired immunity to falciparum malaria in endemic areas

The development of NAI [179] to *P. falciparum* malaria is a gradual process characterized by the ability to control disease as well as parasitemia. Both components show a strong age-dependent effect, but are distinct from each other. Anti-disease immunity is rapidly acquired and reduces the risk of the progression from asymptomatic parasitemia to UM to SM for any particular parasitemia. Protection against SM develops first perhaps as a result of only requiring a few infections to develop [180, 181].

In contrast, anti-parasite immunity is developed over a longer period of time and protects against severe manifestations of malaria by conferring the ability to control parasite density [182]. This ability to prevent high-density parasitemias seems to be the primary mechanism responsible for protection against disease in adults since adults from endemic areas have a decreased relative risk of developing high parasite densities compared to young children. However, adults that develop high parasite densities progress to disease at similar rates to young children [183]. Epidemiological data are consistent with this finding and suggest that antidisease immunity has a shorter half-life (~5 years) in contrast with anti-parasite immunity (>20 years) [184]. Sterile immunity is never achieved even in adults so adults are often asymptomatically carriers of parasites.

Evidence for protection by antibodies against malaria antigens has come from passive transfer experiments as well as longitudinal studies comparing the host's level of antibodies with their subsequent clinical course. Passive transfer of antibodies from immune adults, but not from naïve European individuals, has been shown to be sufficient to reduce parasitemias in children with malaria [185]. Other groups saw similar reductions in parasitemia when treating Thai malaria patients with African immune serum [186, 187]. Furthermore, longitudinal studies shown correlations between various malaria antigens including AMA1, MSP1, and MSP2 and protection against malaria [188-190].

#### 1.1.5.2 Naïve individuals and transmigrants

The effectiveness of the NAI that protects adults in endemic areas is dramatic when comparing to the clinical course of naïve individuals. Even very low parasite densities usually cause clinical symptoms in naïve individuals of any age. Adults from endemic areas who migrate to areas without malaria transmission are susceptible to clinical disease when they return to a malaria-endemic area although the course of their illness may be milder and shorter [191-195]. This observation suggests that frequent and continuous exposure is needed to maintain the almost complete protection against clinical disease seen in adults in endemic areas, but migrants from these areas may retain some level of protection.

#### 1.1.5.3 Immunity in young infants

Young infants are an exception to the general pattern of increasing immunity with age. Infants under 3 months of age (and up to 6 months in some cases) show remarkable protection against high parasitemias, clinical disease, and SM [196, 197]. The protective effect of maternal IgG transferred through the placenta *in utero* is often invoked to explain this widely accepted epidemiological observation. The role of maternal IgG in protecting young infants against malaria is supported by the findings that the levels of IgG are similar in both the mother and cord blood at birth [198] and that the plasma from a 3-month-old living in a malaria-endemic area agglutinated a number of heterologous isolates presumably because the child still carried maternal IgG. However, these findings are complicated by a study that found that there is little correlation between parasite-specific maternal IgG levels and time to first infection, parasite density, and incidence of clinical episodes for the infant [199]. Additional protection may be mediated by either by parasite growth-inhibiting lactoferrin found in breast milk, by parasite growth restriction within cells containing fetal hemoglobin (HbF), by reductions in cytoadherence of parasitized HbF-containing RBCs, or by some combination of these mechanisms [179, 200-202].

#### 1.1.5.4 Immunity to pregnancy-associated malaria

Despite the significant anti-malarial immunity acquired before pregnancy, pregnant women experience a marked increase in susceptibility to *P. falciparum* malaria. Over the course of pregnancy, women acquire immunity to PAM leading to a gestation-dependent decrease in parasitemia and risk of developing PAM in subsequent pregnancies. This susceptibility is primarily the result of the expression of CSA on placental syncytiotrophoblasts in pregnancy which allows the accumulation of infected RBCs in placental tissue through cytoadherence to CSA [203, 204]. Maternal immunosuppression may also play a role in this susceptibility. Immunity to PAM is mediated by acquisition of antibodies that block binding of parasitized RBCs to CSA within the placenta. The importance of these antibodies in immunity to PAM is highlighted by the observation that VAR2CSA-specific antibodies are parity-dependent. The level of antibodies within multigravidae correlate to the number of pregnancies they've experienced [203, 205-207]. Children, men, and primigravidae lack VAR2CSA-specific antibodies [205-208].

## 1.2 Genetic modifiers of malaria risk

Malaria's long history of coevolution alongside its mosquito and primate/human hosts has led to the imprinting of its influence on the human genome. Human genetics have been estimated to account for up to 25% of malaria risk in malaria-endemic areas [209]. In particular, host RBC polymorphisms including Hb variants and thalassemias have evolved as a result of the enormous morbidity and mortality burden caused by *Plasmodium*. The connection between these polymorphisms and protection from malaria was initially proposed because of their overlapping geographic distributions [210-212]. In 1949, Haldane first suggested that the relatively high frequencies of β-thalassemia, which in its homozygous state is associated with severe anemia leading to death within the first two years of life if untreated, might be the result of positive selection of the heterozygous state conferred by protection against malaria [213]. A large body of evidence now supports the idea that RBC polymorphisms have reached their current gene frequencies as a result of this protection. This chapter briefly describes each of the RBC polymorphisms, summarizes the evidence supporting the role of RBC polymorphisms and other genetic modifiers in protection against malaria, and discusses the proposed mechanisms of protection.

### 1.2.1 Hemoglobin variants

Normal adult hemoglobin (HbA) metalloproteins are normally comprised of four globular subunits, two α-globin chains and two β-globin chains, which together transport oxygen from pulmonary capillaries to the rest of the body as well as remove carbon dioxide from these tissues. Single amino-acid replacements in the β-globin gene are responsible for malaria-protective hemoglobin S, C, and E variants as well as others, which have not been studied extensively for their role in malaria protection. The WHO estimates that 7% of the worldwide population carries one or more hemoglobin mutation [214]. The presence of

HbF, the main form of Hb in young infants, has been used to explain the malaria protection in these children and is discussed in this section as well.

#### 1.2.1.1 Sickle hemoglobin (HbS)

HbS is caused by a mutation in the 17$^{th}$ nucleotide of the β-globin gene from thymine to adenine, which causes the sixth amino acid to change from glutamic acid to valine. The change from a hydrophilic glutamic acid to a hydrophobic valine leads to the propensity for interaction between the β1 of one hemoglobin molecule to the β2 of another hemoglobin molecule in the deoxygenated state. The resulting crystallization fills the RBC and transforms the normally extremely deformable sac into a highly rigid and dehydrated sickle-shaped RBC. In the homozygous form HbSS, the sickling of deoxygenated hemoglobin leads to episodic vaso-occlusion with ischemia-reperfusion injury and hemolytic anemia, a condition known as sickle-cell anemia (SCA).

RBCs containing HbS contribute to the pathogenesis of SCA in four main ways. First, the loss of deformability and resulting sickled shape of the RBC in its deoxygenated state leads to vaso-occlusion and ischemia [215, 216]. Infarction may result in multiple organs and lead to painful crises, acute chest syndrome, hyposplenism, osteonecrosis, nephropathy, and acute stroke. Second, while reperfusion restores blood flow and oxygen transport to tissue, it causes further damage to tissues by generation of free radicals and oxidative damage [217, 218]. Third, free Hb released into plasma by hemolysis of RBCs binds to nitric oxide (NO) and creates a functional NO deficiency. Since NO plays an important role in proper vasodilation, its decreased availability leads to increased vascular tone and pulmonary artery hypertension [219, 220]. Fourth, sickled RBCs are able to adhere to endothelium lining microvessels, which not only promotes vaso-occlusion, but also activates MVECs [221]. Activation of MVECs leads to release of cytokines and recruitment of inflammatory mediators.

Two modifiers of the clinical presentation and outcome of SCA are HbF concentrations and co-inheritance of α-thalassemia. The presence of high levels of HbF, which lack β globin chains, in these patients past infancy reduces many of the complications of the disease and has been shown to improve life expectancy by decreasing sickling [222-224]. HbF concentrations in these patients range between 1-30% and are determined by three major loci as well as likely other minor loci yet to be identified. The benefit of increased HbF concentrations to HbSS individuals has led to the development of hydroxyurea and other therapies which aim to shift from the production of HbS to HbF [225, 226]. α-thalassemia also leads to amelioration of SCA due to reductions in Hb concentration within the RBC, which reduce the likelihood of polymerization. Due to the geographical overlap of HbS and α-thalassemia trait, coinheritance may occur in up to 30% of HbSS children [227]. Although individuals with co-inherited α-thalassemia may experience some clinical benefits, pain frequency has not been shown to decrease and may actually increase due to increased hematocrit and blood viscosity [228].

The major causes of death in individuals with SCA include chronic organ failure, acute stroke, acute chest syndrome, and infection [222]. Splenic dysfunction, micronutrient deficiencies, and tissue ischemia likely contribute to these individuals' increased susceptibility to bacterial infection, with *Streptococcus pneumoniae* and *Haemophilus influenzae*, in particular, being of particular concern in children with SCA. As a result, children with SCA should be vaccinated against *S. pneumonia*e and *H. influenzae.* As recently as 1973, the median survival of SCA patients was estimated to be 14.3 years in the United States [62]. The advent of new treatments and therapies in the context of SCA such as blood transfusions and prophylactic penicillin [229-231] led to dramatic increases in life expectancy within the United States to approximately 42 years for men and 48 years for women by 1994 [222] and is likely still increasing with improvements in clinical

management of SCA. Predictably, children living in Africa have yet to realize the benefits of these advances and often die undiagnosed and untreated in early childhood.

Sickle-cell trait, the heterozygous state of HbS (HbAS), is generally believed to be relatively benign. HbAS individuals have normal hemoglobin levels and have normal life expectancies. However, sickle-cell trait is also associated with hematuria, hyposthenuria, splenic infarcts, and exercise-related deaths [232]. Although exceedingly rare, renal medullary carcinoma is found almost exclusively in young HbAS individuals and is an extremely aggressive form of cancer with a median survival of approximately 17 weeks [233].

HbS is a classic example of a balanced polymorphism. Despite the early mortality of HbSS children, HbS has reached polymorphic frequencies as a result of the significant protection HbAS children experience against UM and SM. Direct evidence of protection by HbS was first shown by Allison in 1954 when he inoculated 'sickling' and 'non-sickling' adult African males with *P. falciparum* and found that men carrying the sickle-cell trait were less likely than men without sickle-cell trait to develop detectable parasitemia or malaria symptoms [234].

While studies of other RBC polymorphisms have sometimes led to conflicting or inconclusive results, the selection of HbS as a result of its protective effects against malaria is well established and is based on several lines of evidence. First, HbS is found primarily in African populations and is associated with four region-specific African haplotypes (the Senegal, Benin, Bantu, and Cameroon haplotypes) as well as one Asian haplotype (the Arab-India haplotype) [235]. The presence of the HbS mutation on five separate haplotypes implies that HbS arose up to five separate times [236-238] although gene conversion may have been responsible for the different African haplotypes [239]. Nevertheless, that the HbS mutation arose at least twice indicates that it provides some survival benefit within

each of those settings. Second, that the HbS allele is found at its highest frequencies in Africa, where historically and presently malaria transmission is most intense, suggests that the HbS mutation might have been selected for its protection against malaria [210, 240-245]. Third, epidemiological studies have supported this possibility. Since Allison's initial study, further epidemiological studies have strongly reinforced the role of HbS in protection against both UM and SM [246-256]. HbAS has been estimated by meta-analysis to provide 31% protection against UM and 91% protection against SM [257]. Additionally, a prospective study of Malian children showed HbAS children experienced a delayed time to first malaria episode compared to HbAA children [258]. In contrast, cross-sectional studies examining the prevalence of asymptomatic *P. falciparum* parasitemia and parasite densities in HbAS children have shown conflicting results. Some studies have reported reduced asymptomatic parasitemia prevalence [234, 259-261] in HbAS children, while the majority of studies have reported no difference compared to HbAA children [262-271] with a couple studies reporting increased prevalence [272, 273]. Similarly, some studies reported decreased parasite densities in HbAS children compared to HbAA children [234, 263, 266, 273, 274], while others reported similar parasite densities [262, 267, 269, 272]. Taken together, these findings suggest that HbAS children have similar rates of *P. falciparum* infection, but that they are less likely to progress to UM and very rarely progress to SM.

#### 1.2.1.2 Hemoglobin C (HbC)

HbC is the result of a mutation from glutamic acid to lysine in the same amino acid position as HbS (sixth of the β globin chain). The mutation is primarily found in West Africa where its prevalence approaches 35% in some populations. In contrast to the severe clinical manifestations of HbSS, HbCC individuals experience mild anemia and splenomegaly, but are typically otherwise healthy [275, 276]. Microscopic analysis of blood from HbCC individuals reveals microspherocytes and the formation of hexagonal crystals within HbC-

carrying RBCs known as “target cells” [277-279]. HbAC children typically do not exhibit any of the clinical manifestations typical of HbCC children. Interestingly, children who have simultaneously inherited copies of HbS and HbC (HbSC) experience a moderately severe phenotype despite the relatively minor phenotypes attributable to HbAS and HbAC individually. This phenomena is likely attributable to the dehydrated state of HbSC RBCs due to HbC, which increases the Hb concentration within the RBC and consequently, the frequency of HbS polymerization [280, 281].

HbCC RBCs show increases in cell density and osmotic resistance as well as a loss of cell volume [282-284]. These changes within HbCC RBCs result in progressively elevated mean corpuscular hemoglobin concentration (MCHC) levels within the RBC and a tendency to form the characteristic HbC crystals [279, 285]. These changes in MCHC along with increased formation of hemichromes due to the instability of HbC, lead to accelerated senescence of RBCs. Accordingly, HbCC RBCs have a shortened lifespan of 30-50% of the normal 120-day RBC lifespan, which results in elevated reticulocyte counts in HbCC individuals [286-288].

Although epidemiological studies generally agree that HbC confers protection against SM, the magnitude of protection appears to be less than that conferred by HbS. Its protection against SM in its heterozygous state has been estimated to be a 20% reduction in relative risk and in its homozygous state, a 73% reduction compared to HbAA children based on four case-control studies [246-249, 289]. Studies examining the incidence of UM in HbAC and HbAA children have shown no significant protection by HbC against UM [247, 255, 258, 289-291].

#### 1.2.1.3 Hemoglobin E (HbE)

The mutation of the 26$^{th}$ amino acid from glutamic acid to lysine encodes for HbE, which is found almost exclusively in Southeast Asia and the Indian subcontinent [292-296]. The mutation is common in this region with the prevalence of HbE reaching 50% in some areas [297]. HbEE individuals usually have mild anemia and minor splenomegaly, but are otherwise healthy. HbAE individuals are generally asymptomatic. While HbAE RBCs are morphologically similar to HbAA RBCs, HbEE RBCs are hypochromic and microcytic [298].

The relative prevalence of the polymorphism in populations within Southeast Asia has been linked to the variation of malaria prevalence within the region. For example, the prevalence of HbE was higher in three hill regions, which traditionally also had a higher prevalence of malaria, than the plains [295]. Although this association has led to the hypothesis that HbE protects against malaria in Southeast Asia as HbS and HbC do in Africa, the few epidemiological studies on malaria protection by HbE have yielded conflicting and inconclusive results. One study conducted using Thai patients hospitalized for malaria showed that HbAE individuals were protected against SM [299], but other studies found no significant protection against SM [300, 301].

#### 1.2.1.4 Hemoglobin F (HbF)

Unlike the other Hb variants discussed previously, which are found in only a subset of individuals, HbF is universally used as the main oxygen transport protein during the last 7 months of prenatal development and in the first couple months of infancy. In HbF, the 2 β-globin chains found in HbA are replaced with 2 γ-globin chains, which differ from β-globin chains by either 39 or 40 amino acid residues [302]. This replacement of β-globin chains is responsible for the amelioration of SCA in individuals with high levels of HbF. The presence of the γ-globin chains also results in a left shift of the oxygen saturation curve for HbF as a result of its increased affinity for oxygen. In most individuals, HbF is gradually replaced by HbA as the primary Hb variant over the first year of life, with adults making a residual (<1%)

amount of HbF throughout their lives. Some individuals, however, continue to make 10-50% HbF into adulthood in a condition called hereditary persistence of fetal hemoglobin (HPFH).

In contrast to the other Hb variants, the epidemiological protection due to HbF against malaria cannot be easily assessed since HbF is found exclusively in infants. However, young infants are remarkably protected against malaria, and studies have implicated the presence of HbF within young infants, along with maternal IgG and transfer of lactoferrin through breast milk, in this protection [179, 200-202].

### 1.2.2 Thalassemias

Thalassemias result from reduced synthesis of either the α-globin (α-thalassemia) or β-globin (β-thalassemia) chains. Both α- and β-thalassemias are believed to have reached polymorphic frequencies as a result of their protection from malaria.

#### 1.2.2.1 α-thalassemia

α-thalassemia describes a wide-ranging set of autosomal recessive conditions caused by a deficit in synthesis of the α-globin chain. α-globin is encoded by 4 α-globin genes, 2 on each copy of chromosome 16. Since Hb comprises 30-50% of the RBC content, the reduction in the Hb present in the cell results in paler (hypochromic) and smaller (microcytic) than normal RBCs, with a higher surface area to volume ratio. Approximately 128 different molecular defects in α-globin genes may lead to spectrum of clinical phenotypes, which range in severity from nearly asymptomatic to fatal *in utero* or shortly after birth. The clinical severity of the disorder correlates well with the number of mutated or deleted α-globin genes, with deletional defects generally leading to less clinically severe phenotypes than non-deletional defects [303, 304].

Defects encoding for α-thalassemia are extremely common in the Mediterranean countries, Southeast Asia, Africa, the Middle East, and the Indian subcontinent, with certain

mutations found more frequently within specific populations [305]. For example, the 3.7 kb deletional determinant of α-thalassemia, which results from reciprocal recombination between Z segments, is commonly found in sub-Saharan Africa where its prevalence may reach 50% in some areas [306, 307]. Heterozygotes ($-\alpha^{3.7}/\alpha\alpha$) for this deletion experience the loss of one α-globin gene and are typically healthy while homozygotes ($-\alpha^{3.7}/-\alpha^{3.7}$) lack two α-globin genes and have a mild microcytic anemia. Although this defect is exceedingly common in sub-Saharan Africa with areas that have up to 50% prevalence of this defect within their population, the *cis* deletional forms of α-thalassemia, which result in the loss of both α-globin genes on the same chromosomal copy, are much less common in Africa and more common in Southeast Asia [308]. Therefore, HbH disease (−/−α), which is associated with formation of β4 tetramers due to excess β-globin chains, is found more commonly in Southeast Asia than in sub-Saharan Africa.

α-thalassemia has been shown to protect against SM in a dose-dependent fashion [248, 309-315], possibly due to SMA-specific protection [248, 310]. Studies examining the effect of α-thalassemia on UM have found no significant effects [258, 312, 315, 316]. Interestingly, the co-inheritance of α-thalassemia along with HbS (discussed in **1.2.1.1**) has been shown in epidemiological studies to reduce the protection provided by either trait alone [248, 312]. In one study, HbAS children demonstrated almost complete abrogation of their protection against UM and SM with co-inherited homozygous α-thalassemia [312]. These findings may partially explain the varying frequencies of RBC polymorphisms in different geographic areas [317].

#### 1.2.2.2 β-thalassemia

β-thalassemia is a group of blood disorders characterized by the abnormal synthesis of β-globin chains found primarily in the Mediterranean area, the Middle East, Central Asia, the Indian subcontinent, Southern China, Southeast Asia, northern Africa, and South

America. As a result of its partially overlapping geographical distribution with HbE, the coinheritance of HbE/β-thalassemia is relatively common, particularly in Southeast Asia. In contrast to α-thalassemia, β-thalassemia is usually caused by point mutations within the β-globin gene located on chromosome 11; β-thalassemia resulting from deletions of this gene is less common. These mutations lead to a relative excess of unbound α-globin chains, which cause premature death of erythroid precursors through their precipitation.

The most serious form of this disorder, β-thalassemia major, usually presents within the first 2 years of life and leads to a severe, blood transfusion-requiring microcytic anemia. Although untreated β-thalassemia major leads to growth retardation, pallor, jaundice, and other sequelae, frequent blood transfusions leads to iron-overload and its own set of complications including myocardiopathy, endocrine complications, and liver fibrosis and cirrhosis. Individuals with β-thalassemia intermedia or minor experience a much less severe clinical phenotype, with few clinical symptoms resulting from β-thalassemia minor.

The high prevalence of β-thalassemia in certain populations led Haldane to first propose that RBC polymorphisms might have achieved their frequencies as a result of their protection against malaria [213]. Further studies on a regional level have associated the prevalence of this trait with historical malaria prevalence among Sardinian villages [318] and in Melanesia [319]. Despite these correlations, few epidemiological studies have been done examining the effect of β-thalassemia on malaria protection. However, one case-control study conducted in Liberia showed a reduced relative risk of hospitalization-requiring malaria for β-thalassemic individuals [290].

### 1.2.3 Other malaria-protective RBC polymorphisms and blood groups

In addition to the hemoglobinopathies and thalassemias, groups have noted malaria-protective effects by various other RBC polymorphisms including G6PD deficiency,

South Asian Ovalocytosis, and specific blood groups such as ABO blood group O. Here, I discuss G6PD deficiency and ABO blood group O in light of these findings.

#### 1.2.3.1 Glucose-6-phosphate dehydrogenase (G6PD) deficiency

G6PD deficiency is an X-linked genetic defect in the *G6PD* gene, which leads to reductions in enzymatic activity. In its normal state, G6PD plays a major role in protecting the RBC against oxidative damage through catalyzing the rate-limiting first reaction of the pentose phosphate pathway. In the process of oxidizing glucose-6-phosphate to 6-phosphogluconolactone, G6PD produces the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH, which serves as an electron donor for many critical biosynthetic enzymatic reactions, counteracts the effects of oxidant agents such as hydrogen peroxide and oxygen radicals [113, 114], and preserves glutathione (GSH) in its reduced form [67]. Although G6PD is a housekeeping gene constitutively expressed in all cells types at varying concentrations, the partial loss of G6PD activity in G6PD-deficient individuals is particularly notable for its effects in RBCs. Since RBCs lack organelles including mitochondria, the G6PD-catalyzed reactions serve as the only source of NADPH within RBCs [116].

In 1956, Carson noted that individuals who developed severe hemolytic anemia after administration of primaquine had low levels of G6PD [54, 55]. Later, others noted the similarities between the severe hemolytic anemia experienced by G6PD-deficient individuals after administration of primaquine with the anemia associated with ingestion of fava beans in certain individuals, which led to the discovery that they also had low G6PD activity [62, 63]. It is now appreciated that 400 million people worldwide [40, 163] carry one of the 140 mutations that encode for variation in G6PD activity [115]. The rates of G6PD deficiency are highest in sub-Saharan Africa, the Middle East, and the Southeast Asia, but mutations in the *G6PD* gene are also present in the Mediterranean region, the Indian

subcontinent, Oceania, and South America [28]. As the large number of genetic variants encoding for G6PD deficiency suggests, the condition demonstrates a large degree of biochemical and clinical variability with individuals having anywhere between normal G6PD activity to almost complete obliteration of functional enzymatic activity. This variability is affected not only by the specific G6PD genetic variant, but also the absence of a wildtype copy of the *G6PD* allele in hemizygous males since G6PD deficiency is an X-linked disease. The presence of a wildtype *G6PD* allele in heterozygous females, who as genetic mosaics through X-chromosome inactivation carry both RBCs with normal activity and reduced activity, may ameliorate their clinical phenotype. Individuals may experience clinical manifestations ranging from no symptoms to chronic non-spherocytic hemolytic anemia [28], but the majority of individuals with inherited G6PD deficiency have no anemia or hemolysis until challenged by exogenous oxidative agents such as fava beans or primaquine [320].

As noted previously, the distribution of G6PD deficiency overlaps well with the map of historical malaria prevalence. Although a number of studies have shown protection by G6PD deficiency against SM, there is disagreement on the relative degree of protection by genotype. Studies performed have yielded results that span the entire spectrum including: reduced parasite densities in heterozygous females, but not hemizygous males; equal protection against SM by both heterozygous and hemizygous states; and protection against SM for hemizygous males, but not heterozygous females. This last result in which hemizygous males lacking a wildtype G6PD allele experienced ~80% protection against SM and heterozygous mosaic females experienced none, seems to correspond best to our understanding of how genotype affects the level of G6PD activity.

#### 1.2.3.2 Type O blood group antigen

The ABO locus, which determines an individual's ABO blood group, is found on chromosome 9. Two of its main allelic forms, A and B, encode for glycosyltransferases that attach either α-N-acetylgalactosamine or α-D-galactose to precursor H antigen respectively. The third main allelic form O contains a frameshift mutation, which prevents production of a functional glycosyltransferase. Type O antigen is relatively common in sub-Saharan Africa, possibly as the result of natural selection by malaria [321].Two epidemiological studies have provided evidence that type O antigen protects against malaria. The first study compared 'non-O' (A, AB, and B, taken collectively) antigens against O antigens in a collection of African countries and the Gambia and found 18% and 26% increases in susceptibility to SM for 'non-O' antigens respectively. The second study showed a 65% decrease in risk of SM attributable to O antigen.

### 1.2.4 Mutations in host cytoadherence receptors

The critical role of cytoadherence in the pathogenesis of malaria has led researchers to investigate whether mutations in the host receptors (i.e., CD36 and ICAM-1) might act as genetic modifiers of malaria risk. Studies showed that the presence of either the 1264T→G stop mutation (in Africa) and in3$(TG)_{12}$ allele (in Southeast Asia), which both encode for CD36 deficiency, were associated with protection of SM [322, 323]. However, conflicting studies suggested that there was either no protective effect conferred by the 1264T→G stop mutation or an increased susceptibility to SM [324, 325].

A mutation in ICAM-1, ICAM-$1^{Kilifi}$, which is present in up to 30% of individuals in some African populations, has shown conflicting evidence in its role in modifying malaria risk. One study showed that the presence of the mutation increased susceptibility to cerebral malaria [326]. Another study suggested that it protected against SM [323]. Still other studies have not supported any association between ICAM-1 polymorphism and

disease severity [327-330]. This mutation may also have been evolutionarily selected as a result of its protection against non-malaria febrile illnesses [331].

*In vitro* studies have shown that this lysine to methionine substitution at position 29 results in isolate-dependent reductions in binding to ICAM-1 in both static and flow conditions [43, 332]. In both conditions, A4 parasites showed more dramatic reductions in relative binding to ICAM-1$^{Kilifi}$ compared IT4 parasites. Since parasite binding to ICAM-1 is mediated by PfEMP1, these findings suggest that ICAM-1$^{Kilifi}$ might select for a population of isolates with PfEMP1 repertoires capable of high-avidity binding to ICAM-1. Selection of a restricted subset of isolates or expressed PfEMP1 variants might reduce the overall parasite burden and serve a protective role. On the other hand, the selection of such a subset might also increase susceptibility to malaria through the virulence of such isolates.

### 1.2.5 Proposed mechanisms of protection

Various mechanisms have been proposed to explain protection against malaria by RBC polymorphisms. In general, these mechanisms fall into 4 categories: reduced parasite multiplication, enhanced innate immunity, enhanced acquired immunity, and impaired pathogenic mechanisms. It is important to note that each of these explanations does not necessarily preclude the role of other mechanisms simultaneously providing protection to children carrying these polymorphisms. The relative roles of each of these mechanisms may also depend on the genetic background of these individuals, the historical endemicity of malaria in the areas in which these children live, and the presence of co-inherited polymorphisms.

#### 1.2.5.1 Reduced parasite multiplication rates

Some of the earliest proposed mechanisms of protection by RBC polymorphisms were based on reduced parasite multiplication, either by reduced invasion, reduced growth,

or increased destruction within variant RBCs. These mechanisms would protect by limiting the total parasite load and the downstream effects of high parasite densities in individuals carrying variant RBCs (described in **1.1.3.1**). Studies examining these mechanisms have yielded conflicting results. Various groups have reported reduced RBC invasion for many of the RBC polymorphisms including α-thalassemia minor, HbH disease, HbEE, HbAE, and β-thalassemia/HbE, and reduced growth for HbH disease, β-thalassemia minor, HbSS, HbAS, HbCC, HbEE, HbAE, and HbF. However, other studies have shown no difference in invasion or growth for many of these genotypes.

Another related possibility involves the effects of low oxygen tension experienced physiologically in post-capillary venules. Three separate groups reported that parasitized HbAS RBCs experienced higher levels of sickling and destruction at low oxygen tension than parasitized HbAA RBCs [333-335]. HbAA, HbAS, and HbSS RBCs supported similar growth rates at high oxygen tension (18%) conditions [335]. The unique tendency of HbSS RBCs to sickle under conditions of low oxygen tension within the microvasculature may explain the distinguishing strength of the association between HbS and protection against malaria. In contrast, high oxygen tension conditions result in reduced growth in α-thalassemia minima and β-thalassemia perhaps because of the resulting oxidative stress [336].

#### 1.2.5.2 Enhanced innate immunity

The innate immune response initiates a number of stereotypical pathways in the protective response to infection by microorganisms such as *P. falciparum*, which lead to both pro- and anti-inflammatory effects. While release of pro-inflammatory cytokines and recruitment of immune effector cells are essential factors in combatting foreign invaders, these same steps are also responsible for much of the pathology that results from malaria infection. The subsequent release of anti-inflammatory molecules is crucial to protect against collateral damage. Hence, regulation of these opposing forces is essential for

successfully killing or eliminating *P. falciparum* parasites and yet restricting tissue injury by the host's own immune response.

Heme oxygenase-1 (HO-1) is an important anti-inflammatory and cytoprotective molecule, which exerts its effects by catabolizing free heme, through producing carbon monoxide (CO) and bilirubin, and through inducing ferritin synthesis [337]. Experimental murine models of malaria have demonstrated an important role for HO-1 and downstream CO in prevention of progression to SM [338, 339]. A further study has demonstrated elevated HO-1 levels in CM-protected mice carrying human HbS RBCs and presented evidence for the role of induction of HO-1 production in protection by HbS in mice through a CO-dependent mechanism. While these results suggested that upregulation of HO-1 might be a protective mechanism in human individuals with HbS or other RBC polymorphisms as well, a recent study performed in Gambian children suggests that the relationship between HO-1 and SM in humans *in vivo* might be more complicated. Walther and colleagues demonstrated that HO-1 levels were elevated in UM and further elevated in SM compared to in convalescence. Specific HO-1 promoter polymorphisms were associated with increased inducibility of HO-1 *in vitro* and with increased susceptibility to SM *in vivo* [340]. Taken together, their results suggested that protection by HO-1 might occur in a narrow window and that indiscriminate induction of HO-1 might have deleterious effects rather than protective ones.

#### 1.2.5.3 Enhanced acquired immunity

Enhanced anti-malarial immunity in HbAS and other genetically-protected children is another mechanism invoked to explain the protection of RBC polymorphisms against malaria. As maternal IgG and HbF levels wane at around 3-6 months of age, infants in endemic areas become susceptible to malaria for the first time. Over the next decade of their lives, these children attain functional immunity against malaria, with reductions in the

number of malaria episodes with each subsequent year although sterile immunity is never achieved. The vast majority of immune responses target blood stage parasites, which are responsible for the clinical manifestations of malaria. During this stage, *P. falciparum* parasites display variant surface antigens (VSA), such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), on parasitized RBCs to enable the parasite to sequester in host microvessels of end organs and avoid removal from the bloodstream by the spleen. PfEMP1's essential role in the pathogenesis of malaria and its expression on the RBC surface make it the major target of functional antibodies against blood stage antigens [341]. The multiple variant copies of PfEMP1 and diversity both within a parasite's genome as well as between isolates globally, allow the parasite to evade the host response by switching between its 60 *var* genes per haploid genome, which encode for PfEMP1. Increasing levels of IgG against PfEMP1 as well as other major targets of acquired immunity are believed to be responsible for the age-dependent anti-disease immunity observed in endemic areas.

Although all children might start out with similarly nonexistent levels of antimalarial immunity, the enhanced malaria immunity hypothesis predicts that genetically protected children would acquire anti-disease immunity to malaria at an accelerated rate compared to wildtype children. These children carrying RBC polymorphisms would, therefore, attain immunity to the severe manifestations of malaria and to the symptoms of malaria at a younger age than wildtype children as well as experience a greater level of protection at any given age before reaching functional immunity to disease. Wildtype children would be delayed in reaching these levels of immunity, but once reached, would experience similar levels of overall protection against malaria.

The primary epidemiological evidence supporting this proposed mechanism comes from a study performed in a hypoendemic area in Kilifi, Kenya, where Williams *et al.* compared the malaria incidence rates in HbAS and HbAA children [342]. Consistent with the

enhanced malaria immunity hypothesis, there was no difference in malaria incidence in young children (ages 0-2 years old) and in older children (ages >10 years old). However, HbAS children aged 2-10 years experienced reductions of up to 50% in malaria incidence compared to HbAA children of similar age. These data suggest that HbAS confers a protective advantage against malaria in children until HbAA children acquire sufficient levels of anti-disease immunity.

Follow-up studies that have been performed to test the antibody titers for HbAA, HbAS, and HbAC children against VSAs, merozoite invasion ligands, and a panel of 491 *P. falciparum* antigens have provided conflicting results. An initial study performed in the Gambia showed that plasma from HbAS children agglutinated the mature blood stages of a single wild isolate at higher dilutions than HbAA children [343]. Later experiments performed in Burkina Faso showed that HbAS children in urban low-transmission areas, but not rural high-transmission areas, recognized VSAs expressed by a pool of Kenyan parasite isolates at higher titers than HbAA children [344]. A study performed in a meso- to hyperendemic area of Gabon likewise demonstrated that total IgG responses to the VSAs of two short-term adapted Gabonese parasite isolates were higher in HbAS than in HbAA children [345]. In contrast, plasma from Malian HbAS children recognized a series of merozite invasion ligands (MSP1, AMA1, MSP2, EBA175) at lower levels than plasma from HbAA children [346]. Similarly, HbAS children demonstrated IgG recognition of a panel of 491 antigens from the *P. falciparum* genome, including 7 PfEMP1 domains, at the same or lower levels than plasma from HbAA children by microarray [258].

Another line of investigation has hypothesized that RBC polymorphisms might enhance immune clearance of parasitized RBCs through IgG-mediated phagocytosis. The clearance of parasitized RBCs through IgG-mediated phagocytosis is part of the normal host response to *P. falciparum* infection. Studies have shown that IgG binding to parasitized

wildtype RBCs results in preferential phagocytosis by monocytes compared to nonparasitized RBCs [347]. Its role in protection by RBC polymorphisms was first suggested by the observation that parasitized α-thalassemic [348, 349] and β-thalassemic [348] RBCs bound IgG from hyper-immune sera at higher levels than parasitized wildtype RBCs. Furthermore, other studies have shown that ring-parasitized HbAS [350], β-thalassemic [350], HbH [350], and G6PD deficient [351] RBCs were phagocytosed at a higher rate than parasitized wildtype RBCs.

Finally, the importance of acquired immunity in protection by RBC polymorphisms is highlighted by a case report that suggests that non-immune individuals carrying RBC polymorphisms are not protected against malaria. This case report describes a family of 7 individuals (5 siblings and their parents) that had immigrated from Nigeria to the United States 10 years previous to a return visit to see their relatives. Although all of the siblings (2-11 years old) carried at least one HbS allele (2 HbSS, 3 HbAS), all of the HbAS children and 1 of the HbSS children developed malaria as a result of their visit. Of these, 3 (1 HbSS, 2 HbAS) children experienced severe manifestations of the disease [352]. Although the evidence remains anecdotal, this set of cases implies that malaria-protective IgG might be needed alongside HbS to effectively protect against malaria.

#### 1.2.5.4 Impaired pathogenic mechanisms

As discussed previously in **1.1.3.2**, cytoadherence, the binding of parasitized RBCs to endothelium, is a major step in the pathogenesis of malaria by enabling parasites to sequester in the host's microvasculature and escape removal by the spleen. A related pathogenic mechanism is rosetting, which allows merozoites to easily find and invade non-parasitized RBCs through the binding of parasitized RBCs to multiple non-parasitized RBCs. Both processes are mediated by the parasite-encoded virulence ligand, PfEMP1, and recent studies have implicated both related mechanisms in protection by RBC polymorphisms. In

this model of protection, reductions in surface PfEMP1 levels and altered display of PfEMP1 on 'knobs' on the surface of mature parasitized variant RBCs lead to the weakening of the interaction between PfEMP1 and host receptors on MVECs. Although parasitized variant RBCs would still retain the ability to sequester through adherence to MVECs, the weakened interaction between PfEMP1 and host receptor would result in attenuation of the MVEC activation and inflammation associated with cytoadherence. Reductions in PfEMP1 expression would also lead to a diminished ability to multiply to high parasite densities as a result of impaired rosetting. Both mechanisms might reduce the probability of progression to severe disease in individuals carrying variant RBCs.

Studies examining protection by HbS, HbC, α-thalassemia, β-thalassemia, HbF, and type O blood group antigen have supported a role for PfEMP1-mediated protection by variant RBCs against malaria. Initial studies reported aberrant PfEMP1-displaying 'knob' formation on the surface of HbCC RBCs, characterized by fewer knobs; a larger, more protuberant knob morphology; and an abnormally aggregated distribution of knobs over the RBC surface [353, 354]. A follow-up study demonstrated dose-dependent reductions in cytoadherence to MVECs for HbC under flow conditions and reductions in surface-expressed PfEMP1 by agglutination assay and flow cytoadherence [355]. Similarly impaired cytoadherence to MVECs, reductions in surface PfEMP1, and altered knob display has been demonstrated for HbS [356], HbF [357], and α-thalassemia [358], providing further evidence for a unified mechanism of protection by variant RBCs. Decreased adherence to human umbilical vein endothelial cells (HUVEC) has additionally been reported for α- and β-thalassemia. [359]. Rosetting, as another PfEMP1-mediated process, has also been shown to be reduced in HbC [355], HbF [357], type O blood group antigen [360], α-thalassemia [359], and β-thalassemia [359].

Although the mechanisms underlying this abnormal display of PfEMP1 are still being elucidated, a recent study suggested that abberations in the actin cytoskeleton and Maurer's clefts of HbS and HbC erythrocytes might be responsible for irregular PfEMP1 trafficking to the RBC surface. In particular, it appears that increased formation of hemoglobin oxidation products such as ferryl hemoglobin in parasitized HbS and HbC RBCs might prevent proper remodeling of the actin cytoskeleton by the parasite [361]. Although more work must be done to confirm these findings, such a finding would provide further evidence that a mechanism of altered PfEMP1 display, reductions in surface PfEMP1 levels, and commensurate reductions in cytoadherence to MVEC might be at work *in vivo* to protect HbAS and HbAC children.

## 1.3 Plasmodium falciparum erythrocyte membrane protein 1

*P. falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates cytoadherence to host vasculature and serves as a major player in the pathogenesis of malaria [17-19]. Approximately 60 *var* genes per haploid genome encode for this family of proteins, but the parasite typically only expresses one variant at a time during the mature trophozoite and schizont stages. During each intraerythrocytic cycle, the parasite is able to switch its expressed PfEMP1 variant in a process called antigenic variation. Antigenic variation allows evasion of the host immune response and affects the parasite's organ tropism due to the differential affinity of particular variants for receptors expressed on host endothelium such as CD36 and ICAM-1.

### 1.3.1 PfEMP1 structure and classification

PfEMP1 variants show similar domain architectures both intra- and inter-genomically. *Var* genes have a two exon structure separated by a transmembrane region. The first exon encodes for multiple adhesion domains (i.e., Duffy binding ligand (DBL) and cysteine-rich interdomain region (CIDR) domains), which form the extracellular portion of PfEMP1 and are responsible for binding to various host receptors. The 1.0-1.5kb second exon encodes for the cytoplasmic tail and is both more conserved and shorter than the 3.5-9.0kb first exon.

The high degree of polymorphism within PfEMP1 has led to the development of classification schemes for both individual DBL and CIDR domains and the overall PfEMP1 domain architecture. These methods of PfEMP1 classification allow researchers to begin dissecting the intricacies of PfEMP1 expression and binding phenotype. DBL and CIDR domains have been divided into six types of DBL domains ($\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$, $\xi$) and four types of CIDR domains ($\alpha$, $\beta$, $\gamma$, $\delta$) based on sequence similarities [362]. A subgroup of DBL$\alpha$ domains

(DBL$\alpha_1$ domains) have been associated with the inability to bind to CD36 and the ability to rosette [59, 363]. Although the original divisions and subdivisions were determined based on the 3D7 genome, they have been changed several times based on *var* genomes that have since been sequenced. A recent paper by Rask *et al.* provides the most recent iteration of divisions and subdivisions based on the PfEMP1 variants from seven genomes from globally obtained isolates [364].

The particular combination and order of DBL and CIDR domains within a PfEMP1 variant tend to follow certain patterns. The first domain for all currently published PfEMP1 variant sequences is DBLα, with exception of VAR2CSA. These domains are usually followed by CIDRα or CIDRγ domains, which are in turn usually followed by DBLβ, DBLγ, or DBLδ domains. These patterns have led to the classification of PfEMP1 proteins into 17 domain architectures based on the presence and order of these domains based on the 3D7 genome [365] with further architectures identified with the subsequent sequencing of other parasite isolates [366]. The examination of 399 full and partial *var* gene sequences from the seven sequenced *var* genomes led to further understanding that certain domain types are present together in a disproportionate number of variants [364]. For example, DBLα1.8 domains are often followed directly by a CIDRβ2 domain and then a DBLγ7 domain, which led to their identification as a "domain cassette."

*Var* genes are also divided into four major (A, B, C, and E) and two intermediate (BA and BC) groups based on the 5' upstream (*ups*) promoter sequence and the chromosomal location. Group A *var* genes are subtelomerically located, transcribed towards the telomere, and flanked by an *upsA* promoter sequence. In contrast, group B *var* genes are located in the telomere, transcribed toward the centromere, and flanked by an *upsB* promoter sequence, and group C *var* genes are located in central chromosomal regions and flanked by an *upsC* promoter sequence. Although group BA *var* genes are similar to group B

*var* genes in their telomeric location and transcription toward the centromere, they are located further from the ends of the chromosome than group B *var* genes. Group BC *var* genes have a promoter region similar to group B *var* genes, but are located centrally similarly to group C *var* genes. Their similarities to multiple *var* gene groups have led some researchers to suggest that Group BA and BC *var* genes are transitional groups [367].

*Ups* groups have also been shown to associate with certain domain architectures. For example, group A *var* genes tend to encode for larger, multi-domain PfEMP1s with more complex domain compositions than group B or C *var* genes. Their DBL1-CIDR1 protein head structure (usually DBL1α1-CIDR1α1 or DBL1α1-CIDR1γ) is also generally distinguishable from the head structure of group B or C *var* genes (usually DBL1α-CIDR1α) [366].

### 1.3.2 PfEMP1 trafficking and display on knobs

PfEMP1-encoding *var* genes are transcribed exclusively during the ring stage of the intraerythrocytic cycle [368, 369] and displayed on the RBC surface exclusively during the mature trophozoite and schizont stages. Although PfEMP1 lacks the N-terminal hydrophobic signal sequence typically needed for protein export, it is directed to the endoplasmic reticulum (ER) then through Maurer's clefts to the surface of the parasitized RBC. Although the mechanisms behind PfEMP1 export are still not fully understood, a number of proteins including skeleton binding protein 1 (SBP1) [370, 371], membrane-associated histidine-rich protein 1 (MAHRP1) [372], and Pf332 [373, 374] among others have been implicated in PfEMP1 transport. The presence of lipoproteins, particular those from the low-density lipoprotein fraction, as well as cholesterol-rich membrane microdomains in the RBC membrane are also necessary for proper PfEMP1 display [375, 376]. Additionally, the semi-conserved PfEMP1 head structure of DBLα-CIDRα appears to play an important role in PfEMP1 trafficking since truncation of this structure led to the

retention of PfEMP1 in the cytoplasm. The presence of the head structure in addition to the PfEMP1 transmembrane region and ATS domain were sufficient for trafficking of at least two PfEMP1 variants [377].

At the RBC surface, PfEMP1 is displayed on electron-dense protrusions (~100 nm) called 'knobs.' The protrusions are formed by the self-assembly of knob-associated histidine-rich protein (KAHRP) on the cytoplasmic side of the RBC membrane [378]. Although KAHRP is not essential for PfEMP1 trafficking, it is essential for knob formation [379]. Through its interaction with the cytoplasmic region of PfEMP1, KAHRP concentrates and anchors PfEMP1 on the RBC surface. The loss of KAHRP leads to the reductions in PfEMP1 expression on parasitized RBCs [380] and in adherence to host receptors [379, 381-383].

### 1.3.3 Antigenic variation

Antigenic variation, the systematic switching of *var* gene expression during the blood stages of *P. falciparum* infection, enables parasites to evade host immune responses. In the classic understanding of *var* expression, parasites express diverse *var* genes in a mutually exclusive fashion by trafficking one PfEMP1 variant to the RBC surface during each cycle [18, 384-388]. At each cycle, through a tightly controlled process, parasites may continue to express the same variant or may switch to another variant. Such regulation of *var* gene expression may limit exposure of particular variants to mediators of acquired immunity such as B-cells to extend the persistence of a particular parasite within the host by switching to a new variant when the immune system mounts an effective response against the original variant [389]. A recent finding of a parasite sub-line that co-expresses two different *var* genes simultaneously challenges the absoluteness of mutually exclusive *var* gene expression [390], but the paradigm is still generally believed to hold true.

#### 1.3.3.1 Epigenetic and genetic control of *var* gene expression

While the exact mechanisms underlying antigenic variation are still being elucidated, epigenetic factors through histone modifications are believed to control *var* gene expression. The expression of a single *var* gene at a time, also known as the "counting mechanism," seems to be governed by the cooperative pairing of the *var* gene upstream promoter with a 3' intron, which also contains promoter activity [391-393] although several studies challenge the necessity of the intron for silencing of *var* genes [84, 394].

Within the promoter region of *var* genes, modifications at histone 3 lysine 9 (H3K9), histone 4, and histone 3 lysine 4 (H3K4) seem to be particularly crucial in managing the activity or repression of specific *var* genes. The single active *var* locus in a particular repertoire is typically acetylated at the H3K9 and H4 positions, and either di- or tri-methylated at the H3K4 position. In contrast, the promoter region of a silenced *var* gene generally contains trimethylated H3K9 and hypoacetylated H4 [395-400]. Interestingly, trimethylated H3K9 appears to be specific to antigenically variant genes within *P. falciparum* [397, 398]. Additionally, variant histone H2A.Z is found in the promoter of active *var* genes and is believed to play a role in regulation of expression as well [401, 402]. Although the factors controlling these epigenetic changes are still not well-understood, recent studies have implicated silent information regulators (Sirs) 2A and 2B as trans-acting regulators of *var* gene silencing through their role as nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent histone deacetylases [403, 404].

#### 1.3.3.2 *Var* gene switching and recombination

Although parasites are able to switch their expressed *var* gene between every intraerythrocytic cycle, most active *var* genes will remain activated for a number of cycles, most likely due to the preservation of epigenetic histone modifications through multiple

rounds of DNA replication, leading to 'epigenetic memory.' Individual *var* genes seem to have differing on (switching towards activation) and off rates (switching towards silencing), which may vary according to *ups* group [389, 405-407] or may be an intrinsic property of each *var* gene [408]. These differential rates would lead to an ordered, sequential hierarchy of *var* gene expression [389, 409]. The overall switch rate was estimated to be ~2% per cycle in an *in vitro* study [387], but this rate was 16% in experimentally-infected humans [410].

Fluorescent *in situ* hybridization (FISH) has shown that *P. falciparum* chromosomes are tethered to the nuclear envelope [411]. Silent *var* gene loci are believed to cluster together with telomeric regions forming 'bouquet' structures of ~6-8 telomeres. The alignment and proximity of group A *var* genes and group B *var* genes allows for preferential recombination with other members of the same *ups* group resulting in multiple separately evolving groups of *var* genes [366, 367, 412]. Recombination may also occur by meiosis in the mosquito. Once a *var* gene is activated, it travels to a transcriptionally active site also along the nuclear envelope, but distinct from the cluster of silent *var* genes. The movement of the activated *var* gene to this site may be mediated by *var* intron-regulated actin polymerization [413].

### 1.3.4 PfEMP1 diversity

#### 1.3.4.1 Global PfEMP1 diversity

Despite retaining certain similarities within their domain architecture, PfEMP1 variants show an amazing amount of heterogeneity and genetic complexity both intra- and inter-genomically to allow for effective immune evasion by parasites, with equal levels of diversity within the *var* repertoire of one isolate as between *var* gene repertoires of different isolates. The generation of diversity is mediated by the high rates of

recombination which occur at the nuclear periphery between *var* genes of the same *ups* group (see **1.3.3.2**). Until recently, determining the extent and understanding the nature of this diversity was hindered by the lack of fully-sequenced PfEMP1 *var* gene repertoires to compare. However, a study that examined *var* gene repertoires from three parasite isolates, which by genotyping and parasite population studies originate from separate continents, showed that *var* genes demonstrated some overlap between their domain architectures with seven architectures shared among the three genomes [365]. However, of the 31 architecture types, most were only found once within a *var* gene repertoire. Furthermore, most domains had <50% sequence similarity even when comparing domains within the same domain architecture type. However, the study also identified three *var* genes (*var1csa*, *var2csa*, and Type 3 *var*) that were found to be highly conserved (>75% sequence identity over multiple domains) [366]. Another study examined DBLα sequences from 54 global field isolates and 5 cloned lines originating from diverse locations in Africa, Asia, and South America. From the 1088 DBLα domains sequenced, 895 unique DBLα domains were identified suggesting that although there is some overlap in PfEMP1 *var* gene repertoires, that this overlap is minimal [414].

#### 1.3.4.2 Local PfEMP1 diversity

However, it appears that the *var* gene repertoire within local populations may be restricted. A few studies have reported limited *var* gene repertoires within populations in Asia [414] and South America [415, 416]. Interestingly, the *var* gene diversity within local populations in Africa seems to be at least an order of magnitude greater than those found in either Asia or South America [417-420] and efforts to determine the limits of *var* richness within these African populations have not been successful [417]. The *var* gene diversity does not seem to be linked to the malaria endemicity within the local population, but likely results from the long history of *var* gene recombination in Africa.

### 1.3.5 Contribution to malaria pathogenesis

#### 1.3.5.1 PfEMP1 domains and specificity for host receptors

As discussed previously, the PfEMP1-mediated formation of rosettes and cytoadherence of parasitized RBCs to receptors expressed in host microvessels play key roles in the pathogenesis of malaria (**1.1.3.1** and **1.1.3.2**). The specific affinity of PfEMP1 variants for particular receptors is responsible for preferential sequestration in various organs and may affect the clinical phenotype of infection. This affinity for specific host receptors appears to be encoded by certain DBL and CIDR domains. For example, CIDRα domains are responsible for the ability to bind to CD36 [421-425]; DBLβ domains mediate binding to ICAM-1 [426-429]. Specifically, CIDRα domains from group B and C *var* genes typically mediate binding to CD36 while the CIDRα domains from group A *var* genes do not [363]. Likewise, it appears that a limited subset of DBLβ domains is able to bind ICAM-1. Until recently, this subset was believed to be limited to group B and C *var* genes [426-428]. One study showed that all of the seven ICAM-1 binding DBLβ domains within the IT4 parasite line were from group B and C *var* genes and that none of the nine DBLβ domains expressed by group A *var* genes were shown to bind ICAM-1 [430]. However, recently a group A *var* gene from the 3D7 strain was shown to bind to ICAM-1 with high affinity [431] suggesting that the *ups* group specificity of binding ICAM-1 might be vary between parasite lines. Furthermore, the DBLβ domains capable of binding ICAM-1 may be limited to DBLβ5 and DBLβ3 domains [432]. Rosetting is mediated through binding of DBL1α1 domains to complement receptor 1 (CR1) [59, 433] and type A and B blood group antigens [434-436]. The PfEMP1 variants which are able to bind to these receptors are limited to group A *var* genes [59, 434, 437, 438], which are unable to bind CD36 [59].

#### 1.3.5.2 PfEMP1-specific IgG acquisition

The role of PfEMP1 in the pathogenesis of malaria is underscored both by the importance of PfEMP1-specific IgG acquisition in developing NAI against malaria as well as by the demonstration of a restricted *var* gene repertoire expressed by parasites in SM cases. PfEMP1 has been identified as a major target of IgG recognition during the blood stages of *P. falciparum* infection [341, 343, 439]. Acquisition of PfEMP1-specific IgG likely occurs in an ordered, piecemeal fashion and results in protection against homologous PfEMP1 variants [231, 440-443]. Clinical episodes might result when the PfEMP1 variants expressed during *P. falciparum* infection breakthrough the developing repertoire of PfEMP1-specific immunity [444, 445].

#### 1.3.5.3 Restriction of *var* gene repertoire

Several early studies established through agglutination and flow cytometry studies that a limited set of "common" VSAs are expressed more frequently in SM and in young children [446, 447]. These findings led to the hypothesis that a subset of VSAs might be preferentially expressed in young children who had not experienced many *P. falciparum* infections and therefore, had not developed an immune response against these VSAs [180, 181]. This set of particularly virulent VSAs might be expressed in these children due to a selective advantage conferred to parasites by expression of these variants such as increased cytoadherence or high parasite multiplication rates. After the development of immunity against this subset of variants, parasites would express rare, less virulent variants, which might lead to UM, but would not cytoadhere with sufficient avidity to cause SM. The inclusion of both the virulent common VSAs and the less virulent rare VSAs within a parasite's repertoire is advantageous to the parasite by allowing it to express the common VSAs and cytoadhere efficiently in naïve children, yet still evade the host immune response with the expression of rarer variants in semi-immune hosts [448].

Subsequent studies have supported the idea of a restricted *var* gene repertoire in SM and in young children. One study found that multiple amino acid motifs were over-represented in rosetting parasite isolates and in parasite isolates from SM patients [449]. Although examination of the *var* gene repertoire has been performed almost exclusively on parasites from the peripheral blood, an autopsy study found differential *var* gene expression in the organs of children with fatal malaria [450]. Other studies have examined the relationship between *ups* groups and the severe manifestations of malaria. Although some studies using quantitative PCR have suggested that group B, group BA or group C *var* genes associate with SM [451, 452], the majority of the evidence suggests that group A *var* genes may be more virulent than their group B or C counterparts. Selecting for expression of highly recognized PfEMP1 variants within the 3D7 genome was associated with upregulation of group A *var* genes. Another study found upregulation of both group A and B *var* genes in SM compared to UM as well as malaria compared to asymptomatic parasitemia [453]. These results are corroborated by studies using a classification scheme based on a ~300 nucleotide (nt) sequence tag in the DBLα region of PfEMP1 variants, which show that PfEMP1 variants from SM patients tend to have a lower cysteine count within a DBLα sequence tag [418, 419, 454, 455]. PfEMP1 variants with two cysteines in this region, instead of the more common four cysteines, are typically group A *var* genes. Further investigations have provided evidence for the relationship between motifs and features within this sequence tag and pathogenic mechanisms such as rosetting as well as specific clinical manifestations of malaria such as CM [449, 454, 456, 457]. The most recent evidence supporting the role of group A *var* genes in SM come from a trio of studies that implicated domain cassettes 8 and 13, which are found in group A *var* genes, in the development of SM potentially as a result of an increased binding phenotype to human brain endothelial cells [458-460].

### 1.3.6 Vaccine prospects

PfEMP1's role as the major target of acquired immunity in blood-stage parasites, its importance in the pathogenesis of malaria through cytoadherence, and its display on the RBC surface for approximately half of each intraerythrocytic cycle make PfEMP1 an attractive malaria vaccine candidate. However, the immense diversity of PfEMP1 and the limited cross-reactivity between variants pose major obstacles towards the development of a viable PfEMP1 vaccine. Despite these challenges, a PfEMP1-based vaccine, particularly for the prevention of PAM, may be feasible.

The potential effectiveness of such a PfEMP1-based vaccine has come from several immunization studies in *Aotus nancymaae* monkeys. Immunization of *Aotus* monkeys with a recombinant Malayan Camp CIDRα fragment, which mediates binding to CD36 [424], along with Freund's adjuvant led to protection against homologous challenge despite the parasite's ability to antigenically switch between PfEMP1 variants, but did not protect against heterologous challenge with the FVO parasite line [461]. Immunization with a recombinant FVO CIDRα fragment resulted in delayed onset of parasitemia, but did not protect against homologous challenge. However, immunized monkeys were differentially protected upon a second homologous challenge compared to non-immunized monkeys [462]. Such studies demonstrate that the development of a PfEMP1-based vaccine might successfully protect or, at least, reduce the clinical severity of malaria.

However, a major obstacle in the development of a PfEMP1-based vaccine is the nearly limitless diversity of PfEMP1 variants globally and even locally in some cases. Geographical locations with overlap between circulating *var* gene repertoires still have thousands of *var* gene variants against which a PfEMP1-based vaccine would have to protect. Despite conserved domain architectures and some restriction on recombination based on *ups* groups, the diversity associated with PfEMP1 would make it impossible to

protect against all of these *var* genes without significant strain-transcending cross-reactivity. Some cross-reactivity between variants has been observed in DNA immunization studies in mice especially upon immunization with DNA from multiple CIDR domains [463, 464] among others [465-467]. However, the level of cross-reactive immunity induced in these studies is likely not sufficient to provide protection against all circulating PfEMP1 variants upon immunization with a practically achievable number of independent DNA sequences or domains. This is supported by the previous experiments described as well as others, which showed some protection against homologous isolates, but little protection against heterologous isolates [445, 461, 462].

Despite the vast diversity of PfEMP1 variants, two specific clinical presentations of malaria, PAM and SM, are associated with a limited number of PfEMP1 variants. As a result, development of a PfEMP1-based vaccine targeting one of these presentations would likely be more effective than attempting to prevent all types of malaria. The concept of a PfEMP1-based vaccine against PAM has drawn particular interest. VAR2CSA, the PfEMP1 variant responsible for PAM, demonstrates a higher degree of interclonal conservation than other PfEMP1 variants [468]. Nevertheless, parasite genomes often contain multiple *var2csa* genes [469-471] and highly conserved regions of VAR2CSA appear to be interspersed with areas of substantial amino acid diversity [364, 472]. Fortunately, it appears that IgG-recognized, functionally-significant VAR2CSA epitopes are interclonally conserved [473-475]. Furthermore, IgG which recognize these conserved epitopes appear sufficient to block binding of parasitized RBC to CSA [476-478]. Although significant work must be done before a VAR2CSA-based vaccine might be used in malaria-endemic settings to protect pregnant women against PAM, these findings suggest that such a vaccine might be viable.

Additionally, a restricted set of *var* genes (group A *var* genes) has been implicated in the development of SM (described in **1.3.5.3**). Recombination within *ups* groups has led

to some conservation within group A var genes although the sequence diversity within this group is still much greater than that seen for VAR2CSA [472]. Nevertheless, recent studies have shown induction of strain-transcending antibodies against rosetting group A *var* genes [230, 479]. The presence of shared epitopes among PfEMP1 variants associated with a particular disease-causing phenotype suggests that a PfEMP1-based vaccine targeting SM may be possible.

# 2 Protection by RBC polymorphisms against malaria in a cohort of Malian children

## 2.1 Introduction and aims

Previous studies have shown that RBC polymorphisms including HbS, HbC, α-thalassemia, and G6PD deficiency have been evolutionarily selected due to their ability to prevent the life-threatening manifestations of malaria (described in **1.2**). However, various groups have shown heterogeneity in the magnitude of protection conferred by these polymorphisms against UM and SM. Since *P. falciparum* parasite isolates and unparasitized RBCs from this population were used in subsequent experiments described in this thesis, we sought to characterize the magnitude of protection by these RBC polymorphisms against malaria within our own cohort of Malian children. Consequently, we initiated a 4-year cohort study of 1585 children examining the incidence rate of malaria taking into account a number of covariates including Hb type, α-thalassemia genotype, G6PD genotype, age, ABO blood group, village, ethnicity, year, and gender. Here we describe our findings from this study.

## 2.2 Study site and methods

### 2.2.1 Study site

Kenieroba, Bozokin, and Fourda are three rural villages located approximately 71 km southwest of the capital city of Bamako, Mali, near the Niger River. As is typical of this area, houses are generally arranged in compounds made of several houses, which are generally made of households belonging to an extended family [480]. Of the three, Kenieroba (12.11° N, 8.33° W) is the largest with a population of 2,170 inhabitants in 353 houses (131 compounds). Its location, slightly inland from the river, is conducive to agriculture. In contrast, Bozokin (12.17° N, 8.24° W) and Fourda (12.09° N, 8.34° W) are considerably smaller with populations of 331 in 55 houses (15 compounds) and 309 in 42 houses (13 compounds), respectively. They are located along the river and the main livelihood of inhabitants is fishing.

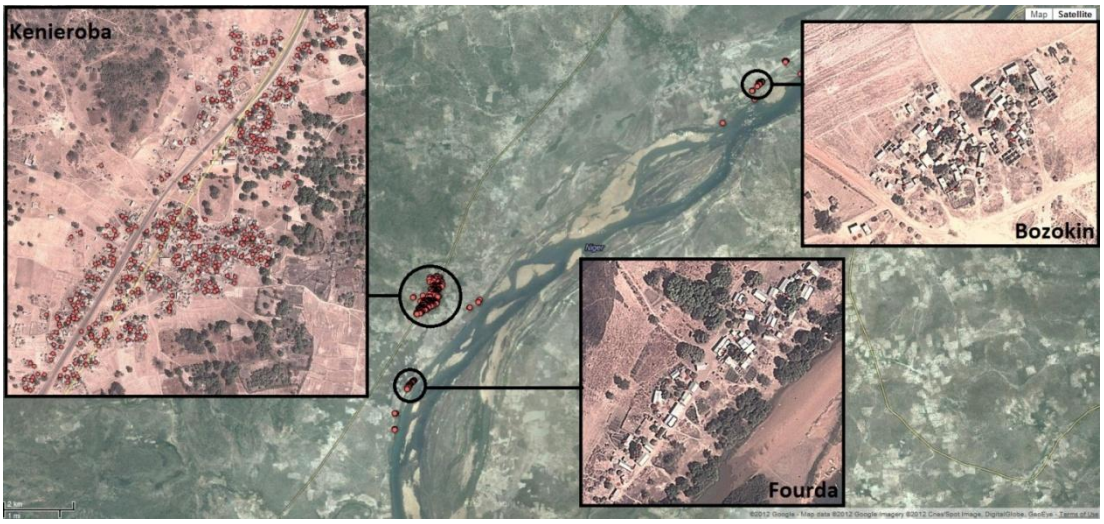



**Figure 2.1 Map of Kenieroba, Bozokin, and Fourda**

The location of Kenieroba, Bozokin, and Fourda along the Niger River are indicated by the three clusters of red circles. Each red circle represents one house.

### 2.2.2 Climate and rainfall

In the three villages, the year is split into rainy (May-November) and dry (December-April) seasons. Almost all of the annual rainfall, which ranges between 750 to 1500 mm, occurs during the rainy season.

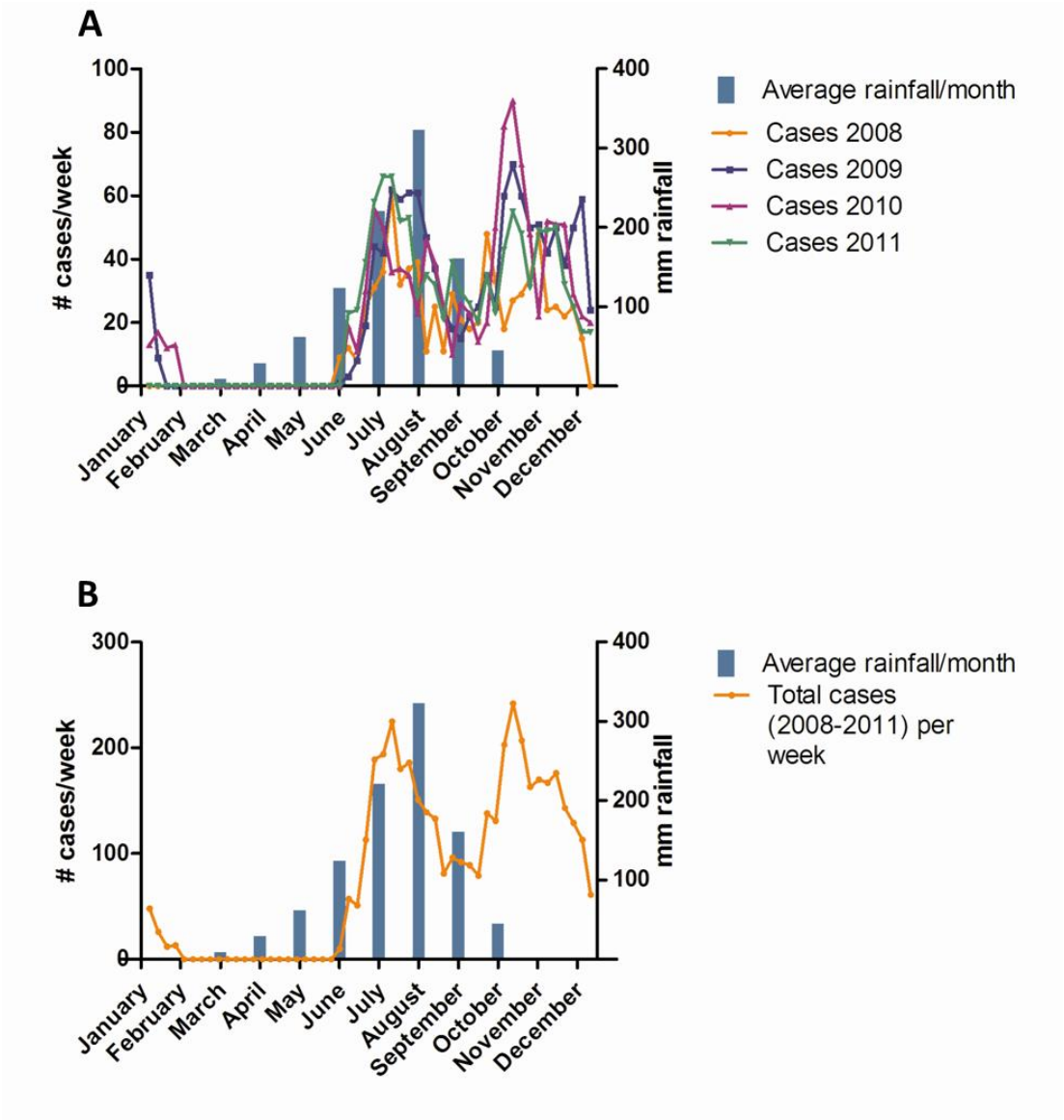



**Figure 2.2 Rainfall and malaria cases in Kenieroba, Mali**

The average rainfall per month (averaged from data from 1994-2008) as well as the **A)** cases per week in 2008, 2009, 2010, and 2011 and **B)** total cases per week for 2008-2011 combined are shown.

### 2.2.3 Malaria transmission

Malaria transmission in these villages is intense and seasonal with an estimated EIR of 21.7 infective bites per individual per month based on entomological data collected in 2006-2008. The patterns of transmission are tied closely to rainfall patterns with the vast majority of malaria episodes occurring between July and November. The peak of transmission occurs at the end of the rainy season in late October with EIRs of between 33.2 and 273.3. The lowest period of transmission occurs during the driest period of the year from January to February with EIRs between 1.3 and 6.2. The primary malaria vector in Kenieroba is *Anopheles gambiae s.l.* which has a 99.4% relative prevalence over *Anopheles funestus,* the other malaria vector in Mali. Within *An. gambiae s.l.* in our site, 95.8% are *An. gambiae s.s.* which can be further divided into 74.6% M and 25.4% S molecular forms. The remaining 4.2% are *An. arabiensis.* The primary *Plasmodium* species in our site is *P. falciparum*.

### 2.2.4 Typing for RBC polymorphisms

From these villages, 1589 children aged 0.5-17 years were enrolled over four transmission seasons (2008, 2009, 2010, 2011). All children were typed for RBC polymorphisms. We performed high performance liquid chromatography (D-10 hemoglobin analyzer; Biorad, Hercules, CA) with 5 µL of hemolysate from each child to determine the Hb type. ABO blood group was determined by agglutination assay as described in [358]. Samples were checked for the 3.7-kb deletional determinant of α-thalassemia (α-thal$^{3.7}$) by nested PCR to identify heterozygotes (-α/αα) and homozygotes (-α/-α) as described previously [258]. The G6PD *A*- allele, which encodes for 50-90% reductions in G6PD enzymatic activity [117, 481], was detected in samples using restriction-fragment length polymorphism analysis of PCR-amplified DNA samples as described previously [482].

### 2.2.5 Patient visits

The parents of children enrolled in our study were educated about the symptoms of malaria and advised to bring their children to our clinic if they exhibited these symptoms. At each visit, children were checked for parasitemia by examination of a thick peripheral blood smear by a trained microscopist and were examined by a clinician, who checked for general appearance, pallor, diminished consciousness, splenomegaly, and respiratory distress by physical examination, and hypoglycemia and severe anemia by laboratory studies. Both the microscopist and the clinician were blinded to the genotypes of each child. Furthermore, since the RBC membranes lyse upon thick smear, the microscopist would not be able to ascertain clues about the individuals' genotypes based on the smear.

### 2.2.6 Case definitions

UM was defined as axillary temperature >37.5°C and parasite density <100,000/µl counted from thick blood smears. SM was defined according to WHO criteria [483]. Children diagnosed with either UM or SM had blood collected by venipuncture into sodium heparin Vacutainers® (Becton-Dickinson, Franklin Lakes, NJ) for laboratory experiments before treatment by oral ACTs (artemisinin plus amodiaquine) for UM patients or intravenous (IV) quinine for SM patients. Interestingly, in *in vitro* assays, parasitized α-thalassemic RBCs have demonstrated decreased sensitivity to artesunate [481]. However, an *in vivo* study performed in Cambodia showed no significant difference in the rate of parasite clearance by artesunate, so α-thalassemic children with malaria were given the same treatment as wildtype treated in our study [320]. The epidemiological cohort study and blood collection protocols were approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (NIAID) and the Ethics Committee of the Faculty of Medicine, Pharmacy and Odontostomatology at the University of Bamako. Informed

consent was obtained from adults or the parent/guardian of children. This study is registered at Clinicaltrials.gov (NCT00669084).

### 2.2.7 Total Time of Exposure

The amount of time each child was tracked on our study and was at risk of acquiring malaria in our study site varied due to several factors. First, some children traveled outside of our study site for a portion of one or more transmission seasons. Second, some children were added or removed from our study over the course of the 4 years due to their age, loss to follow-up, parental withdrawal, or death. Third, administration of antimalarials for the treatment of malaria episodes reduced the length of time that treated children were susceptible to subsequent episodes of malaria. Consequently, these factors were taken into account by adjusting for the total time of exposure in our analysis. Specifically, to account for administration of antimalarial drugs, 3 weeks were removed from a child's "total exposure time" each time they were treated for malaria.

### 2.2.8 Statistics

A Poisson model was used to model the influence of age, Hb type, α-thalassemia genotype, G6PD deficiency genotype, ABO blood group, village, ethnicity, year, and gender on the incidence of malaria (i.e., UM and SM taken together). For the purpose of our analysis, we grouped all 'non-O' blood groups (A, B, and AB) together for comparison against type O blood group, all non-Fulani ethnic groups (Malinke, Bambara, Sarakole, and Dogon) together for comparison against the Fulani ethnic group, and both non-Kenieroba (Bozokin and Fourda) villages together for comparison against Kenieroba. The G6PD genotype was included in the model with a value of 0 for the $A^{+}$ genotype, 1 for the $A^{+/-}$ genotype, and 2 for the $A^{-}$ genotype. The α-thalassemia genotype was included with a value of 0 for the wildtype (αα/αα) genotype, 1 for the heterozygote (−α/αα) genotype,

and 2 for the homozygous (−α/−α) genotype. The effect of each of the covariates was determined in comparison to a reference group that represents a child we would expect to be relatively unprotected: a 6-month-old HbAA, α-thalassemia wildtype, G6PD $A^{+}$, 'non-O' blood group, non-Fulani, non-Kenieroba female in 2008.

## 2.3 Results

### 2.3.1 Description of cohort

Over four years, we were able to enroll almost every child aged 0.5-17 years living in one of these three villages. The vast majority of the children were from Kenieroba (1212 children, 76.5%) with equal contributions from Bozokin (192 children, 12.1%) and Fourda (181 children, 11.4%). Our cohort of 1585 children was evenly split between males (n=788) and females (n=797).

Within our cohort, the most common malaria-protective RBC polymorphisms were HbS, HbC, α-thalassemia (3.7-kb deletional determinant), and G6PD deficiency ($A^-$). The prevalences of HbS and HbC were approximately 15% and 7% respectively [Hb types: 1236 HbAA (78.0%), 228 HbAS (14.4%), 103 HbAC (6.5%), 12 HbSC (0.8%), 2 HbSS (0.1%), 1 HbCC (0.1%)]. We were unable to Hb type three children (0.2%). 409 children (25.8%) were α-thalassemia heterozygotes and 36 (2.3%) were α-thalassemia homozygotes. 87 (5.5%) male children were hemizygous and 149 (9.4%) female children were heterozygous for the G6PD *A*- allele. 18 (1.1%) and 11 (0.7%) of children were not successfully typed for their α-thalassemia or G6PD deficiency genotype, respectively.

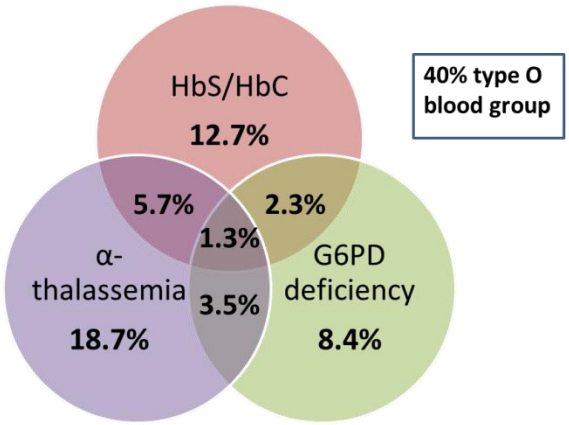



**Figure 2.3 Percentages of RBC polymorphisms within our Malian cohort**

In other studies, individuals carrying type O blood group antigen have been significantly protected compared to 'non-O' individuals (antigens A, B, and AB taken together). 40% of children on our cohort had type O blood group with 604 $O^+$ children (38.1%) and 33 O- children (2.1%). The ABO blood groups of the remaining children on our cohort were 433 A+ (27.3%), 49 A- (3.1%), 120 AB+ (7.6%), 5 AB- (0.3%), 309 B+ (19.5%), and 32 B- (2.0%).

The ethnic breakdown of our cohort was: 85.0% Malinke, 8.5% Fulani, 4.2% Bambara, 1.8% Sarakole, and 0.5% Dogon. Of particular note, Fulani individuals are protected against malaria through a mechanism independent of RBC polymorphisms [484]. Recent studies have suggested that this mechanism of protection might be due to a functional deficit in T regulatory cells [485]. As a result, ethnicity was considered in our analyses to account for any differences that might have arisen through this protection.

### 2.3.2 Protection by RBC polymophisms within our cohort

Over the course of four transmission seasons (2008, 2009, 2010, 2011), we diagnosed a total of 4207 malaria cases in our cohort of 1585 children. The majority of cases (3802 cases, 90.4%) were UM cases and of the remaining 405 SM cases, 38 met at least one of the major SM criteria: CM, SMA, or RD. Of these, 21 were CM only, 12 SMA only, and 1 RD only cases. Three cases had both CM and RD and one case had SMA and RD. The majority of the remaining cases were classified as SM due to prostration, repetitive vomiting, the inability to eat or drink, or a combination of the three criteria. Consequently, given the relatively small number of SM cases within our cohort and the lack of many CM, SMA, or RD cases, in our analyses, we combined UM and CM cases to look at the incidence rate ratio of malaria incidence taking into account the following covariates: age, Hb type, α-thalassemia genotype, G6PD deficiency genotype, ABO blood group, village, ethnicity, year, and gender.

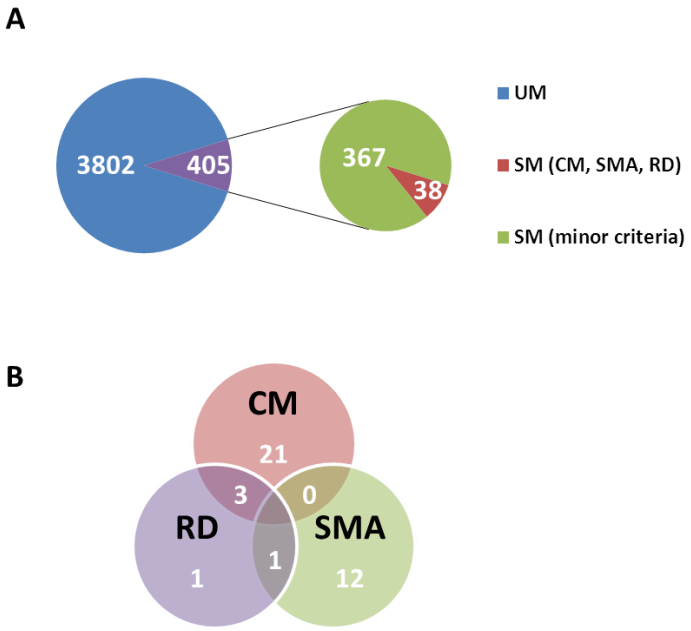



**Figure 2.4 Malaria cases in Kenieroba, Mali from 2008-2011**

Over the course of 4 transmission seasons, a total of 4207 malaria cases presented to our clinic in Kenieroba, Mali. **A)** The distribution of UM, SM (CM, SMA, RD, or a combination), and SM (minor criteria) is shown with the number of cases for each category indicated. **B)** The distribution of the 38 cases that involved CM, SMA, RD, or a combination of the three severe manifestations of malaria.

#### 2.3.2.1 Relative risk of developing malaria decreases with age

In regions of hyperendemic malaria transmission within Africa, such as the three rural villages in our study, children are repeatedly exposed to *P. falciparum* from infancy. As a result of their almost constant exposure to malaria, they gradually acquire anti-disease immunity with protection against SM developing first followed by protection against UM. Consequently, to account for the expected age-dependent protection against malaria, age was included in our model as a categorical covariate with each year of age analyzed as a separate group. Compared to the reference group of 6-month-olds, 1-year-olds had a significant two-fold increase in relative risk of developing malaria (relative risk (RR) 2.12, 95% confidence interval (CI) 1.69 - 2.68, $P < 0.000001$). The risk of developing malaria

continued to increase with age until reaching a peak of 3.5 times increased likelihood at age 3 years old

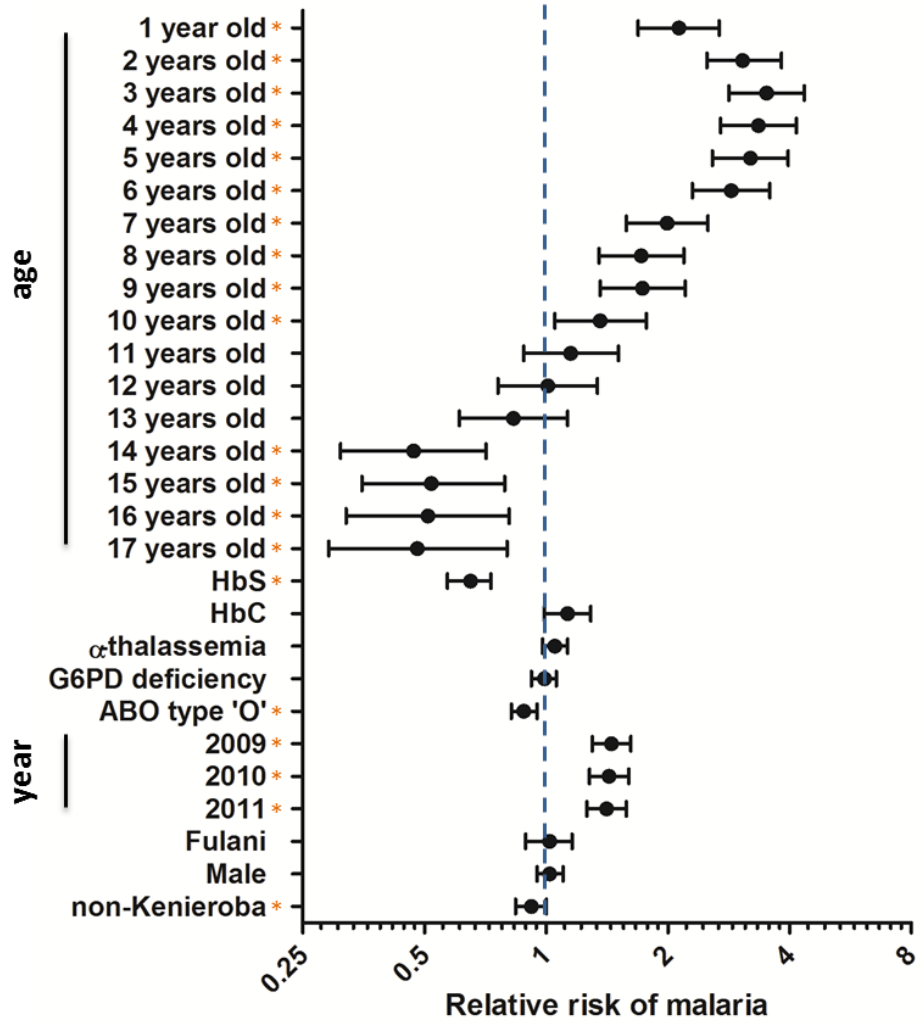



**Figure 2.5 The relative risk of malaria by age, year, Hb type, α-thalassemia genotype, G6PD genotype, ABO blood group, village, ethnicity, and gender**

A relative risk of 1, which would indicate no change in relative risk due to the particular covariate, is denoted by the blue dotted line. Any covariates that were significantly ($P < 0.05$) associated with malaria risk were indicated with an orange asterisk.

compared to the 6 month olds (RR 3.51, 95% CI 2.83 - 4.35, P < 0.000001) before decreasing to an approximate 50% decrease in relative risk for 14 (RR 0.47, 95% CI 0.31 - 0.71, P = 0.0003), 15 (RR 0.52, 95% CI 0.35 - 0.79, P = 0.001), 16 (RR 0.51, 95% CI 0.32 - 0.81, P = 0.003), and 17 year olds (RR 0.48, 95% CI 0.29 - 0.80, P = 0.005). Taken together, these data argue for a strong age-dependent effect on incidence of malaria with older children, in general, experiencing greater protection than younger children.

#### 2.3.2.2 HbS and ABO blood group O demonstrate significant protection against malaria

Our cohort contained a high percentage of children who carried HbS (15%), HbC (7%), α-thalassemia (3.7kb deletional determinant) (28%), or G6PD ($A^-$) deficiency (15%) with a relatively large percentage of children carrying more than one RBC polymorphism (13%). Additionally, 40% of the children in our study carried type O blood group antigen. By our analysis, our model showed a significant 35% protection by HbS (RR 0.65, 95% CI 0.57-0.73, P < 0.000001), but not HbC, which conferred a non-significant 13% increase in relative risk of developing malaria (RR 1.13, 95% CI 0.99-1.29, P = 0.08). α-thalassemia and G6PD deficiency similarly showed no significant effect on relative risk (α-thalassemia RR 1.05, 95% CI 0.98 – 1.1, P = 0.17; G6PD deficiency RR 0.98, 95% CI 0.92 – 1.06, P = 0.67). However, children with blood group O had a 12% decrease in relative risk compared to 'non-O' children (RR 0.88, 95% CI 0.82 – 0.95, P = 0.001).

#### 2.3.2.3 Year and village had significant effects on relative risk of developing malaria

To account for any changes in malaria incidence due to villagers' access to rapid malaria diagnosis and treatment over the course of our study, climate patterns, or other factors, we included a term for "year" within our model. Children had a higher relative risk of similar magnitude for developing malaria during the last 3 years of the study – 2009 (RR 1.45, 95% CI 1.30 – 1.62, P < 0.000001), 2010 (RR 1.43, 95% CI 1.28 – 1.60, P < 0.000001),

and 2011 (RR 1.41, 95% CI 1.26 – 1.58, P < 0.000001) – compared to the first year of the study (2008). Ethnicity and gender had no significant effect (Fulani RR 1.02, 95% CI 0.89 – 1.16, P = 0.77; male RR 1.02, 95% CI 0.95 – 1.10, P = 0.54). However, children from Bozokin and Fourda ('non-Kenieroba'), the two fishing villages directly on the Niger River, were slightly more protected than children from Kenieroba (RR 0.92, 95% CI 0.84 – 1.16, P = 0.05).

#### 2.3.2.4 HbS and age showed no significant interaction

A previous study from Kilifi, Kenya, showed an interaction between HbAS and age, with similar incidence rates between HbAS and HbAA children at ages 0 - 2 years old and > 10 years old, but decreased incidence in HbAS children between 2 - 10 years old. In our cohort, we saw no evidence for a significant interaction between HbS and age (P = 0.45).

#### 2.3.2.5 HbS and α-thalassemia showed no significant interaction

The overlapping geographical distributions of RBC polymorphisms has led to speculation on why, for instance, α-thalassemia is found at remarkably high frequencies in Oceania and South Asia, but at much lower frequencies in Africa. Previous studies have shown that while HbS and α-thalassemia individually have strong protective effects against the development of malaria, that co-inheritance of both polymorphisms results in a negative epistatic effect [248, 312]. However, within our cohort, we saw no evidence of an epistatic effect between HbS and α-thalassemia (RR = 0.97, 95% CI 0.76 – 1.24, P = 0.83).

## 2.4 Discussion

In this study, we have examined the effect of various factors on malaria protection in over 1500 children living in 3 villages. Over four transmission seasons, we identified a total of 4207 UM and SM cases. The overall finding of our study, that HbS demonstrates significant protection against malaria of much greater magnitude than the other polymorphisms investigated, matches previous epidemiological observations (described in **1.2.1** - **1.2.3**). Although our study was designed to examine protection against UM and SM taken together, the vast majority (~90%) of the malaria cases identified in this study were uncomplicated in severity. Of the cases categorized as 'severe,' very few involved the most serious manifestations of malaria (CM, SMA, or RD). Consequently, our results are mostly driven by incidence of UM. When taking this into account, the magnitude of protection by HbS (35%) and the lack of protection by HbC, α-thalassemia, and G6PD deficiency were similar to the results from other cohorts examining protection against UM [289]. Of particular note, HbC children in our cohort had a non-significant increased incidence of malaria, which highlights that the protection HbC affords against life-threatening SM, likely offsets any increased susceptibility to UM, to evolutionarily select for HbC. Understanding the underlying differences in magnitude of protection and specificity of protection by RBC polymorphisms, may yield further insight into their protective mechanisms.

Additionally, our finding that Type O blood group antigen had a small, but significant protective effect within our cohort was surprising since malaria protection by blood group O has previously only been shown for SM. Previous studies have implicated rosetting as a potential mechanism of protection against SM through blood group O. Our finding that blood group O protects against both UM and SM taken together, suggests that blood group O might protect against the progression not only from UM to SM, but also from asymptomatic parasitemia to UM either by rosetting or by a novel mechanism.

As we expected, protection was highly age-dependent with older children generally experiencing a greater degree of protection than younger children. An exception to this trend was for children from ages 6 months to 3 years old who had increasing incidence with each successive year. Other studies that have observed similar increases in malaria incidence with age for young children have speculated that parents may be more vigilant in implementing malaria-protective methods such as use of ITNs for infants and young toddlers resulting in malaria protection for these children. Additionally, 6-month-old infants may be partially protected by maternal IgG, by the presence of HbF and by lactoferrin in breast milk. The other exception to the trend of increasing protection with age was for older children who acquired no additional protection against malaria with each successive year after age 14 years old. These data suggest that 14 year old children in our villages have acquired functional immunity against malaria such that they are no longer actively acquiring anti-disease immunity.

The year was a significant factor in malaria incidence within our cohort, with a reduced relative risk in 2008 compared to the succeeding years. In our study design, an accurate determination of incidence in our study was dependent on parents bringing their children to our clinic when they exhibited potential malaria symptoms for evaluation by our staff. Parents may have been less likely to come to our clinic during the first year of the study due to unfamiliarity with our staff and clinic or they might have been less adept at identifying malaria symptoms. The incidence remained steady between 2009 through 2011, suggesting that this may have been the case. An alternative possibility is that since we treated virtually every malaria case in these three villages, we shortened the length of time children were parasitemic and symptomatic, and also potentially decreased the malaria transmission within the villages. These changes may have altered normal acquisition of malaria immunity in the form of IgG resulting in a paradoxical increase in malaria incidence. This possibility is supported by the observation that the greatest number of SM cases; CM,

SMA, and RD cases; and all four cases with RD in addition to CM or SMA, which predicts for poor outcome, occurred in 2011.

We saw a slightly higher incidence of malaria within Kenieroba, the inland, agricultural village, than in Bozokin or Fourda, the two villages along the river. Due to their location by the river and a previous study, which showed that Fourda might act as a reservoir for *Anopheles* mosquitoes during the dry season [480], we had predicted that individuals from Bozokin or Fourda might experience increased malaria incidence. Conversely, the children from these villages may have acquired immunity at an accelerated rate due to the persistent presence of *P. falciparum*-carrying vectors throughout the year. Therefore, these children might have had higher levels of immunity compared to children of comparable age in Kenieroba upon enrollment and therefore, experienced decreased incidence of malaria over the course of our study. Additionally, the distance of the two fishing villages from the clinic in Kenieroba may have made them less likely to come into the clinic although a vehicle was sent to each village every day to diagnose any children who exhibited malaria symptoms by blood smear and to drive them to the clinic.

In contrast to previous studies [248, 312, 342], we saw no evidence within our cohort for differential protection by HbS due to age or α-thalassemia genotype. Both of the original studies reporting the interaction between these factors were conducted in Kilifi, a hypo- to meso-endemic area of costal Kenya. Differences in genetic background between our West African Malian population and their East African Kenyan population as well as differences in the endemicity of our sites may account for the apparent discordance between our epidemiological findings. Although a study performed in Ghana, where the genetic background and malaria endemicity more closely resemble our site, also demonstrated negative epistasis between HbS and heterozygous α-thalassemia (–α/αα) in SM, they did not examine whether this effect existed within their population for UM.

Furthermore, the negative epistasis between HbS and α-thalassemia appears to be greater in SM than UM, making it unclear whether we might have seen this effect in our population if we had a greater number of SM cases. Further studies that provide finer resolution for areas and populations where this interaction exists may provide greater insight into the nature of the interaction and the mechanisms driving it.

In conclusion, this study provides evidence for significant protection by HbS against malaria in hyperendemic region of rural Mali, where children appear to achieve adult levels of malaria immunity by age 14. We also provide the first reported evidence of protection by ABO blood group O against UM taken together with SM. Although we did not see an interaction between HbS and either α-thalassemia or age within our cohort, genetic or environmental differences between the populations may explain this discordance. Future investigations into the mechanisms driving these epidemiological differences may provide insight into the underlying mechanisms of protection by RBC polymorphisms and their relationship to acquired immunity.

# 3 Cytoadherence phenotype of parasites from HbAA, HbAS, and HbAC children

## 3.1 Introduction and aims

The mechanism of malaria protection by variant RBCs has been widely investigated over the last half century. Cytoadherence, the binding of mature blood forms of *P. falciparum* to host endothelium in postcapillary venules, is a major step in the pathogenesis of malaria and is mediated through the interaction between PfEMP1 and host receptors, such as CD36 and ICAM-1. Recent findings implicate impaired cytoadherence through reduced surface PfEMP1 levels and abnormal display of PfEMP1 on knobs as a potential mechanism of protection against malaria (discussed further in **1.2.5.4**). This proposed mechanism would reduce the avidity of interaction between parasitized RBC and host endothelium, thereby decreasing the damaging downstream effects of cytoadherence-mediated endothelial cell activation, release of pro-inflammatory cytokines, and activation of tissue factor. Therefore, we hypothesized that to overcome the effects of abnormal PfEMP1 display in HbAS children, parasites in these children are selected to switch to a subset of PfEMP1 variants that encode for high-avidity binding to MVECs. We also tested whether parasites from HbAC children exhibited an increased binding phenotype to MVEC. Expression of such variants might increase a parasite's overall avidity for host endothelium by increasing the parasite's affinity for a particular receptor or by encoding for binding to more than one receptor simultaneously.

## 3.2 Materials and methods

### 3.2.1 Study site, participants, and case definitions

Described in **2.2** and **2.3.1**.

### 3.2.2 Parasite culture and inoculation into wildtype RBCs

Blood samples from HbAA, HbAS, and HbAC children with UM and SM were obtained by venipuncture in Kenieroba, Mali and collected in sodium heparin Vacutainers® (Becton-Dickinson, Franklin Lakes, NJ). To avoid any switching in *var* expression which has been shown to occur with freezing and thawing parasite isolates [80, 486, 487], fresh parasite isolates were used for all comparisons. After transporting at 4°C to Bamako, Mali (~75km, 3 hours by car), RBCs were washed 3 times in RPMI 1640 (Life Technologies, Grand Island, NY) before placing into culture in complete medium [RPMI 1640 supplemented with 2 mg/ml sodium bicarbonate, 50 μg/ml gentamicin, and 0.5% Albumax II (Life Technologies)] at either 1 or 2% hematocrit based on the parasitemia by thin smear at time of initial culture (>1% parasitemia cultured at 1% hematocrit, <1% parasitemia cultured at 2% hematocrit). Blood samples from uninfected 'wildtype' children (HbAA, normal αα/αα genotype, and no *G6PD**(A-) allele) were collected similarly by venipuncture into sodium heparin Vacutainers®, but were resuspended at 50% hematocrit after 3 washes in RPMI 1620 and stored at 4°C until use within 3 days. All cultures were maintained in a humidified 5% $CO_2$ environment at 37°C and cultured in 0.2-μm-vented flasks (Corning, Inc. Corning, NY).

The development of parasites was tracked by thin smears taken at 8-hour intervals. Once parasites had reached mid to late trophozoite stage ( ~24 hours) (**Figure 3.1a**), they were passed over MACs LS columns (Miltenyi Biotec, Auburn, CA), which purify and enrich for mature trophozites based on the paramagnetic properties of hemozoin (**Figure 3.1b**).

The resulting eluate of ~99% mature trophozoites was then inoculated into wildtype donor uninfected RBCs at 2.5% hematocrit. Once invasion of uninfected RBCs was confirmed by the appearance of new ring-stage parasites by thin smear approximately 16 hours after inoculation, complete media was added to the culture to reduce the hematocrit to 0.8%.

### 3.2.3 Maintenance of dermal microvascular endothelial cells

Dermal human MVECs (HMVECs-d, Lonza, Walkersville, MD) were grown in the manufacturer's EGM-2MV media for up to 5 passages with media changes every 2 days. 1-3 days prior to the cytoadherence assay, HMVECs-d were trypsinized (Lonza, Walkerville, MD) and seeded into 8-well LabTak CC2-coated chamber slides (Nalge Nunc International, Rochester, NY). Cytoadherence comparisons were performed on endothelial cells that were ~30% confluent.

### 3.2.4 Cytoadherence assay

Cytoadherence comparisons to MVEC were performed in parallel once two or more parasite isolates from HbAA and either HbAS or HbAC children that had been cultivated, purified, and inoculated into the same wildtype cells, simultaneously matured to trophozoites (**Figure 3.1c**). When this occurred, the parallel parasite cultures were enriched for mature forms by magnetic purification. The parasitized RBCs were resuspended at 3-5% parasitemia and 0.5% hematocrit using uninfected RBCs in binding medium (BM; RPMI 1640, 0.5% BSA). For each HbAS or HbAC parasite isolate, 200 µL of parasitized RBC suspension per well was added to MVEC-coated chamber slides in duplicate wells, where possible, alongside the duplicate wells for the HbAA control (**Figure 3.1d**). After incubating on an orbital shaker (100 rpm) for 1 hour at room temperature, the parasitized RBC suspensions were removed from each well, the gaskets were detached from the slide, and the slide was washed by dipping horizontally in BM four times. Slides were fixed overnight

in 2% glutaraldehyde (Sigma, St. Louis, MO) at room temperature, then stained in 10% Giemsa for 30 minutes. The number of parasitized RBCs bound to ~350 MVECs was counted for each well.

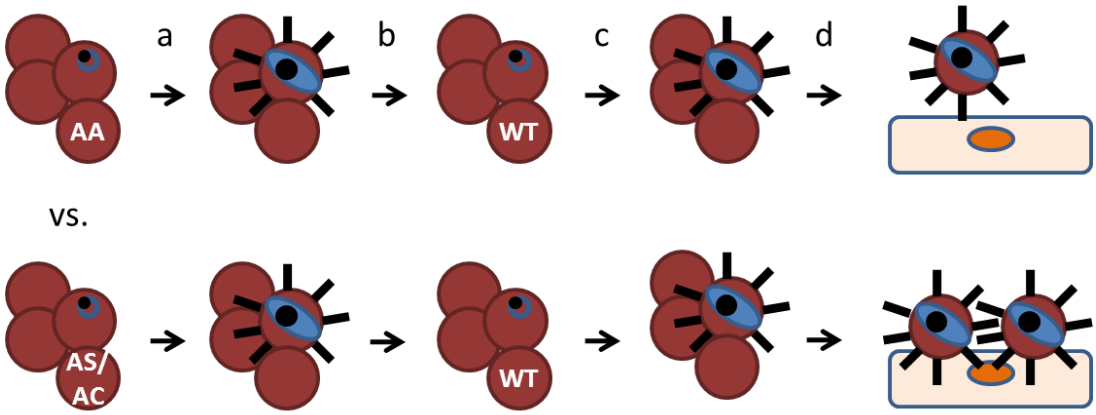



**Figure 3.1 Schematic representation of cytoadherence assay.**

Ring-stage parasites from HbAA, HbAS and HbAC children were allowed to mature to trophozoite stage (a), then purified by magnetic column and inoculated into wildtype donor RBCs (b). After invasion and maturation to trophozoite stage (c), parasites from HbAS or HbAC children were compared for binding to MVECs in parallel with those from HbAA controls (d).

### 3.2.5 Statistical analysis

An overdispersed Poisson regression was used to model the effect of Hb type (HbAS and HbAC) and age (≤5 and >5 years) on the binding of parasitized RBCs to MVECs, controlling for any 'slide' and 'location within slide' effects in our data set and for any multiple comparisons done with an individual parasite isolate. Analyses were done in Proc GLMMIX in SAS Version 9.2.

## 3.3 Results

Recent studies implicating reduced cytoadherence as a mechanism of malaria protection by HbS and HbC led us to hypothesize that parasite isolates from symptomatic HbAS or HbAC children express high-avidity PfEMP1 variants. To test this hypothesis, we compared parasite isolates from either HbAS or HbAC children in parallel with those from HbAA children for their binding to MVEC in a semi-static cytoadherence assay. To remove any confounding effects of RBC type, isolates compared in parallel were inoculated into the same wildtype cell before comparison.

### 3.3.1 Effect of Hb type on binding phenotype

**Parasites from HbAS children show non-significantly increased binding to MVEC**

In 16 paired comparisons between parasites from HbAS and HbAA children (**Supplemental Table 3.1**), we found that parasites from HbAS children showed a ~50% increase in binding to MVECs compared to those from HbAA children, but this difference did not reach statistical significance (fold change = 1.46, 95% CI 0.97 – 2.19, P = 0.07) (**Figure 3.2**). However, parasites from HbAC children clearly showed no difference in binding compared to those from HbAA children (fold change = 1.19, 95% CI 0.77 – 1.84, n = 15, P = 0.43).

### 3.3.2 Effect of age on binding phenotype

**Parasites from younger children and older children showed no difference in their binding to MVEC**

Within our cohort, age played a significant role in protection against malaria with older children experiencing significantly lower incidence of malaria than younger children (see **2.3.2.1**) suggesting that they have acquired higher levels of anti-disease immunity to malaria. Furthermore, since another study has shown that age, as a surrogate for immunity,

may affect the expression of PfEMP1 variants [419], we included it in our analysis as a categorical covariate. Parasites from younger children (≤5 years) showed no difference in binding compared to those from older children (>5 years) (fold change = 1.21, 95% CI 0.83-1.77, n=31, p=0.31) (**Figure 3.2b, Supplemental Table 3.1**).

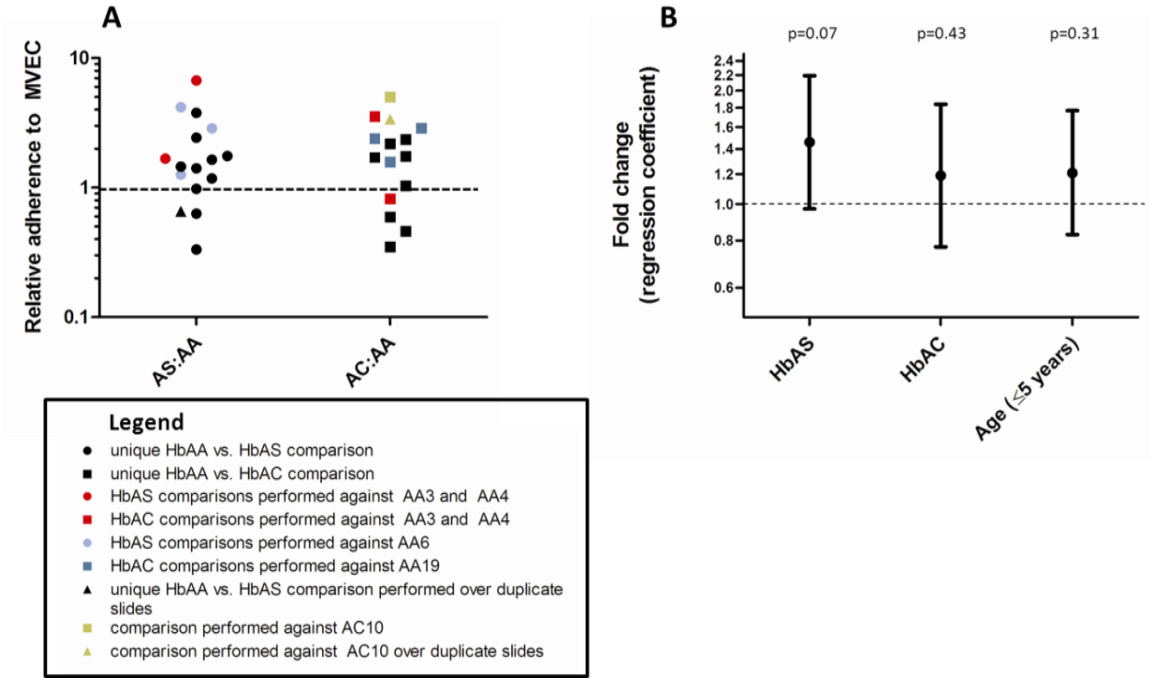



**Figure 3.2 Relative adherence of parasitized HbAA, HbAS, and HbAC red blood cells to microvascular endothelial cells**

**A)** Parasites from HbAS and HbAC children were inoculated into wildtype donor RBCs and compared for binding to MVECs in parallel with parasites from HbAA children. The number of parasitized RBCs bound per MVEC was calculated for each isolate (binding ratio). Ratios of relative adherence of were determined for each of these comparisons by normalizing the binding ratio of the HbAS- or HbAC-derived parasites to the binding ratio of the HbAA-derived parasites tested in parallel. As a result of the multiple comparisons conducted for several of the samples, statistical analyses taking this into account were performed. **B)** Relationship between cytoadherence, hemoglobin type and host age. A Poisson regression model was constructed to examine the effect of Hb type (HbAS and HbAC) and host age on the cytoadherence of parasitized RBCs to microvascular endothelial cells. The fold changes and 95% confidence intervals of the relative binding compared to parasites from HbA (for HbS and HbC) and parasites from children >5 years old (for age ≤5 years) are indicated.

## 3.4 Discussion

Recent studies have implicated impaired cytoadherence due to reductions in surface PfEMP1 levels and altered display of PfEMP1 in the protection by variant RBCs against malaria. Consequently, we hypothesized that parasites from symptomatically *P. falciparum*-infected HbAS and HbAC children display an enhanced binding phenotype towards MVECs in order to overcome this protective mechanism and cause disease in genetically protected individuals. Our results, although not conclusive, support the possible selection of parasites with increased avidity to MVEC in HbAS children. In 31 total comparisons between parasites from HbAS and HbAC children against parasites from HbAA children, we observed a trend of 46% increased binding phenotype to MVECs in HbAS children, but this difference did not reach significance (P = 0.07). There was clearly no association between binding phenotype and either HbAC or age.

Further follow-up studies are needed to confirm these findings since the difference in binding phenotype between HbAA and HbAS children did not reach significance possibly due to a number of limitations. One limitation was the number of comparisons we were able to perform. Multiple challenges inherent to this study prevented us from performing more comparisons despite working within a cohort of ~1500 Malian children over 3 transmission seasons in an area with intense, seasonal transmission and relatively high percentages of HbS (15%) and HbC (7%). First, the cytoadherence assay required large numbers of parasites to achieve significant binding to MVECs. Since we wanted to capture the binding phenotype displayed by these parasites *in vivo* before significant PfEMP1 antigenic switching could occur, we conducted the assay with fresh isolates within two culture cycles. Therefore, since isolates could not be expanded in culture, only isolates from children with high parasite densities could be used. Second, since fresh isolates were used in comparisons, the ability to compare parasites from wildtype and variant RBCs was

dependent on HbAA and either HbAS or HbAC children reporting to our clinic on the same day for treatment of malaria. After their isolates were placed into culture, parasites from each isolate had to synchronously mature for parallel comparison of binding to MVEC. However, isolates displayed variability in their rate of maturation, which prevented successful comparison of some isolates. Third, since HbAS confers significant epidemiological protection against malaria within our cohort (see **2.3.2.2**), HbAS children were less likely to report to our clinic with malaria and fewer parasite isolates were available from these children.

Another limitation of our study may have been corrected with a greater number of comparisons. Age, coinherited polymorphisms such as α-thalassemia and G6PD deficiency, and ABO blood group may affect malaria protection and influence cytoadherence [358]. The severity of infection may also be associated with cytoadherence. We included age in our model by comparing younger (≤5 years old) to older (>5 years old) children based on the observation that children older than 5 appear to gain some immunity against malaria with each year within our cohort (see **2.3.2.1**). However, more comparisons would allow us to divide the ages into more categories to achieve a better understanding of age's potential role in affecting cytoadherence. 42% of our comparisons may also have been confounded by co-inherited α-thalassemia or G6PD deficiency and 63% by the presence of blood group O in only one of the isolates compared in parallel. Blood group O, in particular, may have significantly affected our findings due to the high percentage of comparisons between isolates from O and non-O children as well as the significant protective effect by blood group O within our cohort (see **2.3.2.2**). Although 4 of our comparisons included isolates from 'severe' episodes, these episodes were categorized as 'severe' based on the inability to drink, prostration, repetitive vomiting or a combination of these three criteria. Since none of these episodes involved CM, SMA, or RD, we might predict that the severity of infection would have a relatively minor effect on binding phenotype within our study.

Additionally, the assay used in our experiments was performed in conditions which differ from physiological conditions in several important ways. First, the assay was performed at room temperature, which may have prevented optimal binding between parasitized RBCs and MVECs. Second, the MVECs in our assay were 30% confluent, which is significant since MVECs are known to undergo changes in their expression of receptors, their morphology, and the composition of the surrounding extracellular matrix upon formation of a confluent monolayer. Third, the MVECs in our assay were not stimulated with TNF, which would normally be present in an acute malaria episode and would induce expression of ICAM-1. Without TNF stimulation, MVECs express high levels of CD36, but low levels of most other PfEMP1 receptors, most notably ICAM-1, but also VCAM-1 and PECAM. Despite these drawbacks of our assay conditions, we chose to use them to most closely replicate the conditions used in the original experiments that observed decreased cytoadherence in HbAS and HbAC RBCs.

Lastly, the large numbers of parasites required to conduct the cytoadherence assay limited the parasite samples used in our assay to those from children with relatively high parasite densities, as discussed above. However, parasite isolates from symptomatic children with low parasite densities caused malaria with relatively few parasites, and therefore, may have had increased pathogenicity. Since one potential mechanism of increased pathogenicity would be through increased binding avidity to MVECs, exclusion of these parasites may have biased our experiment parasites with lower avidity binding to MVECs.

Despite the limitations of our study, the trend of increased binding in parasites from HbAS children suggests that alterations in PfEMP1 presentation might restrict disease-causing parasites in these individuals to those expressing a limited subset of high-avidity PfEMP1 variants. Selection of a specific group of expressed PfEMP1 variants in HbAS

children with malaria would have several important implications. First, it would provide *in vivo* evidence that a mechanism of reduced cytoadherence protects children carrying variant RBCs and that parasites modulate their expressed *var* gene repertoire in adaptive response. Although parasites expressing high-avidity PfEMP1 variants might 'break-through' the PfEMP1-dependent protective mechanism to cause malaria in an HbAS child, the resulting clinical episode might be less severe than in an HbAA child of similar age. Additionally, HbAS children might develop immunity to malaria at an earlier age since acquisition of IgG against a limited number of PfEMP1 variants, which mediate increased binding, would be sufficient to protect against disease. Second, characterization of the restricted subset of PfEMP1 variants expressed in HbAS children might result in the discovery of 'pathogenicity motifs' (e.g., particular PfEMP1 domains, or conserved sequences within domains). PfEMP1 variants containing 'pathogencity motifs' may only cause uncomplicated malaria in older children with HbAS, but they presumably cause severe malaria in younger HbAA children who lack both immune- and genetically-mediated resistance to this disease. Therefore, this strategy for identification of 'virulent' PfEMP1 variants complements efforts to determine *var* genes preferentially expressed in SM. A related alternative strategy might be to simulate the process of selecting high-avidity PfEMP1 variants *in vitro* through panning isolates with sequenced *var* genomes in HbAS RBCs followed by identifying selected variants by quantitative PCR.

Our findings from this study may also aid in explaining the apparent discordance between the degree *in vitro* reductions in cytoadherence compared to the *in vivo* protection afforded by HbS and HbC. Although both HbAS and HbAC RBCs demonstrate similar cytoadherence reductions of approximately 50% *in vitro*, the degree of *in vivo* protection afforded by HbAS against SM is significantly greater than that afforded by HbAC and only HbAS is thought to protect against UM. Within our cohort study, HbS showed a 35% protection against malaria (UM and SM taken together), while HbC demonstrated a

non-significant decrease in malaria protection (**2.3.2.4**). Our results from comparing the cytoadherence of parasites from HbAS and HbAC children to those from HbAA children demonstrated that while neither Hb variant resulted in a significant difference in cytoadherence compared to parasites from HbAA children, parasites from HbAS children trended towards increased binding while those from HbAC children clearly showed no significant difference in binding. These results imply that the differences in *in vitro* reductions and *in vivo* protection might be due to a greater impairment of cytoadherence by HbAS *in vivo* than *in vitro* relative to HbAC. However, our results suggest that while both Hb variants may result in reductions in surface PfEMP1 levels and *in vivo* cytoadherence, these reductions may not be sufficient to select for high-avidity binding PfEMP1 variants. The lack of statistical significance in both sets of comparisons suggests that other mechanisms (e.g., PfEMP1-specific IgG) are also likely working in concert with reduced cytoadherence to produce clinical protection against UM and SM.

In this study, we have examined the binding phenotype of parasites from HbAA. HbAS, and HbAC children to MVEC under semi-static conditions. While this approach has the advantage of examining binding to receptors displayed on physiologically relevant cells, previous experiments have demonstrated that cytoadherence may differ under semi-static and flow conditions [488] and that isolates may show differential binding to specific host receptors [75, 432, 489]. Examining binding to MVEC under flow conditions, to individual immobilized host receptors (i.e., CD36, ICAM-1, and gC1qR), or to endothelial cells derived from the brain or lung would further define the binding phenotype of parasites from HbAA, HbAS, and HbAC children. Additionally, multiple receptors have been shown to work synergistically to mediate binding of parasitized RBCs with ICAM-1 and CD36 promoting rolling and static adhesion respectively [88, 89, 91]. Although MVEC normally express high levels of CD36 and low levels of ICAM-1, simultaneous expression of CD36 and ICAM-1 on MVECs can be achieved through incubation with TNFα [89].

In conclusion, *P. falciparum* parasites from HbAS children may demonstrate an increased binding phenotype to MVEC although within our study, the difference in binding to MVEC between parasites from HbAA and HbAS children did not reach statistical significance. Further studies, which are adequately powered to properly account for potential confounders such as age, severity of malaria episode, and co-inherited RBC polymorphisms, should be performed to confirm our findings. If confirmed, our result implies that parasites select for high avidity PfEMP1 variants to circumvent the altered PfEMP1 display which normally protects HbAS children against malaria. Identifying domains which are selectively found in high avidity PfEMP1 variants or mutations which confer intrinsically higher avidity binding to MVEC, may help us better understand he role that PfEMP1 variation plays in modulating the virulence of parasite isolates. However, the extensive diversity of PfEMP1 and technical challenges pose barriers in using selection in HbAS children to identify these domains and mutations.

# 4 Expressed *var* gene repertoire in HbAA, HbAS, and HbAC children

## 4.1 Introduction and aims

The expression of PfEMP1 variants on the surface of mature blood-stage *P. falciparum* parasites confers the ability to bind to specific host receptors in the microvasculature and influences the clinical severity of infection. Since PfEMP1 expression, although crucial to parasite survival, leaves the host vulnerable to recognition by the host immune system, parasites escape detection by switching between their ~60 diverse *var* genes, which encode for PfEMP1. However, several studies have reported a restricted subset of expressed PfEMP1 variants in parasites from children with SM (described in **1.3.3**), suggesting that a limited number of virulent variants may cause SM through increased avidity of cytoadherence or by other undetermined mechanisms. The association of longer PfEMP1 variants with increased sequence conservation and the ability to bind ICAM-1 suggests that longer, multi-domain PfEMP1 variants may have been evolutionarily selected for their ability to bind to multiple host receptors simultaneously, which would increase the overall avidity of the interaction.

The recent finding that protection against malaria by HbS and HbC might be mediated through PfEMP1-dependent reductions in cytoadherence led us to hypothesize that parasites from HbAS and HbAC children with malaria might express a similarly restricted *var* gene repertoire. In particular, since the ability to bind to host receptors is mediated through specific PfEMP1 domains, we predicted that the parasites from HbAS and HbAC children would express longer, multi-domain PfEMP1 variants. Here, we have tested whether parasites from HbAS and HbAC children show increased homogeneity of expressed

*var* gene repertoires, increased expression of particular features and motifs within the DBLα region of PfEMP1, or increased length of PfEMP1 proteins compared to HbAA children.

## 4.2 Materials and methods

### 4.2.1 Study site, participants, and case definitions

See **2.2** and **2.3.1**.

### 4.2.2 Isolation of RNA and reverse transcription

RNA was extracted directly from 250-500 µL of whole blood from 26 HbAA, 13 HbAS, 9 HbAC, 1 HbSC, and 1 HbCC Malian children with *P. falciparum* malaria between July and September 2009 for cloning and sequencing (**Supplemental Table 8.2**) and from 45 HbAA, 36 HbAS, and 15 HbAC Malian children between July and September 2010 for length analysis by Northern blot (**Supplemental Table 8.3**). RBCs from whole blood were treated with 10% saponin then washed 3 times in 1 x PBS before resuspending in 1 mL Trizol (Invitrogen, Grand Island, NY), and storing at -80°C for up to 3 months until all samples from the transmission season had been collected. All samples from a transmission season were thawed and processed in parallel. A biphasic mixture was formed by addition of chloroform and centrifugation at 13000 rpm at 4°C for 20 minutes. After transferring the upper aqueous phase to a new tube, RNA was precipitated by addition of isopropanol then washed with 70% ethanol. The pellet was air-dried, then resuspended in 20 µL DEPC-treated water.

For cloning and sequencing, RNA was reverse transcribed into cDNA using a Qiagen QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). ~1 µg of RNA was treated using the gDNA wipeout buffer then reverse transcribed according to manufacturer's instructions.

### 4.2.3 Amplification of DBLα sequence tag, cloning of sequences, and sequencing

A ~300 nt region of the DBLα domain was amplified from 50 cDNA samples from children with malaria in 2009 using universal primers DBLαAF', GCACG(A/C)AGTTT(C*/T)GC,

and DBLαBR, GCCCATTC(G/C)TCGAACCA with PCR conditions: 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 42°C for 30 sec, 65°C for 1 min then one cycle of 65°C 7 min [454, 490]. A negative control of water and positive control of 3D7 gDNA were also performed in parallel. PCR products were run on 1.5% agarose gels at 50V to confirm a ~300 nt product. PCR products of the correct length were ligated into a pCR-TOPO vector using a TOPO TA cloning kit (Invitrogen, Grand Island, NY) according to manufacturer's instructions. Cloned products were then transformed into DH5α-T1$^{R}$ competent cells (Invitrogen, Grand Island, NY) by heat shock at 42°C for 45 sec, incubated at 37°C for 1 hour in SOC media, then plated at two volumes (50 µL and 200 µL) onto Luria Broth (LB) + Ampicillin (Amp) (100mg/L) + (0.3%) X-gal plates and incubated at 37°C overnight. White colonies were picked from the plates and grown in 2 x TY with 100mg/L ampicillin. DNA was purified from the resulting culture using Wizard Plus SV Miniprep (Promega, Madison, WI) then sequenced in both the forward and reverse directions using a 4:1 mix of BDT:dGTP on ABI 3730 sequencing machines (Applied Biosystems, Carlsbad, CA). We aimed to sequence the ~300 nucleotide (nt) DBLα sequence tag from 50 clones from each parasite sample. The number we were successfully able to sequence ranged from 17 to 87 although for the majority of parasite samples (41/50) we successfully sequenced 30 to 50 clones.

### 4.2.4 Characterization of DBLα sequences

For each forward and reverse read, the vector was stripped using a web-based program (Primers: 5' GCACGAAGTTTTGC 3' and 5' TGGTTCGAATGGGC 3') with up to 10% mismatch. The sequences were then translated into all three forward reading frames using web-based program Transeq (http://www.ebi.ac.uk/Tools/st/) and visually inspected for expected motifs (DIGDI at the 5' end, PFQLR at the 3' end) from the ~300 nucleotide DBLα sequence tag. Only sequences from reading frames that had both of these motifs were retained and trimmed on both ends to start with DIGDI and end with PFQLR. The same was

repeated for the reverse complement primers in the three reverse reading frames. The corresponding forward and reverse sequences were then compared. Identical sequences were retained. Consensus sequences were formed for any non-identical sequences with > 96% similarity. All sequence tags were then classified by the number of cysteine residues within the DBLα sequence tag and motifs within positions of limited variability (PoLV) as described previously by Bull and colleagues [454] using a Perl script described in a subsequent paper [491].

### 4.2.5 Construction of phylogenetic tree

The three most predominant unique DBLα sequence tags were determined for each isolate according to their prevalence. In the cases of ties, if the inclusion of the tied sequences would lead to more than three unique sequences for a particular isolate, the tied sequences were included only if they comprised > 5% of the isolate's sequenced DBLα tags. Each sequence was named by its sample's unique identifier (Hbtype + number) followed by the sequence's prevalence rank (1 = most predominant). Any ties were indicated by a "." followed by an arbitrary number to distinguish the sequence tag. A phylogenetic tree was constructed with these sequences using the neighbor-joining method [492] in MEGA5 [493] by p-distance and displayed using the Interactive Tree of Life (iTOL) [494, 495].

### 4.2.6 Modeling PfEMP1 length by PoLV motifs

PfEMP1 sequences from 7 genomes: 3D7, HB3, IT4, DD2, RAJ116, IGH-CR14, and PFCLIN, were included in our analysis. To eliminate partial genes or pseudogenes, only PfEMP1 variants that either had reported phenotypic data or a fully sequenced exon 1, as determined by the presence of both an *ups* sequence and an ATS, were included in our analyses. Of the total 399 PfEMP1 variants reported in Rask et al. [364], we included 199. For each of the PfEMP1 sequences, we determined the number of domains reported as well

as the number of cysteine residues and the PoLV motifs within the DBLα sequence tag as described in **4.2.4**.

### 4.2.7 Northern blots

#### 4.2.7.1 Size separation of RNA fragments by 0.8% denaturing Northern blot agarose gel

For each sample, ~1 µg of RNA suspended in 20 µL diethylpyrocarbonate (DEPC)-treated water was precipitated in 50 µL EtOH and 2 µL NaOAc with 9 µL 20 mg/mL glycogen added to each sample before incubation at -80°C for 15-20 min. After centrifugation for 30 min at 4°C, the supernatant was removed and the RNA samples were denatured through addition of 5 µL formamide and incubation at 60°C for 5 min. Samples were then stored on ice until they were loaded onto 0.8% agarose gels [0.8 g in 100 mL 1 x Tris-Borate-EDTA (TBE)] containing 5mM guanidine thiocyanate. A 0.25-9 kB Gibco ladder and A4 control were run alongside RNA samples in loading dye (50% glycerol, 49.6% DEPC $H_2O$, 0.4% bromophenol blue). Gels were run at 110V for 10-15 min, then at 75V for 3 hr. Gels were stained in 1 µL EtBr in 50 mL 1 x TBE for 10 min and destained in 1 x TBE before ultraviolet (UV) visualization for confirmation of RNA quality, verification of parasite RNA purity and lack of contaminating human RNA, and determination of the approximate total amount of RNA in the sample.

#### 4.2.7.2 Transfer to Hybond $N^+$ membrane and neutralization

Size-separated RNA samples were transferred from agarose gels to Hybond $N^+$ nitrocellulose membranes by capillary transfer in 7.5mM NaOH overnight. After transfer, the blots were neutralized in 2 x saline sodium citrate (SCC) (0.3M NaCl, 0.03M sodium citrate) and air-dried. Samples were immobilized by covalent linkage to Hybond $N^+$ nitrocellulose membranes by UV light.

#### 4.2.7.3 Probe construction and hybridization

PfEMP1 variants were detected using a varC probe designed to detect a variety of semi-conserved exon2 sequences [496]. To create this probe, primers flanking a ~1kb region of exon 2: 5' AAAAAACAAAATCATCAGTAGGAAATTTATT(C/T)C 3' and 5' TATCCCATAAATCTGC(A/T)AT(A/T)GG(A/G)TA 3' were used with PCR conditions: 92°C for 120 sec followed by 35 cycles of 92°C for 20 sec, 41-52°C for 20 sec, 70°C for 30 seconds on 3D7 gDNA. Random prime labeling (GE Healthcare Amersham Megaprime DNA labeling system) was used to radioactively label the varC PCR products. After denaturing the varC PCR product with random primers by boiling for 5 min, 'Klenow' polymerase and cold deoxynucleotide triphosphates (dNTPs) (dC, dG, and dTTP) were added alongside $\alpha^{32}$P dATP and incubated at 37°C for 30 minutes before denaturing the labeled DNA by boiling for 2 min.

In preparation for hybridization, the blot was incubated with prehybridization church buffer (7% sodium dodecyl sulfate (SDS)/0.5M $NaH_2PO_4$ pH 7.2/2% dextran sulfate) at 50°C in a hybridization bottle with rotation. After 3-5 hrs, the prehybridization buffer was removed and replaced by church buffer with the varC probe added. After incubating with rotation overnight at 50°C, the blot was washed in 0.5x SSC/0.1% SDS. The radioactive blot was transferred to film at -80°C overnight and developed.

### 4.2.8 Statistics

#### 4.2.8.1 Homogeneity calculation

As a measure of homogeneity, the degree to which PfEMP1 variants were identical to each other within the expressed *var* repertoire for each parasite sample, we used the Herfindahl-Hirschman index [497]. The homogeneity index was calculated for each parasite sample using the following equation where H represents the homogeneity of the sample, m

is the number of unique DBLα sequences typed within a sample, X represents the number of typed copies of that particular DBLα sequence within a sample, and N represents the total number of DBLα sequences typed for a sample.

$$H = \sum_{k=1}^{m_i} \left(\frac{x_{ik}}{N_i}\right)^2$$

**Equation 4.1 Homogeneity index (Herfindahl-Hirschman index)**

A value of 1 would indicate that all typed sequences within a particular parasite isolate were identical (>96% sequence similarity) suggesting complete homogeneity of the expressed *var* gene repertoire within that isolate. A value of $1/N_i$ would indicate that all typed sequences were unique.

#### 4.2.8.2 Ratio of unique 'sequence types' to total sequences

To determine the homogeneity of the expressed *var* gene repertoire on a population level, we calculated a ratio of the total number of DBLα sequence tags expressed in HbAA, HbAS, or HbAC children to the number of unique 'sequence types' within the corresponding Hb type. Sequences with >96% similarity were considered identical. A ratio of 1 would indicate that there was no overlap in the sequenced DBLα tags expressed within children of a particular Hb type. Higher ratios indicate greater degrees of overlap within the *var* gene repertoires expressed in particular Hb types.

#### 4.2.8.3 Pairwise distance calculations

A measure of the differences between DBLα sequence tags of PfEMP1 variants expressed by parasites in HbAA, HbAS, and HbAC children was determined based on pairwise distances, which represent the average number of amino acid differences per site. Using these pairwise distances, the average distance was determined for sequences

expressed in HbAA, HbAS, and HbAC children. A distance of 0 would indicate that all sequences were identical and a distance of 1 would indicate sequences that were completely different. These calculations were performed in Mega5 [493] using the 'pairwise deletion' setting.

#### 4.2.8.4 Regression models

To determine the effect of Hb type on the percentage of *cys2* sequences expressed by parasite isolates, a linear regression model was constructed with host Hb type, host age, parasitemia, and severity of infection included as covariates. The coefficients of such a model represent the percentage increase or decrease in *cys2* sequences attributable to each covariate. To determine the effect of Hb type on the percentage of expressed variants from each group (1-6), a logistic regression model was constructed for each group using the same covariates. To take into account the multiple comparisons we performed, the level of significance was corrected by a Bonferroni correction. Analyses were done in Proc GLMMIX in SAS Version 9.2.

#### 4.2.8.5 Association of PfEMP1 length by PoLV motifs

Univariate quasi-Poisson linear models were used to model the number of PfEMP1 domains based on the four-letter motifs located in the PoLV1, PoLV2, PoLV3, PoLV4 as well as individual amino acids located within these PoLVs. Any amino acid locations with a conserved residue in all sequences were not included in the analysis. For any PoLV or individual amino acid positions that were significantly associated with the number of PfEMP1 domains, further univariate quasi-Poisson linear models were performed on each individual motif or amino acid residue within that position. Chi-squared tests were performed to determine whether any associations were statistically significant. To take into account the multiple comparisons we performed, the level of significance was

corrected by a Bonferroni correction for all analyses. Analyses were done in Proc GLMMIX in SAS Version 9.2.

## 4.3 Results

### 4.3.1 Homogeneity of PfEMP1 variants expressed in HbAA, HbAS, and HbAC children

We attempted to determine the homogeneity of PfEMP1 variants expressed in HbAA, HbAS, and HbAC children both within an isolate's expressed *var* repertoire and within the expressed *var* repertoire of all sequenced isolates from children of each Hb type. We hypothesized that parasite isolates from HbAS and HbAC children would show restriction in their expressed *var* gene repertoire and, therefore, would demonstrate increased homogeneity by both of these measures. The immense diversity and 3-9kb length of sequences encoding the extracellular portion of PfEMP1 pose obstacles to comparing the full-length *var* gene sequences. Consequently, almost all efforts to characterize expressed PfEMP1 variants from field isolates have focused on sequencing a region of the DBLα domain, which demonstrates much higher levels of conservation (40-50% sequence conservation) than other PfEMP1 domains. Using universal primers to amplify a ~300 nucleotide region of DBLα, we compared the homogeneity of *var* genes expressed in parasites from 26 HbAA, 13 HbAS, 9 HbAC, 1 HbSC, and 1 HbCC children with malaria.

**Hb type does not affect the homogeneity of the expressed *var* repertoire within each parasite isolate**

We determined the homogeneity of the expressed *var* gene repertoires within each isolate using the Herfindahl-Hirschman index. We predicted that if parasites from HbAS and HbAC children were restricted in the PfEMP1 variants they expressed, that they would be more likely to express fewer variants, and thus would have a greater homogeneity index. However, parasites from children of different Hb types (HbAA, HbAS, HbAC, HbSC, and HbCC) showed no significant difference in the homogeneity of their expressed *var* genes ($P = 0.21$) (**Figure 4.1**).

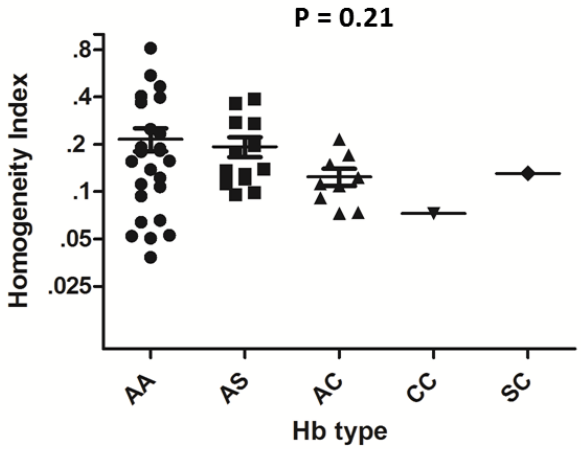



**Figure 4.1 Homogeneity index for PfEMP1 variants expressed by HbAA, HbAS, HbAC, HbCC, and HbSC children**

The homogeneity index, which represents the uniformity of expressed PfEMP1 variants within each sample, was calculated as described in **4.2.8.1** for each sample. Each symbol represents the calculated homogeneity index for the parasite isolate from an individual HbAA, HbAS, HbAC, HbCC, or HbSC child. A value of 1 indicates the highest degree of homogeneity (i.e., all sequences are > 96% similar to each other) and a value of 1/N (where N equals the total number of sequenced DBLα tags), indicates that all sequenced samples are unique.

**Hb type does not affect the homogeneity of the total population of expressed *var* genes**

We also wanted to determine the *var* gene homogeneity on a population level within the expressed PfEMP1 repertoire for all sequenced isolates from HbAA children compared to those in all HbAS or HbAC children. If the expressed *var* gene repertoire is restricted in HbAS and HbAC children, we would expect greater homogeneity of expressed PfEMP1 variants in the population of parasites from HbAA children than those from HbAS or HbAC children. We examined this question using two approaches. In the first approach, we determined the total number of DBLα sequences tags expressed in HbAA, HbAS, or HbAC children and divided this by the number of unique 'sequence types' expressed in children of the corresponding Hb type. This ratio was similar between the three Hb types with a ratio of 1.06 for parasites from HbAA children (418 sequences, 394 'sequence types'), 1.10 for

HbAS children (154 sequences, 144 'sequence types'), and 1.07 for HbAC children (184 sequences, 167 'sequence types'). In the second approach, we determined the homogeneity of sequences expressed within HbAA, HbAS, and HbAC children based on pairwise distance calculations, which represent the average number of amino acid differences per site ($aa_{diff}$) between sequences. By this measure, the 422 unique 'sequence types' in HbAA children had an $aa_{diff}$ of 0.477, the 184 'sequence types' in HbAS children had an $aa_{diff}$ of 0.485, and the 154 'sequence types' in HbAC children had an $aa_{diff}$ of 0.479. Taken together, these results suggest that Hb type does not affect the homogeneity of the expressed *var* gene repertoire within the population of each Hb type.

**Predominant DBLα sequence tags demonstrate no clustering by Hb type in a phylogenetic tree**

Another way to determine whether parasites in HbAS or HbAC children express a limited *var* gene repertoire is to construct a phylogenetic tree using the predominant DBLα sequence tags. We selected the three most highly expressed unique sequence tags from each parasite sample and constructed a phylogenetic tree using the neighbor-joining method (**Figure 4.2**). From this analysis, there appears to be no clustering of PfEMP1 variants expressed in HbAS or HbAC children in the most distinct groupings of similar variants (AS4-1 to AC6-2; AA13-3.2 to AC4-2). Although several sequences from HbAS children appear to be very similar, such as AS4-1 and AS2-2 as well as AS13-2 and AS12-2, comparable similarities are present between HbAA children (AA4-1 and AA13-3.2) as well as between HbAA and HbAS children (AA3-2 and AS5-1).

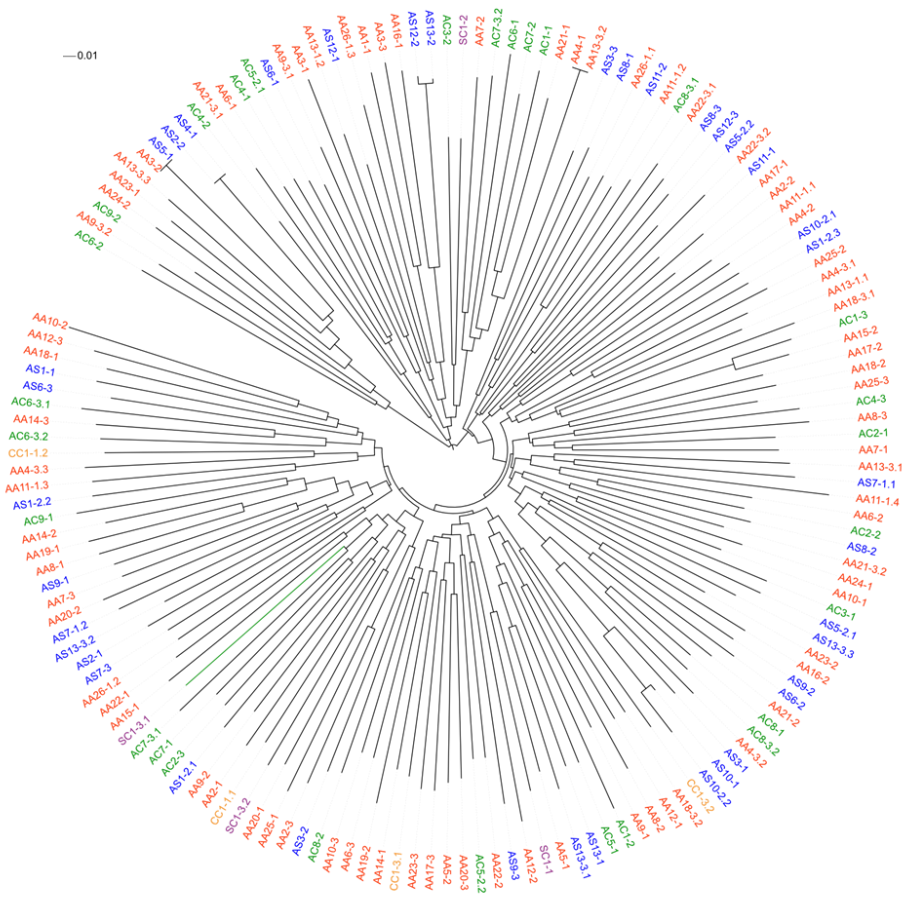



**Figure 4.2 Phylogenic tree of 3 predominant sequence tags for each parasite sample**

A phylogenic tree was created using the 3 most highly expressed DBLα sequence tags for each parasite sample using the neighbor-joining method with p-distances. The tree is drawn according to scale with the branch lengths representing the computed evolutionary distance between sequences. A distance of 0.01, which is equivalent to 0.01 differences in amino acid residues per site ($aa_{diff}$), is indicated. Labels are colored according to host Hb type with red = HbAA, blue = HbAS, green = HbAC, purple = HbSC, and orange = HbCC. Each of the sequences tags is named with the parasite sample ID (host Hb type + arbitrary identifying number) followed by a number representing whether the sequence tag was the most (1), the second-most (2), or the third-most (3) predominant tag with any ties followed by a "." and an arbitrary number to distinguish the sequence tag.

### 4.3.2 Presence of predictive motifs within DBLα sequence tags expressed in HbAA, HbAS, and HbAC children

Although the ~300 nt DBLα sequence tag represents less than 3% of even the shortest PfEMP1 extracellular region, predictive motifs within the tag have been identified, which associate with other PfEMP1 characteristics such as the ability to bind to CD36 or to rosette, *ups* group classification, and PfEMP1 length. We sought to determine whether any of these motifs were differentially expressed in parasites from HbAS or HbAC children compared to HbAA children.

**Hb type does not affect the percentage of *cys2* PfEMP1 variants expressed by isolates**

The DBLα sequence tag typically contains either 2 or 4 cysteines. The presence of 2 cysteines within the tag, *cys2*, has been associated with the inability to bind CD36, the ability to rosette, *upsA var* genes, and longer, multi-domain PfEMP1 variants [454]. On the other hand, the presence of 4 cysteines, *cys4*, is associated with opposite characteristics: the ability to bind CD36, the inability to rosette, *upsB* and *upsC var* genes, and shorter PfEMP1 variants. Additionally, *cys2* variants have been associated with severe disease and expression in young children who lack immunity to malaria [419]. As a result, we sought to determine the percentage of *cys2* variants within the tags sequenced for each parasite isolate and to correlate this percentage to host Hb type while accounting for host age, parasitemia, and disease severity by including these covariates in our linear regression model. The coefficient associated with each covariate predicts the change in percentage *cys2* for a given parasite isolate (i.e., a coefficient of 10 would predict a 10% increase in *cys2* percentage for isolates with that particular covariate). Our hypothesis would predict that *cys2* variants would be overrepresented in PfEMP1 variants expressed in HbAS and HbAC children. However, by this analysis, host Hb type had no significant association with the percentage of *cys2* variants within parasite isolates' expressed *var* repertoire (HbS

coefficient = 8.4, P = 0.22; HbC coefficient = 13.9, P = 0.08) (**Figure 4.3a**). Host age and severity of infection also did not correlate with *cys2* percentage (age coefficient = 1.5/year of age, P = 0.17; severity coefficient = -13.8, P = 0.16). However, parasite density showed a weak, but significant, association with *cys2* percentage. Each $\log_{10}$ increase in parasite density was associated with a 25% in *cys2* percentage (coefficient = 25.1, P = 0.01) in our multivariate analysis and an 18% increase in *cys2* percentage by univariate analysis (coefficient = 18.4, $R^2$ = 0.09) (**Figure 4.3a**, **Figure 4.4**).

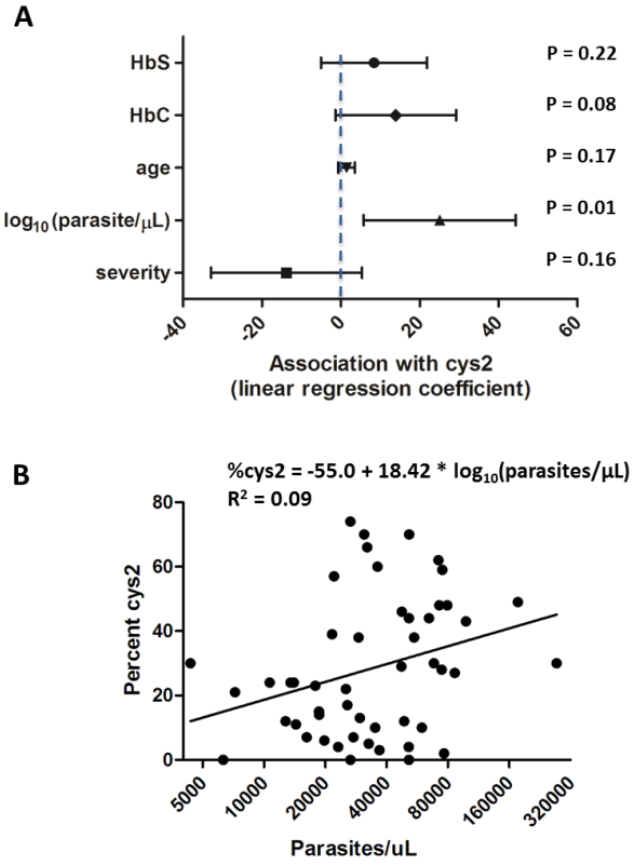



**Figure 4.3 Linear regression for association with cys2 motif**

**A)** A multivariate linear regression model was constructed to determine the association between the percent of *cys2* variants expressed in an isolate and Hb type, age, $\log_{10}$(parasite density), and clinical severity. The coefficients represent the percentage increase or decrease in *cys2* associated with each factor. **B)** A univariate linear regression analysis of the relationship between parasite density (parasites/µL) and percent *cys2* was performed.

**Hb type does not affect the percentage of PfEMP1 variants belonging to each PoLV group expressed by isolates**

Variants were further subdivided into 3 *cys2* groups, 2 *cys4* groups, and 1 *cysX* group based on the presence or absence of amino acid motifs: MFK* and *REY within four 4-amino acid (aa) regions of the sequence tag called 'positions of limited variability' (PoLVs) as defined in [454]. Groups 1-3 contained *cys 2* variants with $PoLV1_{MFK*}$ (group 1), $PoLV2_{*REY}$ (group 2), or neither motif (group 3). Group 4-5 contained *cys4* variants with (group 4) or without (group 5) $PoLV2_{*REY}$. Lastly, group 6 contained all *cysX* variants. These groups may represent independent recombination groups since there is greater sequence similarity within each group than with members of other groups. They may also associate with functional phenotypes as well since group 2 *var* genes have been associated with rosetting. Within our samples, parasites from HbAS children demonstrated a lower percentage of group 4 PfEMP1 variants (coefficient = -0.71, P = 0.02) and a higher percentage of group 6 PfEPM1 variants (coefficient = 1.32, P = 0.005). After adjusting for multiple comparisons, there were no significant associations in between any groups and host Hb type although the increase in group 6 PfEMP1 variants expressed in HbS children was marginally significant (coefficient 1.32, P = 0.05) (**Figure 4.4**).

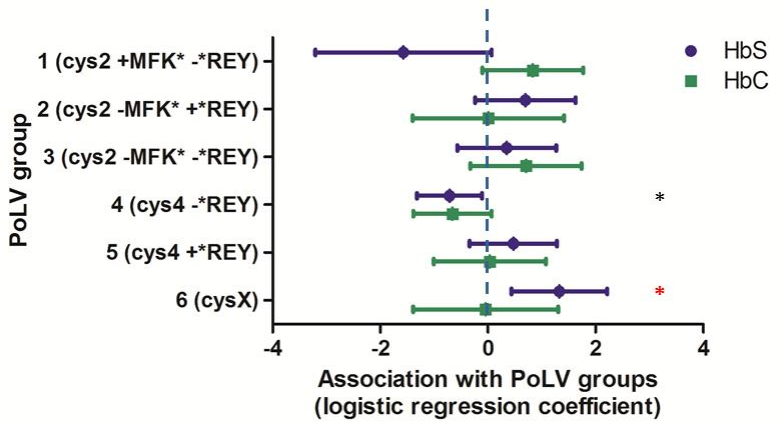



**Figure 4.4 Logistic regression for association with PoLV group**

The association between PoLV groups and Hb type were determined by logistic regression analysis. Blue and green dots represent the coefficients associated with PoLV groups and HbS or HbC, respectively. The bars indicate the 95% confidence intervals before adjustment for multiple comparisons. * indicates PoLV groups that were significantly associated ($P < 0.05$) with HbS or HbC before adjustment for multiple comparisons. * indicates PoLV groups that were significant associated ($P < 0.05$) with HbS or HbC both before and after adjustment for multiple comparisons.

### 4.3.3 Length of PfEMP1 variants expressed in HbAA, HbAS, and HbAC children

#### 4.3.3.1 Analysis of PoLV motifs and PfEMP1 length

Additionally, we hypothesized that HbAS and HbAC children might express longer, multi-domain PfEMP1 variants, which would confer increased avidity of binding through the ability to bind to multiple host receptors simultaneously. Although *cys2* sequences have been associated with longer *upsA* PfEMP1 variants (≥ 5 domains), some *cys4* sequences also contain 5 or more domains. Since the number of cysteine residues within the DBLα sequence tag and the presence of particular PoLV motifs have been shown to associate with other PfEMP1 characteristics, we wanted to determine whether particular PoLV motifs might predict PfEMP1 length. Using data from the 7 sequenced genomes from Rask and colleagues [364], we attempted to determine whether any significant correlations existed between PoLV motifs within DBLα and the number of DBL and CIDR domains.

**Expressed motifs at PoLV4 and amino acid residues at PoLV4L3 are correlated to PfEMP1 length**

By univariate analysis, only PoLV4 and specifically, the 3$^{rd}$ amino acid position of PoLV4, were significantly correlated to PfEMP1 length as measured by the number of PfEMP1 domains after adjusting for multiple comparisons (PoLV4 P = 0.04, PoLV4L3 P = 0.02) (**Figure 4.5a**). The number of cysteine residues within the DBLα sequence tag and the group (as described in **4.3.2**) were not significantly correlated to PfEMP1 length (number of cysteine residues P = 0.10, group P = 0.08). PoLV4 was predicted to account for approximately 9% of the variability in the number of PfEMP1 domains ($R^2$ = 0.094). Most of this ability to account for the variability was attributable to the 3$^{rd}$ position of PoLV4 ($R^2$ = 0.087). Additionally, PoLV4 and specifically, PoLV4L1 and PoLV4L3 were predictive of 'long' (> 4 domains) PfEMP1 variants (PoLV4 P = 0.02, PoLV4L1 P = 0.02, PoLVL3 P = 0.02) (**Figure 4.5b**). Since PoLV4 and specifically PoLVL3 were identified by both univariate analyses

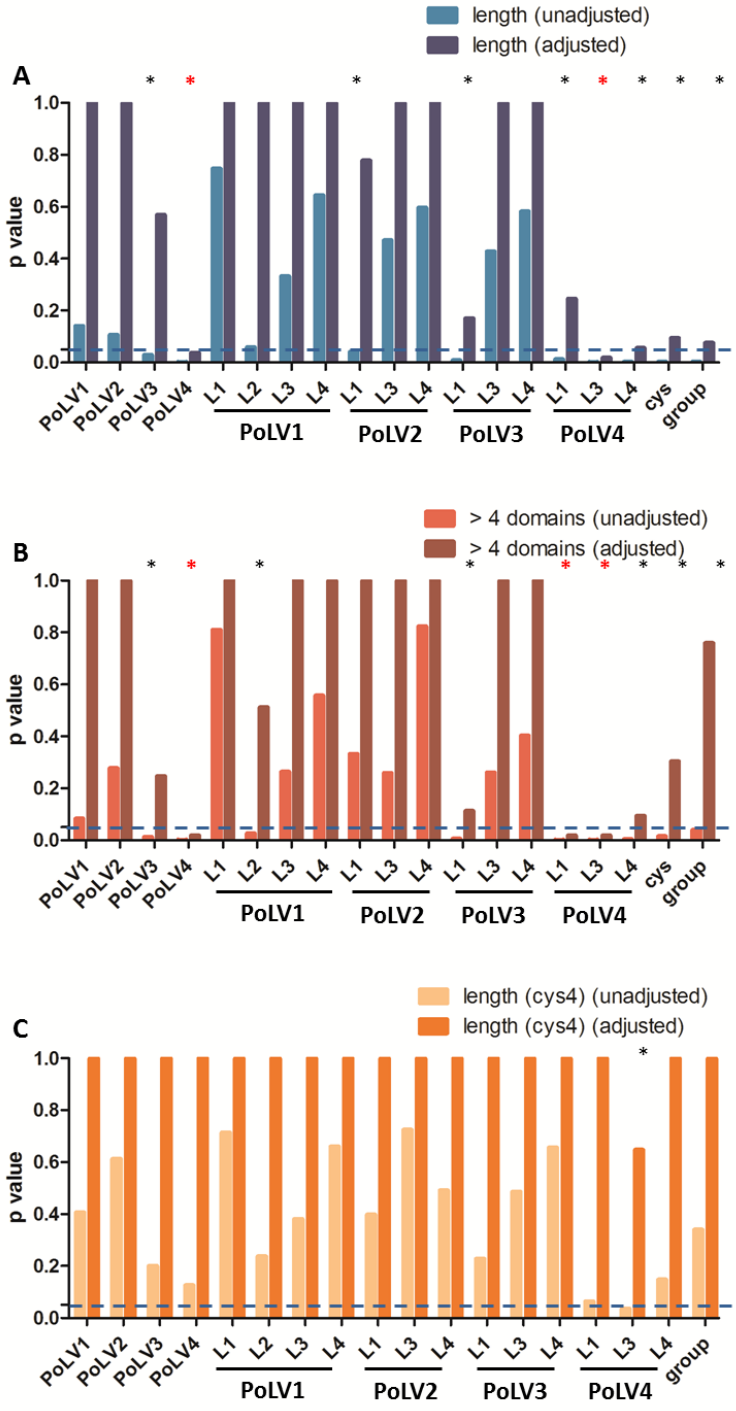



**Figure 4.5 P values for associations between PoLV groups and PfEMP1 length**

The associations between **A)** PfEMP1 length (as measured by number of domains), **B)** 'long' PfEMP1s (> 4 domains), and **C)** PfEMP1 length for *cys4* PfEMP1 variants with PoLV or amino acid positions, the number of cysteines within the DBLα sequence tag, and the PoLV group were tested by univariate analysis of 199 fully sequenced PfEMP1 variants [364]. The p values for these associations are reported here. * indicates a significant association ($P < 0.05$) before adjustment for multiple comparisons. * indicates a significant association ($P < 0.05$) before and after adjustment for multiple comparisons.

(number of PfEMP1 domains and 'long' PfEMP1 variants), we focused on this PoLV motif and amino acid residue in subsequent analyses.

**The presence of an asparagine (N) residue at PoLV4L3 predicts for longer PfEMP1 variants**

Within 199 PfEMP1 variants from these 7 genomes, PoLV4 contained 9 unique amino acid motifs: PTYF, PTNL, LTNF, PTNF, ATNL, PTNI, PTNM, LTNL, TTYF. Of these motifs, PTYF was the most common PoLV4 motif (n = 162) among the 199 PfEMP1 sequences included in our analysis. Sequences containing the PTYF motif were associated with an average of 4.5 PfEMP1 domains. Sequences containing the second most common motif, PTNL, were associated with significantly more domains (average number of domains = 5.4, N = 15, P = 0.009) than sequences containing PTYF (**Figure 4.6**). Although the PTNL motif was the only motif that was significantly correlated with a different number of domains than PTYF motifs, in general, motifs containing an asparagine (N) in the 3$^{rd}$ position of PoLV4 predicted for longer PfEMP1 variants than those associated with the PTYF motif (LTNF # domains = 5.7, N = 6, P = 0.06; PTNF # domains = 5.0, N = 6, P = 1; ATNL # domains = 5.5, N = 2, P = 1; PTNI # domains = 5.0, N = 2, P = 1; LTNL # domains 7.0, N = 1, P =0.14) with exception of the PTNM motif which predicted 4.0 domains (# domains = 4.0, N = 2, P = 1). Only one other motif, TTYF, contained a tyrosine (Y) at the 3$^{rd}$ position (# domains = 4.0, N = 1, P = 1). Overall, the presence of asparagine (N) at the 3$^{rd}$ position of PoLV4 (PoLV4L3-N) predicted that the corresponding PfEMP1 variant would have 5.3 domains (N = 34, P = 0.002). In contrast, the presence of tyrosine at the same position predicted that the corresponding PfEMP1 variant would have 4.5 domains (N = 163, P = 0.002).

**The predictive value of PoLV4L3-N differs between *cys2* and *cys4* variants**

Overall, PoLV4L3-N had 53.1% sensitivity and 89.8% specificity in determining whether a PfEMP1 variant was "long" (> 4 domains) within these 7 genomes. However, the

sensitivity and specificity varied significantly between *cys2* and *cys4* sequences. For *cys2* sequences, the sensitivity was 86.9% with 38.1% specificity. On the other hand, for *cys4* sequences, the sensitivity was only 15.8% and the specificity was 97.3%. Consequently, we predicted that PoLV4L3-N would mostly detect long *cys2* variants within our Malian DBLα sequence tags, but would falsely identify some short *cys2* variants as 'long' PfEMP1 and would miss identifying most long *cys4* PfEMP1 variants.

**PoLV motifs do not associate with PfEMP1 length for *cys4* variants**

Since the vast majority of *cys2* variants have >4 domains, we decided to focus on determining whether PoLV motifs might associate with longer PfEMP1 variants within *cys4* sequences. In considering both the percentage *cys2* variants as well as the percentage of long *cys4* variants based off of PoLV motifs, we hoped to determine an overall estimate of the percentage of long variants. However, given the number of sequences available to us, none of the PoLV or amino acid residue locations significantly associated with the PfEMP1 length (P value range = 0.51 – 1) (**Figure 4.5C**).

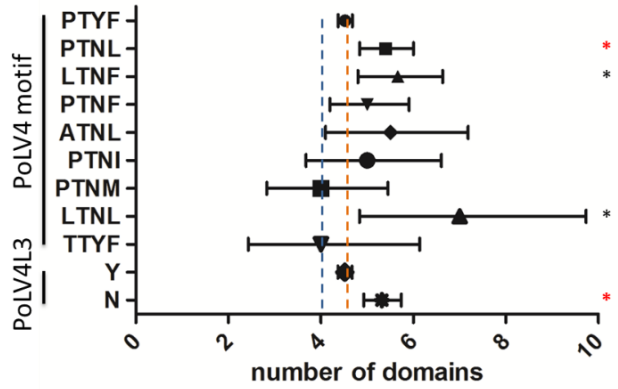



**Figure 4.6 Associations between PoLV4 motifs and PfEMP1 length**

The association of PfEMP1 length and the presence of specific PoLV4 motifs or specific amino acid residues at the 3rd position of PoLV4 (PoLV4L3) was tested by univariate analysis. The predicted number of domains associated with each PoLV4 motif or amino acid residue was compared against PTYF, the most common PoLV4 motif, or Y, the most common PoLV4L3 residue, respectively, to determine the p value. * indicates a significant association ($P < 0.05$) before adjustment for multiple comparisons. * indicates a significant association ($P < 0.05$) before and after adjustment for multiple comparisons. The dotted orange line indicates the predicted 4.5 domains for variants containing PoLV4 motif PTYF or PoLV4L3-Y. The dotted blue line indicates 'long' PfEMP1s (> 4 domains).

#### 4.3.3.2 Northern blot analysis to determine PfEMP1 length

Since we were unable to define any correlations between PoLV motifs that predicted PfEMP1 length independently from the number of cysteine residues within the DBLα sequence tag, we performed Northern blots on RNA isolated in 2010 from 45 HbAA, 36 HbAS, and 15 HbAC children with malaria to determine whether the length of PfEMP1 variants expressed by a parasite was associated with host Hb type. Of the 97 samples on which we attempted to perform a Northern blot analysis, we were able to distinguish distinct bands representing different PfEMP1 variants for 60 samples (33 HbAA, 18 HbAS, 9 HbAC). Our positive control, A4, had been serially selected for variant A4var using monoclonal Ab BC6 and was run on every gel alongside RNA samples from field isolates in our study. This particular variant, which contains 5 DBL domains and typically is structured as follows: DBL1α, CIDR1, DBL2β, DBL3δ, CIDR2, DBL4γ, DBL5β, was represented by a strong

band closer to the top edge of the gel due to its multi-domain nature and resulting length. Of the 60 samples that were detected successfully on our Northern blots, 48 had discrete bands representing both longer and shorter PfEMP1 variants reflecting the heterogeneity of PfEMP1 variants expressed in a particular parasite isolate population. However, there were no clear differences between the contributions of longer (≥ 5 domains) and shorter (< 5 domains) due to host Hb type. Representative examples are shown in **Figure 4.7** with all Northern blots shown in (**Supplemental Figure 8.1**). To our knowledge, this is the most comprehensive reported set of Northern blots for PfEMP1 variants expressed *in vivo*.

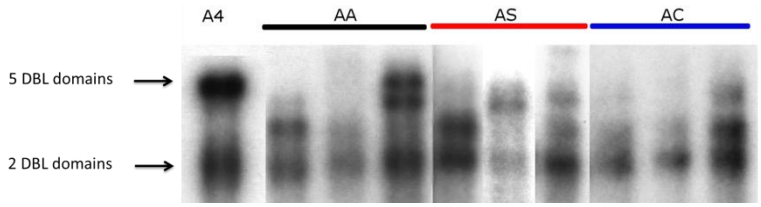



**Figure 4.7 Representative Northern blots from HbAA, HbAS, and HbAC children**

The lengths of expressed PfEMP1 variants were determined by Northern blot with an *upsC* probe for a semi-conserved region of exon2. Three representative samples for HbAA, HbAS, and HbAC children each are shown here alongside an A4 positive control, which expresses a long, 5 DBL domain-containing (7 total domains) PfEMP1 variant A4var as well as shorter 2 DBL domain-containing (4 total domains) PfEMP1 variants. The attempted Northern blots from 45 HbAA, 36 HbAS, and 15 HbAC children are shown in **Supplemental Figure 8.1**.

## 4.4 Discussion

We hypothesized that parasites from HbAS and HbAC children would express a restricted set of longer, multi-domain PfEMP1 variants, which would mediate increased avidity for MVEC by simultaneously binding multiple host receptors. To test this hypothesis, we examined the (i) homogeneity of the expressed *var* gene repertoires in both individual parasite isolates and within the circulating parasite population of HbAA, HbAS, and HbAC children; (ii) expression of variants containing particular features and motifs within a ~300-nt DBLα sequence tag, which predict other PfEMP1 characteristics; and (iii) length of expressed PfEMP1 variants.

By our analysis, we found no significant evidence for restriction of the PfEMP1 repertoire using a DBLα sequence tag and PfEMP1 length. Although our hypothesis would predict increased homogeneity (and decreased diversity) of expressed *var* genes within both individual parasite isolates' PfEMP1 repertoire as well as within the circulating parasite population for parasites from HbAS and HbAC children, we saw no significant differences by any of these measures. Phylogenetic analysis of the predominantly expressed sequences in each isolate revealed no clustering by Hb type. There was no significant association between host Hb type and the percentage of *cys2* PfEMP1 variants or variants belonging to specific PoLV groups expressed within an isolate. We had also hypothesized that longer multi-domain PfEMP1 variants might mediate simultaneous binding to multiple receptors. However, there was no significant difference in the contribution of 'long' and 'short' PfEMP1s to the repertoire of expressed PfEMP1 variants in HbAS and HbAC children compared to HbAA children by Northern blot.

Interestingly, unlike a study performed in Kilifi, Kenya [419], we saw no relationship between proportion cys2 and host age in our sample, but saw a weak, but significant association between proportion cys2 and parasitemia. The discordance in our findings may

be explained by differences in the genetic background and in the transmission intensity at the sites. Furthermore, the majority of children included in the Kenyan study are aged 0-3 years. In contrast, in addition to this younger age group, our study included a large number of children aged 4-6 years.

Taken together, these results suggest that contrary to our initial hypothesis, all PfEMP1 variants may be expressed in HbAS and HbAC children. Previous studies have shown that a restricted *var* gene repertoire is expressed in SM patients. Our findings imply that HbAS and HbAC children are not selecting for expression of this same repertoire of *var* genes. Several possibilities might reconcile our findings with these previous studies as well as *in vitro* evidence that HbS and HbC might protect by altered PfEMP1 display.

One possibility is that parasites that cause malaria in HbAS or HbAC children mediate increased binding to host receptors through either PfEMP1- or non PfEMP1-dependent mechanisms. Parasites from HbAS or HbAC children with malaria might express diverse PfEMP1 variants, but may be selected for their ability to form normal 'knobs' or to traffic wildtype amounts of PfEMP1 to the RBC surface even in HbAS or HbAC children. In normal PfEMP1 trafficking, PfEMP1 is trafficked to the RBC surface through Maurer's clefts and loaded onto 'knobs' comprised in part of KAHRP (see **1.3.2**). Although the mechanisms which result in abnormal knob distribution and reduced PfEMP1 levels on parasitized HbS- and HbC-containing RBCs are still being elucidated, a recent study implicates aberrations in the actin cytoskeleton and in Maurer's clefts in causing these alterations. Parasites expressed in HbAS or HbAC children might be able to prevent or reverse these aberrations and instead, display wildtype amounts of PfEMP1 on 'knobs' of normal size, distribution, and morphology.

Increased binding might also be mediated by other proteins such as rifins and stevors or yet unidentified molecules expressed on the surface of parasitized RBCs. Rifins

and stevors are other VSAs expressed on the surface of parasitized RBCs along with PfEMP1. Although their function has not been well-defined, studies have suggested that rifins may be cofactors in rosetting or binding to CD31. Alternatively, although PfEMP1 appears to be the parasite's main cytoadherence ligand, parasites might upregulate an undiscovered cytoadherence ligand to increase avidity of binding in HbS- or HbC-containing RBCs. Such a ligand may not have been identified in previous studies if its expression only provides an advantage in children with significant genetic protection against malaria, which would result in relatively rare expression. In young genetically unprotected children, expression of PfEMP1 alone may mediate parasite sequestration and may result in sufficient inflammation to cause malaria symptoms. In older HbAS or HbAC children, parasites may encounter alterations in PfEMP1 display in addition to high levels of anti-PfEMP1 IgG, which might impair cytoadherence or lead to opsonization of parasites. Therefore, a restricted subset of parasites which express particular rifins, stevors, or an unknown surface ligand might cause disease in these children by compensating for reduced and altered PfEMP1 expression to bind endothelium with high avidity.

Another possibility is that *var* gene restriction within HbAS and HbAC children does occur, but that the methods used in this study were not capable of detecting it. Several limitations of our study may have led to this result. First, although we did not observe any significant differences between the DBLα sequence tags expressed in HbAA, HbAS, and HbAC children or in the lengths of expressed PfEMP1 variants, differences might exist in the downstream DBL or CIDR domains expressed by parasites in HbAS or HbAC children. Although the DBLα sequence tag provides useful information on PfEMP1 characteristics such as the ability to bind CD36 or rosette and *ups* group, a mutation within a downstream domain such as CD36-binding CIDRα or ICAM-1-binding DBLβ might mediate increased binding to host receptors without associating with any motifs or features within the sequence tag. Other studies have demonstrated that only a subset of DBLβ domains bind to

ICAM-1 [426-428, 431, 432]. Therefore, parasites that contain similar domain types may display significantly different binding signatures. Parasites from HbAS and HbAC children may display PfEMP1 variants that are similar to those displayed in HbAA children in length and domain types, but exhibit an increased avidity for endothelium due to the ability to bind to multiple receptors simultaneously. This ability may also be due to 'spacer' domains, which do not bind host receptor, but instead position other domains in interaction-promoting conformations [432]. Expanding technological limits may soon lead to the ability to fully sequence and assemble entire *var* genes quickly and easily. Application of these advances to fully sequence expressed PfEMP1 variants in HbAA and HbAS children will be necessary to definitively determine whether restriction in expressed PfEMP1 repertoire occurs in HbAS children.

Second, HbAS children are significantly protected against malaria within our cohort. As a result, they are less likely to present to our clinic or to be treated with parasite-eliminating treatments. Consequently, over a transmission season, they may be more likely to accumulate parasite clones and to simultaneously express PfEMP1 variants from multiple clones resulting in a greater apparent diversity of expressed variants. One way to test this possibility is to determine the number of clones within an isolate by microsatellite typing. Also, although the population of parasites may simultaneously express multiple PfEMP1 variants within its host, it is impossible to determine which expressed variants are responsible for disease manifestations. Although most studies examining the relative contributions of individual variants in children with malaria, including ours, assume that variants that are more highly expressed contribute to a child's outcome, this may not always be true. HbAS or HbAC children might be able to tolerate relatively large numbers of parasites expressing PfEMP1 variants that bind host receptor with enough avidity to allow multiplication of parasites expressing the variant to high densities, but do not bind with enough avidity to cause symptoms. However, a particularly virulent variant may cause

disease with relatively few expressed copies due to its ability to activate endothelial cells leading to downstream effects.

Third, co-inherited RBC polymorphisms or host immunity may have confounded our findings. As with the cytoadherence comparisons performed in **Chapter 3**, many of the samples used in our experiments in this chapter had co-inherited polymorphisms. 30% of the samples that we cloned and sequenced (**Supplemental Table 8.2**) and 40% of the samples we ran on a Northern blot (**Supplemental Table 8.3**) were confounded by either α-thalassemia or G6PD deficiency. Furthermore, the average ages by Hb type for each set of samples also were different since they were not matched (sequenced: average age HbAA – 5.2 years old, HbAS – 4.8, HbAC – 3.5; Northern: HbAA – 4.7, HbAS – 7, HbAC – 5.9). Since age and other RBC polymorphisms may affect protection against malaria, they may also affect the expressed *var* gene repertoire.

Fourth, the parasite isolates used for both DBLα sequence tag and Northern blog analysis were biased towards those from episodes with high parasitemia in order to have enough starting RNA material. In biasing our selection of parasite isolates, as discussed in **3.4**, we may have inadvertently excluded isolates with increased pathogenicity, which cause malaria symptoms at lower densities. We would have expected that these isolates would have demonstrated an increased percentage of virulent PfEMP1 variants. Therefore, excluding them may have skewed our analysis to those isolates expressing less virulent PfEMP1 variants.

The alternative to these possibilities is that other proposed mechanisms of malaria protection by HbS and HbC might be more influential *in vivo* than PfEMP1-mediated reductions in cytoadherence. Other studies have suggested that differences in multiplication rate, innate immunity, or acquired immune responses might protect HbS or HbC children against malaria. These mechanisms might protect these children to such an

extent that even though parasites may demonstrate reduced cytoadherence to MVECs, these reductions might not be sufficient *in vivo* to cause significant differences in parasite virulence. Therefore, the altered display of PfEMP1 in HbS- and HbC-containing RBCs would not shape the expressed *var* repertoire. A related possibility is that since the degree of protection conferred by HbS may be dependent on host age as a surrogate of acquired immunity against *P. falciparum* malaria in some populations, the *var* gene repertoire may be restricted in younger relatively malaria-naïve children, but not in older children. Although we do not see this epidemiological interaction between Hb type and age within our cohort (see **2.3.2.4**), this relationship within a study population in Kenya [342] demonstrates that multiple mechanisms of protection may interact with each other in yet unexplored ways.

Studies that provide further insight into the *var* gene repertoire expressed in HbAS and HbAC children are needed. High-avidity binding parasites may be selected *in vitro* by panning parasites with a fully sequenced *var* genome on MVECs in HbAS RBCs followed by testing for overexpressed variants by quantitative PCR. Quantitative PCR may also be used to assay for the expression levels of particular cassettes of PfEMP1 domains that are frequently found in the same variant. Recent studies implicate cassettes 8 and 13 in SM [458-460]. Expression of these cassettes is unlikely to be associated with HbAS and HbAC since by our analysis, any restriction in the expressed *var* repertoire in these children is likely different than that observed in SM. However, this analysis may reveal other cassettes that are frequently expressed within HbAS and HbAC children.

In conclusion, we found no evidence for the restriction of the PfEMP1 repertoire in HbAS or HbAC children as measured by the homogeneity of a DBLα sequence tag, the motifs or features in the sequence tag, or the length of expressed PfEMP1 variants. Although this finding does not preclude malaria protection by PfEMP1-mediated

mechanisms, it emphasizes that multiple mechanisms may simultaneously work together to effect protection in genetically protected individuals. Further studies examining the interaction between these protected mechanisms are needed to understand the evolutionary selection of RBC polymorphisms such as HbS and HbC.

# 5 Acquisition of PfEMP1-specific IgG responses in HbAA, HbAS and HbAC Malian children

## 5.1 Introduction and aims

We sought to examine IgG recognition to specific PfEMP1 DBL and CIDR domains for HbAS and HbAC children in comparison with HbAA children. Enhanced acquisition of malaria immunity has been proposed as a mechanism to explain malaria protection by HbS discussed in **1.2.5.3**. Various groups have compared the reactivity of HbAS children against VSAs, merozoite invasion ligands, and other *P. falciparum* proteins to that of HbAA children. However, despite PfEMP1's role as a major target of protective acquired immunity against falciparum malaria [341], no group to our knowledge has reported comparisons of IgG recognition by wildtype children and children carrying RBC polymorphisms against a repertoire of PfEMP1 variants. We would predict that if expression of a restricted subset of high-avidity PfEMP1 variants was required for parasites to cause malaria symptoms in HbAS or HbAC children, these parasites would have to switch their expressed PfEMP1 multiple times before reaching a PfEMP1 variant capable of binding with sufficient avidity to cause disease. Additionally, HbAS children are more likely to experience chronic *P. falciparum* infections since they are less likely to progress from asymptomatic parasitemia to malaria, and therefore are treated less often than their HbAA counterparts. Chronic infection would also lead to the presentation of additional PfEMP1 variants to the host immune system. As the parasite presents each new PfEMP1 variant on the surface of the RBC, activated B-cells in HbAS children might produce IgGs to a broader repertoire of PfEMP1 domains and at higher IgG titers than HbAA children.

Therefore, we hypothesized that HbAS children would demonstrate accelerated acquisition of PfEMP1 domain-specific IgGs. Here, we have tested the plasma from 73 HbAS and 30 HbAC Malian children against plasma from matched HbAA children for recognition of 91 PfEMP1 domains to test this hypothesis.

## 5.2 Materials and Methods

### 5.2.1 Study population

This study was conducted in two rural villages in southwest Mali – Kenieroba and Fourda – using a selected subset of children within our larger cohort study in 2009 as described in **Chapter 2.3.1**. Children from Bozokin were not included on our study due to the village's distance from the other two villages. In our larger cohort study, in 2009, we had enrolled 109 HbAS children and 48 HbAC children aged 3-11 years (**Table 5.1**). Of these, we were able to enroll onto our sub-cohort study 73 HbAS and 30 HbAC children aged 3-11 years as well as 103 HbAA children who were matched by age, gender, α-thalassemia genotype, G6PD deficiency genotype, ABO blood group, village, and ethnicity (**Table 5.2**) and obtained written informed consent from each child's parent or guardian. The average age and the percentage of children who fit into each group by each of the criteria used for matching were similar between the three groups. As a result, all analyses were conducted by grouping all HbAS, all HbAC, and all HbAA children together.

Whole blood samples (5-8 mL) were collected by venipuncture into sodium heparin Vacutainers® (Becton-Dickinson, Franklin Lakes, NJ) in May 2009 (pre-transmission season) and in December 2009 (post-transmission season). Plasma was isolated from these samples and stored at -80C until use. Plasma was collected from all 206 children from our sub-cohort in May, but we were able to collect a second plasma sample in December from only 189 children. The 6 HbAA, 4 HbAS, and 4 HbAC children who were unavailable for follow-up in December were included in the May analyses, but excluded from December analyses and from analyses examining the boosting of PfEMP1-specific IgG over the transmission season. A thick film blood smear was also prepared, stained with Giemsa, and examined by an

expert microscopist for the presence of *Plasmodium* parasites. Of the 206 children who participated in this study, only 2 HbAA, 1 HbAS, and 1 HbAC were asymptomatically

**A)**

| | Hb type | # | % | avg age | % male | α-thal | | | G6PD def | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | WT | HE | HO | A+ | A+/- | A- |
| all 2009 | HbAA | 990 | 78.3 | 8.1 | 50 | 71.1 | 26.2 | 2.2 | 83.7 | 9.8 | 6 |
| | HbAS | 185 | 14.6 | 8 | 52.4 | 67 | 30.8 | 1.1 | 84.3 | 9.2 | 6.5 |
| | HbAC | 78 | 6.2 | 8.1 | 52.6 | 71.8 | 25.6 | 2.6 | 82.1 | 11.5 | 6.4 |
| | HbSC | 10 | 0.8 | 7.2 | 40 | 80 | 10 | 0 | 100 | 0 | 0 |
| | HbSS | 1 | 0.1 | 16 | 100 | 0 | 100 | 0 | 100 | 0 | 0 |
| | HbCC | 1 | 0.1 | 5 | 0 | 100 | 0 | 0.0 | 100 | 0 | 0 |
| ages 3-11 | HbAA | 601 | 79.3 | 6.6 | 48.9 | 71.4 | 26.1 | 1.8 | 82.9 | 10 | 6.7 |
| | HbAS | 109 | 14.4 | 6.5 | 49.5 | 68.8 | 29.4 | 0 | 85.3 | 9.2 | 5.5 |
| | HbAC | 48 | 6.3 | 7 | 54.2 | 64.6 | 31.3 | 4.2 | 83.3 | 10.4 | 6.3 |
| | HbSC | 7 | 0.9 | 7.7 | 57.1 | 85.7 | 14.3 | 0 | 100 | 0 | 0 |
| | HbSS | 0 | 0 | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| | HbCC | 1 | 1.3 | 5 | 0 | 100 | 0 | 0.0 | 100 | 0 | 0 |

**B)**

| | Hb type | ABO blood group | | | | | | | | Village | | | Ethnicity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A+ | AB+ | B+ | O+ | A- | AB- | B- | O- | Ken | Boz | Fou | Mal | Fula | Bam | Sar | Dogon |
| all 2009 | HbAA | 26.8 | 6.1 | 19.6 | 39.2 | 3.5 | 0.3 | 2.2 | 2.3 | 74.2 | 12.7 | 13.0 | 83.6 | 8.6 | 4.9 | 2.3 | 0.5 |
| | HbAS | 28.1 | 8.6 | 17.8 | 36.2 | 4.3 | 1.1 | 2.2 | 1.6 | 80.0 | 13.0 | 7.0 | 95.1 | 4.3 | 0.5 | 0 | 0 |
| | HbAC | 26.9 | 2.6 | 25.6 | 32.1 | 2.6 | 0 | 6.4 | 3.8 | 89.7 | 5.1 | 5.1 | 83.3 | 11.5 | 3.8 | 1.3 | 0 |
| | HbSC | 50.0 | 0 | 10.0 | 20.0 | 20.0 | 0 | 0 | 0 | 60.0 | 30.0 | 10.0 | 100 | 0 | 0 | 0 | 0 |
| | HbSS | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| | HbCC | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| ages 3-11 | HbAA | 26.0 | 5.5 | 21.3 | 38.4 | 4.0 | 0.3 | 2.7 | 1.8 | 75.2 | 11.8 | 13.0 | 83.4 | 8.2 | 5.3 | 2.7 | 0.5 |
| | HbAS | 31.2 | 9.2 | 20.2 | 33.0 | 3.7 | 0.9 | 0.9 | 0.9 | 78.9 | 14.7 | 6.4 | 94.5 | 4.6 | 0.9 | 0 | 0 |
| | HbAC | 29.2 | 2.1 | 18.8 | 39.6 | 4.2 | 0 | 2.1 | 4.2 | 93.8 | 4.2 | 2.1 | 81.3 | 16.7 | 2.1 | 0 | 0 |
| | HbSC | 57.1 | 0 | 14.3 | 14.3 | 14.3 | 0 | 0 | 0 | 71.4 | 28.6 | 0 | 100 | 0 | 0 | 0 | 0 |
| | HbSS | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| | HbCC | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |

**Table 5.1 Clinical data for larger cohort in 2009**

Clinical information for both the larger cohort and children between the ages of 3-11 years old. The numbers presented for **A)** % male, α-thalassemia genotype (α-thal), G6PD deficiency genotype (G6PD def), **B)** ABO blood group, village, and ethnicity are represented as a percentage of the total for that particular Hb type. We were unable to successfully type 5 HbAA samples (0.5%) for α-thalassemia genotype and 5 others for G6PD genotype (0.5%). Of these, 4 of the children (0.7%) whose samples we were unable to type were for α-thalassemia genotype were between the ages of 3-11, and 3 (0.5%) for G6PD deficiency. We were unable to genotype 2 HbAS children who were between the ages of 3-11 years old for α-thalassemia (1.1% of total cohort, 1.8% of 3-11 year old cohort) and 1 SC child (10% of total cohort), but this child was not between the ages of 3-11 years old. Ken = Kenieroba, Fou = Fourda, Boz = Bozokin, Mal = Malinke, Bam = Bambara, Fula = Fulani, Sar = Sarakole.

**A)**

| | | | | | α-thal | | | G6PD def | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Hb type** | **#** | **%** | **avg age** | **% male** | **WT** | **HE** | **HO** | **A+** | **A+/-** | **A-** |
| **HbAA (paired with HbAS)** | 73 | 35.4 | 6.6 | 47.9 | 75.3 | 23.3 | 1.4 | 90.4 | 5.5 | 4.1 |
| **HbAS** | 73 | 35.4 | 6.6 | 54.8 | 72.6 | 24.7 | 0 | 87.7 | 8.2 | 4.1 |
| **HbAA (paired with HbAC)** | 30 | 14.6 | 6.9 | 53.3 | 73.3 | 26.7 | 0 | 86.7 | 13.3 | 0 |
| **HbAC** | 30 | 14.6 | 6.9 | 46.7 | 73.3 | 23.3 | 3.3 | 76.7 | 16.7 | 6.7 |
| **HbAA total** | 103 | 50 | 6.7 | 49.5 | 74.8 | 24.3 | 1.0 | 89.3 | 7.8 | 2.9 |

**B)**

| | ABO blood group | | | | | | | | Village | | | Ethnicity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hb type** | **A+** | **AB+** | **B+** | **O+** | **A-** | **AB-** | **B-** | **O-** | **Ken** | **Fou** | **Boz** | **Mal** | **Bam** | **Fula** | **Sar** | **Dogon** |
| **HbAA (paired with HbAS)** | 27.4 | 8.2 | 19.2 | 34.2 | 6.8 | 0 | 2.7 | 1.4 | 90.4 | 9.6 | 0 | 93.2 | 5.5 | 1.4 | 0 | 0 |
| **HbAS** | 28.8 | 8.2 | 20.5 | 37.0 | 4.1 | 0 | 1.4 | 0 | 90.4 | 9.6 | 0 | 100 | 0 | 0 | 0 | 0 |
| **HbAA (paired with HbAC)** | 33.3 | 3.3 | 13.3 | 43.3 | 3.3 | 0 | 0 | 3.3 | 90 | 10 | 0 | 96.7 | 3.3 | 0 | 0 | 0 |
| **HbAC** | 33.3 | 0 | 13.3 | 46.7 | 3.3 | 0 | 0 | 3.3 | 96.7 | 3.3 | 0 | 96.7 | 3.3 | 0 | 0 | 0 |
| **HbAA total** | 29.1 | 6.8 | 17.5 | 36.9 | 5.8 | 0 | 1.9 | 1.9 | 90.3 | 9.7 | 0 | 94.2 | 4.9 | 1.0 | 0 | 0 |

**Table 5.2 Clinical data for sub-cohort**

73 HbAS and 30 HbAC children aged 3 to 11 years were paired to 103 HbAA children from our Kenieroba, Mali cohort discussed in chapter **2**. Each pair was matched by **A)** age, gender, α-thalassemia genotype (α-thal), and G6PD deficiency genotype (G6PD def) as well as **B)** ABO blood group, village, and ethnicity. We were unable to successfully type 2 HbAS samples (2.7%) for α-thalassemia genotype. Ken = Kenieroba, Fou = Fourda, Boz = Bozokin, Mal = Malinke, Bam = Bambara, Fula = Fulani, Sar = Sarakole.

parasitemic (50-200/µL whole blood) with *P. falciparum* in May 2009. The study protocols were approved by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odontostomatology, University of Bamako, and the Institutional Review Board of the NIAID. Further details about consent can be found in **2.2.5**.

### 5.2.2 Analysis of IgG recognition of PfEMP1 domains by Luminex

The 206 HbAA, HbAS, and HbAC children were tested for their IgG reactivity towards 91 PfEMP1 domains (28 CIDR domains, 63 DBL domains), positive control EBA-175, and negative control tetanus toxoid using a Luminex assay according to manufacturer's instructions as previously described in detail [441]. In short, 63 DBL and 28 CIDR domain-encoding fragments were amplified by PCR from 3D7, FCR3, and HB3 genomic DNA (**Figure 5.1**, **Table 5.1**) and cloned into a baculovirus vector pAcGP67-A (BD Biosciences) containing a histidine tag and V5 epitope. The vector was then co-transfected along with linearized Bakpak6 baculovirus DNA into Sf9 insect cells. The histidine-tagged PfEMP1 domains were purified by $Co^2$ metal-chelate agarose columns and their identity confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting against V5. Additionally, antibodies were generated against each domain in rabbits and were found to be reactive towards the initial immunizing antigen by enzyme-linked immunosorbent assay (ELISA).

The purified domains were then covalently conjugated onto Luminex microspheres ($1.25 \times 10^7$ microspheres/mL) through the interaction between the carboxyl group on the microspheres and the amino groups on the proteins. Each individual Luminex microsphere had been tagged using two dyes to form a dye profile, which served as a unique identifier of that particular microsphere. Each microsphere population was incubated with a specific domain to covalently link the domain to an identifiable

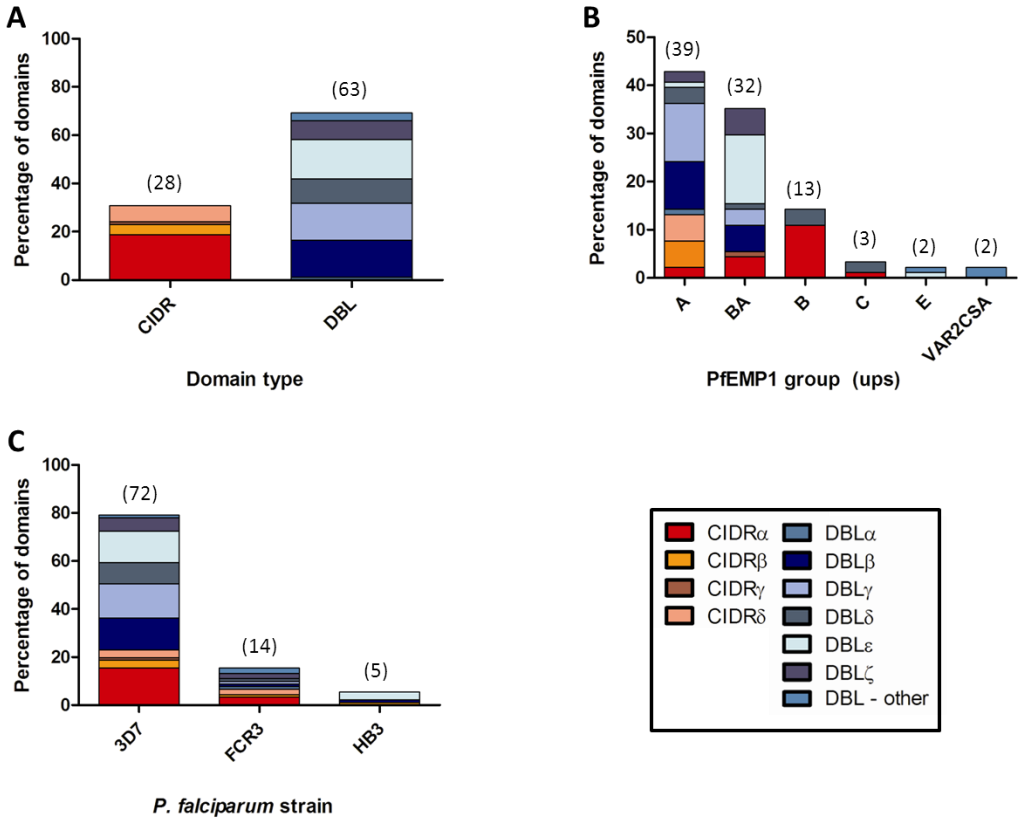



**Figure 5.1 Characteristics of 91 PfEMP1 domains included in analysis**

91 DBL and CIDR domains were expressed in a baculovirus vector and conjugated onto Luminex microspheres to test against plasma from HbAA, HbAS, and HbAC children. Percentage breakdowns of **A)** domain type **B)** PfEMP1 group and **C)** *P. falciparum* clone are represented by the height of the bars. The number of domains included for each category is indicated in parentheses above each bar.

| Plex 4 PfEMP1 domains | | | | | | Plex 5 PfEMP1 domains | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein ID | PfEMP1 | Genome | PfEMP1 group (ups) | Domain type | Cassette/ Type | Protein ID | PfEMP1 | Genome | PfEMP1 group (ups) | Domain type | Cassette/ Type |
| GK2 (39) | PF11_0521 | 3D7 | A | CIDRa14 | 13 | 61/62 (22) | MAL6P14 | 3D7 | BA | DBLb5 | 14 |
| 2324 (63) | PFD0020c | 3D7 | A | DBLg6 | 8 | 167/168 (23) | HB3var22 | HB3 | BA | DBLe2 | 7 |
| 2122 (77) | PFD0020c | 3D7 | A | DBLb12 | 8 | 53/54 (26) | PFL0020w | 3D7 | BA | DBLz5 | 6 |
| 127128 (76) | PFE1640w | 3D7 | A | DBLb1 | 1 | 171/172 (27) | MAL6P1316 | 3D7 | BA | DBLg4 | 6 |
| GK26 (59) | PFL2665c | 3D7 | C | CIDRa2.3 | type 1 | 189/190 (28) | IT4var21 | FCR3 | B | CIDRa3.1 | none |
| 199200 (90) | IT4_var2 | FCR3 | A | CIDRd1 | 16 | 199/200 (29) | IT4var02 | FCR3 | A | CIDRd1 | 16 |
| GK28 (57) | MAL6P11 | 3D7 | B | CIDRa4 | type 1 | 230/231 (33) | PF11_0008 | 3D7 | A | DBLg12 | 5 |
| GK23 (60) | PFL0005w | 3D7 | B | CIDRa2.2 | type 1 | 232/233 (34) | PF11_0008 | 3D7 | A | DBLd5 | 5 |
| GK25 (41) | PFC0005w | 3D7 | B | CIDRa2.4 | type 1 | 234/235 (35) | PF11_0008 | 3D7 | A | CIDRb4 | 5 |
| 129130 (67) | PFE1640w | 3D7 | A | DBLg15 | 1 | 236/237 (36) | PF11_0008 | 3D7 | A | DBLb9 | 5 |
| VAR5CIDR1 (37) | PF11_0008 | 3D7 | A | CIDRb4 | 5 | 238/239 (37) | IT4var02 | FCR3 | A | DBLg12 | 5 |
| 155156 (65) | MAL6P1316 | 3D7 | BA | DBLz3 | 12 | 240/241 (38) | IT4var02 | FCR3 | A | DBLd5 | 5 |
| 189190 (51) | IT4var21 | FCR3 | B | CIDRa3.1 | type 1 | 242/243 (42) | IT4var02 | FCR3 | A | CIDRb3 | 5 |
| 167168 (61) | HB3var22 | HB3 | BA | DBLe2 | 7 | 244/245 (43) | IT4var02 | FCR3 | A | DBLb9 | 5 |
| 8788 (52) | PFD0005w | 3D7 | B | DBLd1 | none | 266/267 (44) | HB3var35 | HB3 | A | CIDRd1 | 16 |
| SM1112 (58) | PFL1955w | 3D7 | B | CIDRa3.4 | 19 | 274/275 (45) | IT4var20 | FCR3 | BA | CIDRa11 | 8 |
| GK17 (40) | PFD0005w | 3D7 | B | CIDRa2.10 | type 1 | 290/291 (46) | PF13_0003 | 3D7 | A | CIDRd1 | 16 |
| 6970 (73) | MAL6P14 | 3D7 | BA | DBLe2 | 7 | 296/297 (47) | PFD0020c | 3D7 | BA | CIDRa11 | 8 |
| 5556 (43) | PFL0020w | 3D7 | BA | DBLe4 | 6 | 382/383 (52) | HB3var05 | HB3 | A | DBLb9 | 5 |
| E22-3 (55) | PF11_0008 | 3D7 | A | DBLd5 | 5 | 394/395 (53) | MAL6P1316 | 3D7 | BA | DBLe12 | 12 |
| D20-24 (42) | PF11_0008 | 3D7 | A | DBLg12 | 5 | 396/397 (54) | PF08_0141 | 3D7 | BA | DBLe4 | 6 |
| 179180a (46) | PFD0615c | 3D7 | C | DBLd1 | type 1 | 410/411 (61) | IT4var46 | FCR3 | BA | DBLz3 | none |
| 733 (92) | PF13_0003 | 3D7 | A | CIDRd1 | 16 | 412/413 (62) | HB3var22 | HB3 | BA | DBLe3 | 7 |
| 660C (68) | PFD1235w | 3D7 | A | DBLg13 | none | 476/477 (65) | IT4var64 | FCR3 | A | DBLa15 | 16 |
| 171174 (34) | MAL6P1316 | 3D7 | BA | DBLg4 | 6 / 8 | 496/497 (66) | IT4var08 | FCR3 | A | DBLz6 | 10 |
| 5354 (64) | PFL0020w | 3D7 | BA | DBLz5 | 6 | var2dbl4 (73) | PFL0030c | 3D7 | E | DBLpam3 | 2 |
| 6768 (89) | MAL6P14 | 3D7 | BA | CIDRg1 | 43 | var2dbl5 (74) | PFL0030c | 3D7 | E | DBLpam4 | 2 |
| 6566 (36) | MAL6P14 | 3D7 | BA | DBLd4 | none | var4dbl4 (51) | PFD1235w | 3D7 | A | DBLb3 | none |
| 6162 (69) | MAL6P14 | 3D7 | BA | DBLb5 | 14 | 155/156 (20) | MAL6P1316 | 3D7 | BA | DBLz3 | 12 |
| GK37 (49) | PF08_0103 | 3D7 | B | CIDRa2.2 | type 1 | 49/50 (11) | PFL0020w | 3D7 | BA | DBLb5 | 17 |
| 1516 (75) | PF08_0141 | 3D7 | A | DBLz5 | 6 | 91/92 (14) | PFA0765c | 3D7 | B | DBLd1 | type 1 |
| 1112 (74) | PF08_0141 | 3D7 | A | DBLb6 | none | 87/88 (15) | PFD0005w | 3D7 | B | DBLd1 | none |
| 1314 (66) | PF08_0141 | 3D7 | A | DBLg14 | 6 | var5cidr (16) | PF11_0008 | 3D7 | A | CIDRb4 | 5 |
| MP319 (86) | it4var04 | FCR3 | VAR2CSA | DBLepam5 | 2 | D20-24 (17) | PF11_0008 | 3D7 | A | DBLg12 | 5 |
| MP353 (87) | it4var04 | FCR3 | VAR2CSA | DBLpam1 | 2 | 43/44 (19) | PF08_0140 | 3D7 | BA | CIDRa16 | none |
| 101102 (33) | PF07_0049 | 3D7 | C | DBLd1 | type 1 | 21/22 (21) | PFD0020c | 3D7 | A | DBLb12 | 8 |
| 7374 (45) | MAL6P14 | 3D7 | BA | DBLe3 | 7 | 37/38 (24) | PF08_0140 | 3D7 | BA | DBLb12 | 8 |
| 6364 (84) | MAL6P14 | 3D7 | BA | DBLg13 | none | 13/14 (25) | PF08_0141 | 3D7 | A | DBLg14 | 6 |
| GK34 (50) | PFL1955w | 3D7 | B | CIDRa3.4 | 19 | 55/56 (76) | PFL0020w | 3D7 | BA | DBLe4 | 6 |
| 7172 (72) | MAL6P14 | 3D7 | BA | DBLe7 | 7 | 69/70 (75) | MAL6P14 | 3D7 | BA | DBLe2 | 7 |
| 4344 (53) | PF08_0140 | 3D7 | BA | CIDRa16 | none | GK25 (63) | PFC0005w | 3D7 | B | CIDRa2.4 | type 1 |
| 3738 (56) | PF08_0140 | 3D7 | BA | DBLb12 | 8 | 131/132 (13) | PFE1640w | 3D7 | A | DBLe1 | 1 |
| P310 (54) | PFD1235w | 3D7 | A | CIDRa16 | 4 | 129/130 (12) | PFE1640w | 3D7 | A | DBLg1 | 1 |
| P308 (47) | PFD1235w | 3D7 | A | DBLg13 | none | 733 (56) | PF13_0003 | 3D7 | A | CIDRd1 | 16 |
| | | | | | | 789 (77) | PF13_0003 | 3D7 | A | DBLb9 | 5 |
| | | | | | | 71/72 (57) | MAL6P14 | 3D7 | BA | DBLe7 | 7 |
| | | | | | | 73/74 (60) | MAL6P14 | 3D7 | BA | DBLe3 | 7 |

**Table 5.3 91 domains included in analysis**

91 CIDR and DBL domains based off of the 3D7, HB3, and FCR3 genomes multiplexed into two separate plexes and tested against 206 HbAA, HbAS, and HbAC children for their recognition. Domain types were colored according to their domain type as reported in [364].

microsphere. Since each microsphere could be individually identified, large numbers of microspheres could be multiplexed together to detect recognition of multiple domains simultaneously.

Microspheres were multiplexed into two plexes (plex 4 and plex 5) by combining equal volumes of each individual microsphere conjugated to its unique domain. Plex 4 contained 44 domains and plex 5 contained 47 (**Table 5.3**). Multiplexes were lyophilized into single-use aliquots (AdVantage, Wizard 2.0; VirTis), and stored at -80°C until use.

50 µL of diluted plasma (1:80) was added to 1.2-µm filter bottom 96-well microtiter plates along with 50 µL of either plex 4 or plex 5 (1:333). Plates were incubated in the dark on a shaking platform (1100 rpm for 30 sec, then 300 rpm for 30 min) then subsequently incubated under the same conditions with 25 µL of biotinylated human IgG (Sigma-Aldrich,) (1:500) added to each well, and then with 50 µL of streptavidin-PE (Sigma-Aldrich) (1:500) added to each well. All dilutions were performed in assay buffer E (ABE buffer) (0.1% BSA, 0.05% Tween 20, 0.05% sodium azide in PBS (pH 7.4). The microspheres were analyzed on the Luminex instrument, which is able to detect both the unique dye signature of each bead as well as the amount of streptavidin-PE bound to each bead. A minimum of 100 beads was read for each domain to determine the mean fluorescence intensity (MFI). The MFI was converted to a value between 0-1000 we refer to as “titer” using a standard curve generated using a hyperimmune pool of Tanzanian adults, with 1000 set as the MFI obtained using a 1:80 dilution of the hyperimmune pool.

### 5.2.3 Fold change and fold change ratio calculations

Fold change values for individual domains and for ‘the PfEMP1 repertoire’ by age were calculated by log base 2 transforming the titers for HbAA children and plotting these values by age. After calculating the slope of the linear regression, this value was converted

to a fold change by taking the antilog (**Figure 5.2a**). Fold change ratios were calculated to compare the rates of PfEMP1-specific IgG acquisition by Hb type by dividing the HbAS or HbAC fold change by the HbAA fold change (**Figure 5.2b**).

### 5.2.4 Heat map construction

The R project for statistical computing was also used to construct heat maps for May and December, which represent the recognition of all HbAA, HbAS, and HbAC children for the 91 domains tested. The program clusters the children and domains based on similarities between their patterns of recognition independent of any clinical data. Clusters A-G representing children with similar patterns of IgG reactivity towards PfEMP1 domains were determined by grouping children whose pattern of recognition was similar. Likewise, Clusters 1-8 representing domains with similar IgG recognition by individual children were grouped in a similar manner.

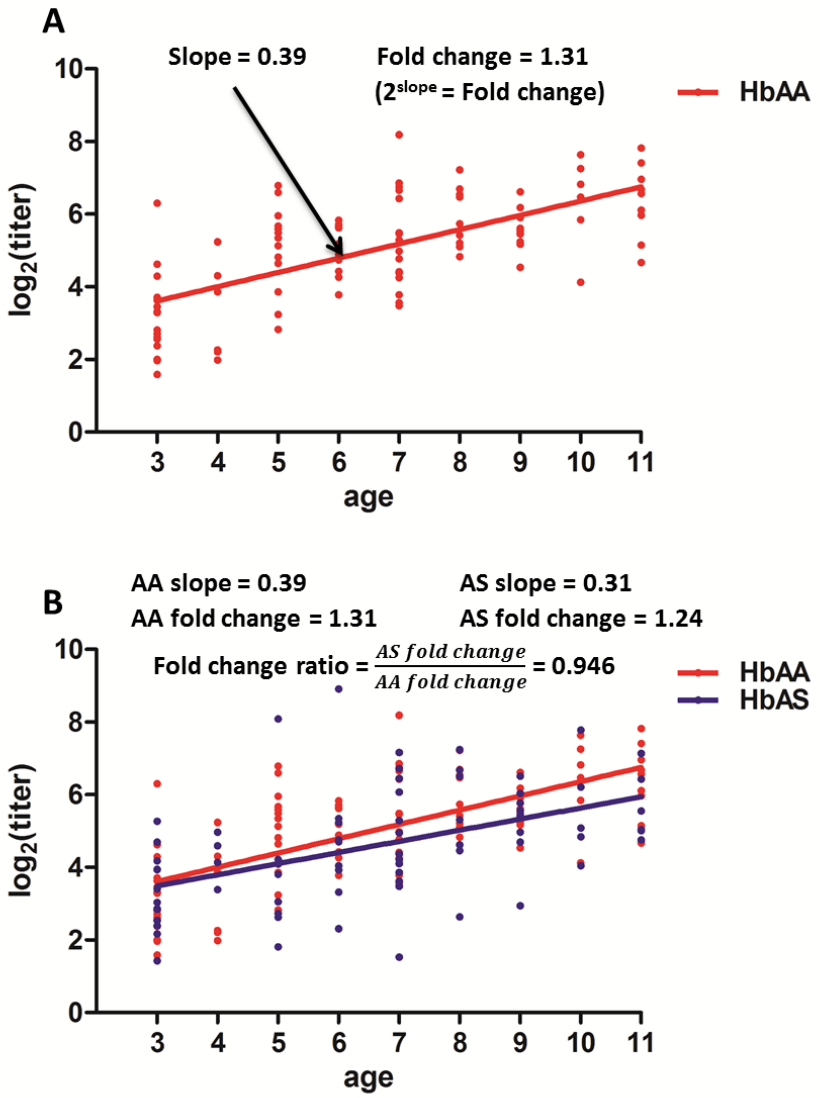



**Figure 5.2 Sample fold change and fold change ratio calculation**

**A)** Fold changes for each of the 91 domains were determined by first calculating the linear regression line for the log base 2 transformed titers of HbAA children plotted by age. The slope of the linear regression line was then transformed into a fold change by taking the antilog. These calculations were repeated for each individual domain. **B)** Fold change ratios for each of the 91 domains were calculated by first determining the fold change by age for HbAA children and for HbAS children. The fold change ratio was then calculated by dividing the fold change for HbAS by age by the fold change for HbAA by age. These calculations were repeated for each individual domain for HbAS and HbAC.

### 5.2.5 Statistics

The titers were normalized using a robust linear model [62] to account for any differences between plates or plexes. An F test, which tests whether the slopes of linear regression lines are significantly different, was used to test the significance of all differences between fold changes and fold change ratios. P values for statistical tests performed on the 91 individual PfEMP1 domains were corrected for multiple comparisons using false discovery rate adjustment [498, 499]. A measure of each child's recognition of 'the PfEMP1 repertoire' was determined by averaging each child's IgG titers towards the 91 PfEMP1 domains tested in our assay. A paired Wilcoxon signed rank test was performed on IgG titers to individual domains in May and December to determine whether each child had boosted their IgG titers over the transmission season.

The ages of children whose samples were clustered together were compared first by Kruskal-Wallis for May (A-D) and December (E-G). For any comparisons that were significant, Mann-Whitney tests were performed to compare each pair of clusters individually. The frequencies of each Hb type in each cluster (A-D in May, E-G in Dec) and the frequencies of each *ups* group in each cluster (1-4 in May, 5-8 in Dec) were compared by a chi-squared test. Since few domains representing *upsC, upsE,* or VAR2CSA PfEMP1 variants were tested, these were combined together in the chi-squared analysis. Additionally, clusters 1 & 2, 3 & 4, and 7 & 8 were combined for analysis since clusters 2, 4, and 8 did not contain many domains.

## 5.3 Results

### 5.3.1 Acquisition of PfEMP1 domain-specific IgG by age

**Older HbAA children recognize PfEMP1 domains at higher IgG titers than younger HbAA children**

To examine the effect of age on the recognition of individual PfEMP1 domains apart from any confounding effects RBC polymorphisms might have, we calculated the fold change in IgG titer against each of the 91 DBL and CIDR domains per year for 103 HbAA children before (May 2009) and after the transmission season (December 2009) (**Figure 5.2a**). In the rest of this chapter, these collection time points before and after the transmission season will be referred to simply as May and December. For each domain, the titers were log base 2 transformed and plotted by age. The slope of the linear regression line was then converted to a fold change by taking the antilog. The fold changes for 90/91 domains in May and 80/91 domains were significantly >1, which indicates that the vast majority of the domains we tested showed a statistically significant increase in IgG titer with increasing age (**Figure 5.3**). The mean fold change for May was 1.32 and for December was 1.26. The remaining 12 domains (1 May, 11 December) also had fold change values >1, but these did not reach statistical significance. EBA-175 had a significant increase in IgG titer by age (May FC = 1.22, P = 0.00003; Dec FC = 1.18, P = 0.003). Tetanus toxoid showed no significant effect by age (May FC = 0.97, P = 0.60; Dec FC = 0.98, P = 0.71).

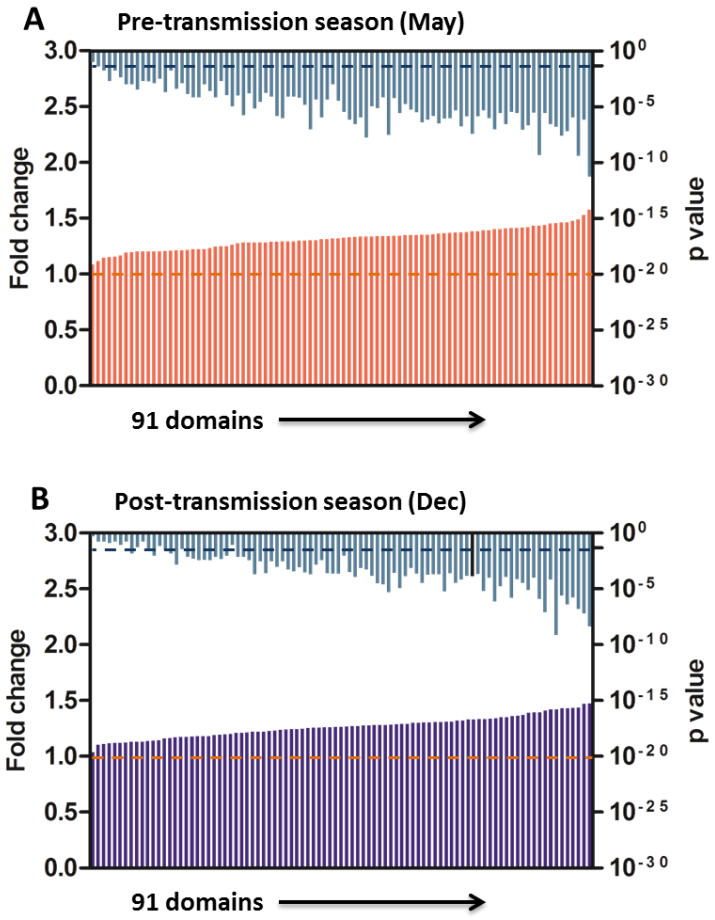



**Figure 5.3 Fold changes in PfEMP1 domain-specific IgG titers and p values for HbAA children by age**

The fold changes in PfEMP1 domain-specific IgG titer per year for 103 HbAA children were calculated for each of the 91 PfEMP1 domains indicated in **A)** red (May) and **B)** dark blue (December) displayed in ascending order. The corresponding p-values for each domain are indicated in light blue. The dotted orange lines indicate a fold change value of 1.0 and the dotted blue line indicates the level of significant p values after correction for multiple comparisons.

### 5.3.2 Acquisition of PfEMP1 domain-specific IgG by Hb type

**HbAS and HbAC children acquire PfEMP1 domain-specific IgGs at a similar rate to HbAA children**

To then compare the rates of PfEMP1 domain-specific IgG acquisition by Hb type, we calculated the fold change in IgG titer by age for HbAS and HbAC for each domain. We then calculated the fold change ratio for each individual domain by dividing the HbAS or HbAC fold change by the HbAA fold change (**Figure 5.2b**). A fold change ratio >1 would imply that HbAS or HbAC children acquire domain-specific IgGs at an accelerated rate while a fold change ratio of <1 would imply that HbAS or HbAC acquire these IgGs at a diminished rate.

None of the fold change ratios for either HbAS or HbAC in May or December were significantly different from 1 except for two PfEMP1 domains in HbAS children in December (a DBLγ domain from 3D7 *upsA var* gene PF08_0141 and a DBLζ domain from 3D7 *upsBA var* gene MAL6P1.316) (**Figure 5.4**, **Figure 5.5**). These results suggest that there is no difference between the rate at which HbAS and HbAC children acquire domain-specific IgGs compared to HbAA children. However, interestingly, the vast majority of fold change ratios tended to be < 1. 79/91 domains had a fold change ratio of < 1 for HbAS children in May (mean fold change ratio = 0.94), 88/91 for HbAS children in December (mean fold change ratio = 0.89), 53/91 for HbAC in May (mean fold change ratio = 0.99), and 81/91 for HbAC in December (mean fold change ratio = 0.90). These results indicate that while HbAS and HbAC children acquire domain-specific IgGs at a similar rate to HbAA children, HbAS and HbAC children might acquire IgGs towards the entire repertoire of 91 domains, 'the PfEMP1 repertoire,' at a slower rate than HbAA children.

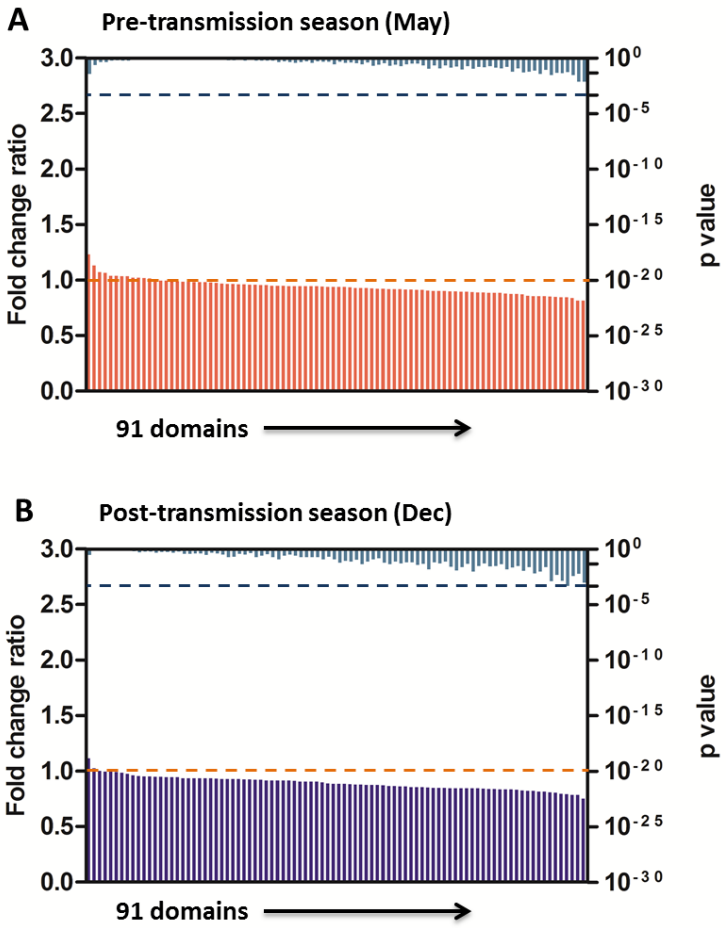



**Figure 5.4 Fold change ratios in PfEMP1 domain-specific IgG titers and p values for HbAS children to HbAA children by age**

The fold change ratios in PfEMP1 domain-specific IgG titer per year for HbAS children compared to HbAA children were calculated for each of the 91 PfEMP1 domains indicated in **A)** red (May) and **B)** dark blue (December) displayed in descending order. The corresponding p values for each domain are indicated in light blue. The dotted orange lines indicate a fold change value of 1.0 and the dotted blue line indicates the level of significant p values after correction for multiple comparisons.

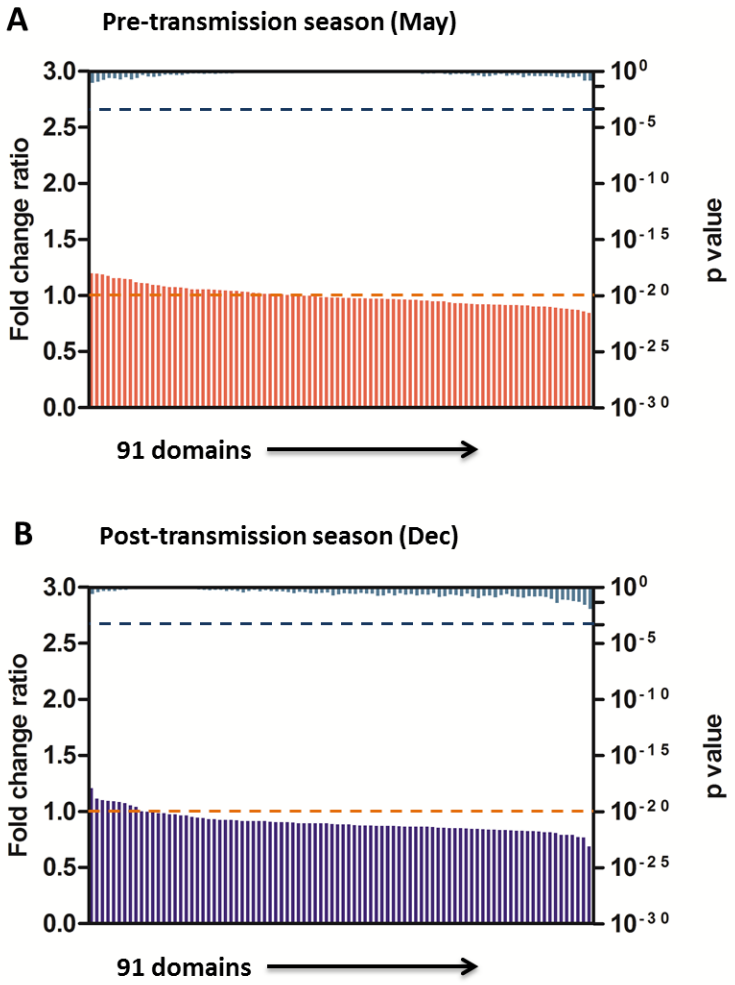



**Figure 5.5 Fold change ratios in PfEMP1 domain-specific IgG titers and p values for HbAC children to HbAA children by age**

The fold change ratios in PfEMP1 domain-specific IgG titer per year for HbAC children compared to HbAA children were calculated for each of the 91 PfEMP1 domains indicated in **A)** red (May) and **B)** dark blue (December) displayed in descending order. The corresponding p values for each domain are indicated in light blue. The dotted orange lines indicate a fold change value of 1.0 and the dotted blue line indicates the level of significant p values after correction for multiple comparisons.

### 5.3.3 Acquisition of IgG against 'the PfEMP1 repertoire' by Hb type

**HbAS children acquire IgGs against 'the PfEMP1 repertoire' at a decreased rate compared to HbAA children in December**

Therefore, to investigate the rate of IgG acquisition against 'the PfEMP1 repertoire' by Hb type, we averaged the IgG titers from the 91 PfEMP1 domains for each child as a measure of a child's overall PfEMP1 recognition by IgG. Using these averaged IgG titers, we calculated the fold change for HbAA, HbAS, and HbAC children by age, then used the fold changes to determine the fold change ratios of HbAS to HbAA and HbAC to HbAA. The fold change ratio for HbAS in May was 0.93 (P = 0.21) and in December was 0.89 (P = 0.01) (**Figure 5.6**) suggesting that HbAS children acquire IgG against 'the PfEMP1 repertoire' at a slower rate than HbAA children. In contrast, HbAC children showed no statistically significant difference in their rate of IgG acquisition against 'the PfEMP1 repertoire' in either May or December (**Figure 5.7**).

### 5.3.4 Boosting of IgG titers against PfEMP1 over a transmission season

**The majority of children boosted their PfEMP1 domain-specific IgG titers over the transmission season**

We next wanted to determine whether children significantly boosted their IgG titers against PfEMP1 from May to December. For the rest of this chapter, "boosting" refers to the difference between IgG titers against PfEMP1 over the 2009 transmission season (from May to December). We compared PfEMP1 domain-specific titers at these two time points and found statistically significant boosting of IgG titers against PfEMP1 in 129/192 children (67.2%).

**Boosting in PfEMP1 domain-specific IgG titers did not change with age in HbAA children**

Our next aim was evaluate whether a child's age affected how much boosting of PfEMP1 domain-specific IgG titers occurred over a transmission season. Therefore, we

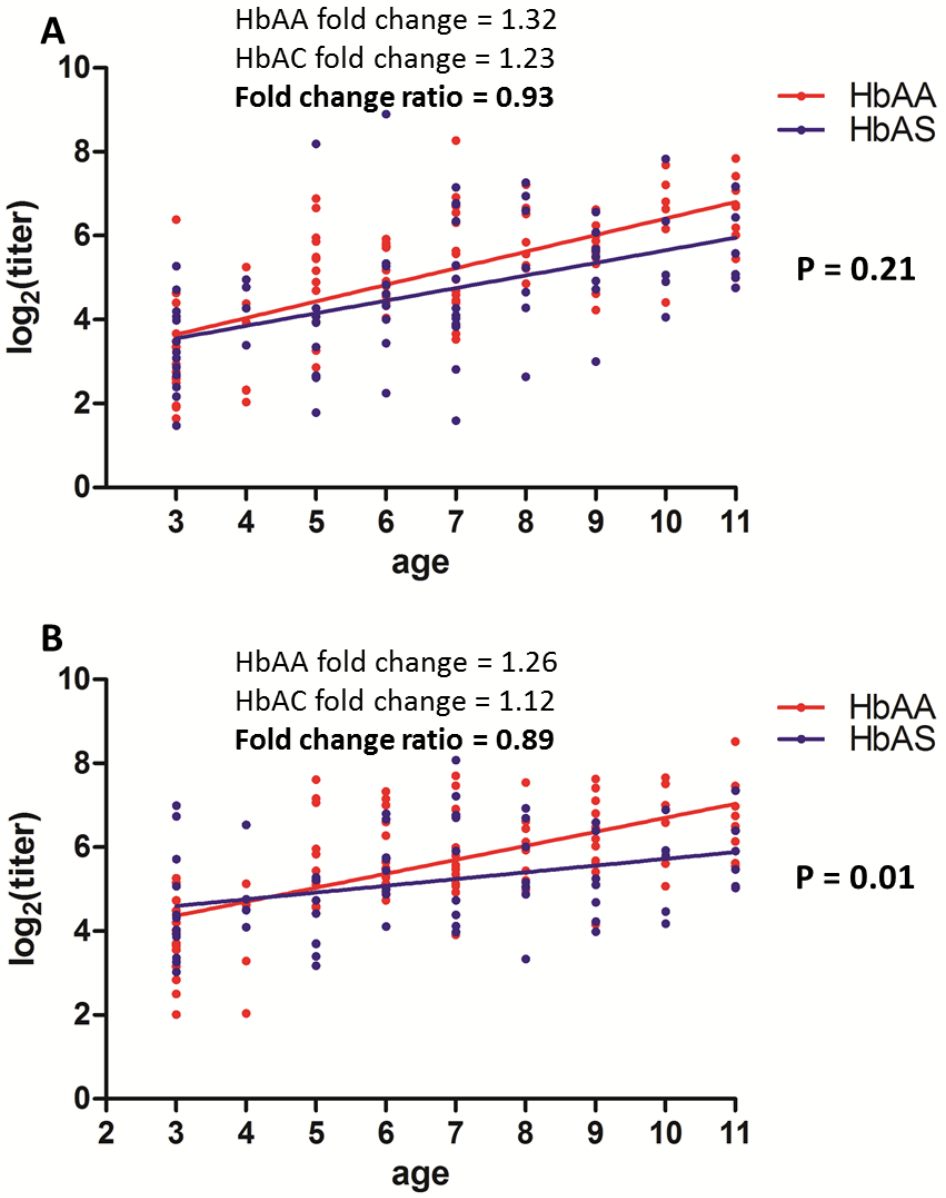



**Figure 5.6 Linear regression analysis for IgG titers against 'the PfEMP1 repertoire' for HbAS and HbAA by age**

The fold change ratio in IgG titer against 'the PfEMP1 repertoire' per year for HbAS children compared to HbAA children was calculated using the average titers of all 91 domains for each child's **A)** May and **B)** December plasma.

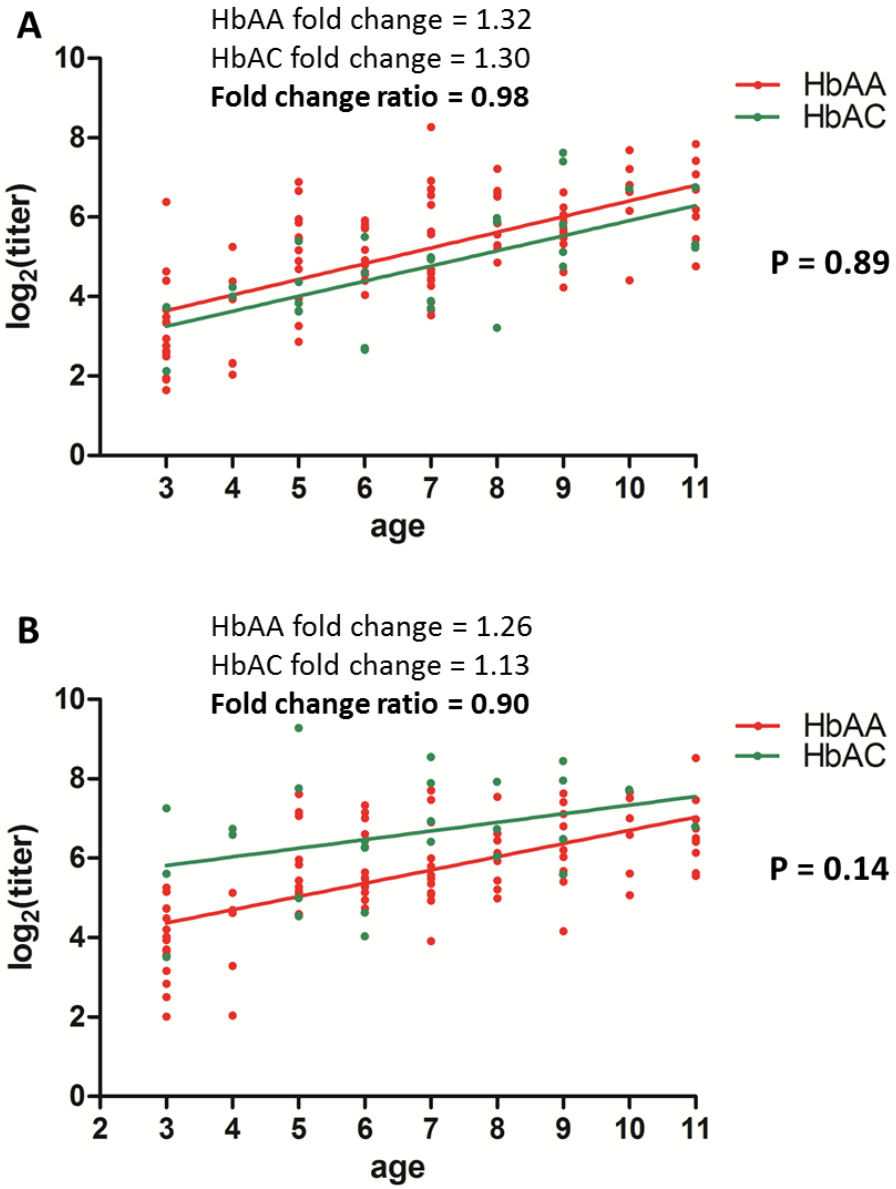



**Figure 5.7 Linear regression analysis for IgG titers against 'the PfEMP1 repertoire' for HbAC and HbAA by age**

The fold change ratio in IgG titer against 'the PfEMP1 repertoire' per year for HbAC children compared to HbAA children was calculated using the average titers of all 91 domains for each child's **A)** May and **B)** December plasma.

calculated the difference in PfEMP1 domain-specific IgG titers between December and May for each child to each domain. We plotted these differences for HbAA children by age and calculated the fold change to determine if the boosting of these IgG titers was age-dependent. A fold change value of > 1 would indicate that older children had a greater boosting in their domain-specific IgG response than younger children, and a fold change < 1 would indicate that they had less. Although 78/91 PfEMP1 domains had a fold change of < 1 with an overall mean fold change of 0.96, none of these were significant indicating that age had no effect on the boosting of domain-specific IgGs (**Figure 5.8a**).

**Age-related boosting in PfEMP1 domain-specific IgG titers did not change by Hb type**

To compare age-related boosting of PfEMP1 domain-specific IgG titers for HbAS and HbAC children compared to HbAA children, we calculated the fold change ratio by dividing the fold change in boosting by age for HbAS or HbAC children by the fold change for HbAA children. A fold change ratio of > 1 would indicate that HbAS or HbAC children had increased boosting in domain-specific IgGs by age compared to HbAA children while a ratio of < 1 would indicate decreased boosting by age compared to HbAA children. Again, although 84/91 domains for HbAS children and 87/91 domains for HbAC had fold change ratios < 1 (HbAS mean = 0.95, HbAC mean = 0.90), none of these differences were significant indicating that Hb type had no significant effect on age-related boosting of domain-specific IgG (**Figure 5.9a, b**).

**Older HbAA children boosted their IgG titers against 'the PfEMP1 repertoire' less than younger HbAA children**

We next wanted to see whether children's age affected how much they boosted their IgG titers against 'the PfEMP1 repertoire.' We calculated the difference between December and May IgG titers against 'the PfEMP1 repertoire.' There was no significant effect of age on boosting of IgG titers against 'the PfEMP1 repertoire' by age (FC = 0.96, P = 0.12) (**Figure 5.10a**).

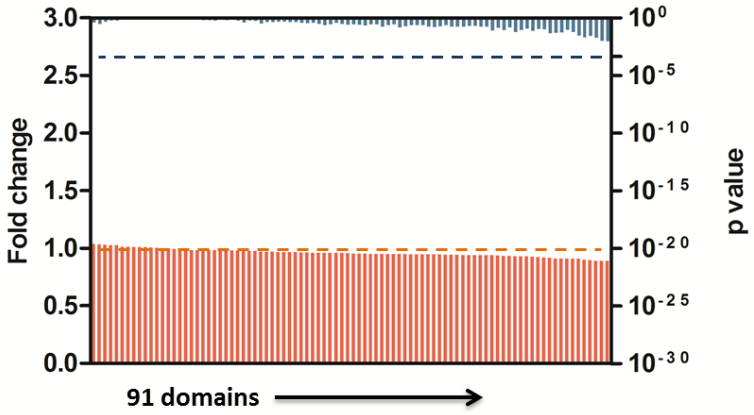



**Figure 5.8 Fold change in boosting of PfEMP1 domain-specific IgG by age**

The fold changes in boosting of PfEMP1 domain-specific IgG per year for HbAA children were calculated for each of the 91 domains. The fold changes are displayed in red in descending order. The corresponding p values for each domain are indicated in light blue. The dotted orange lines indicate a fold change value of 1.0 and the dotted blue line indicates the level of significant p values after correction for multiple comparisons.

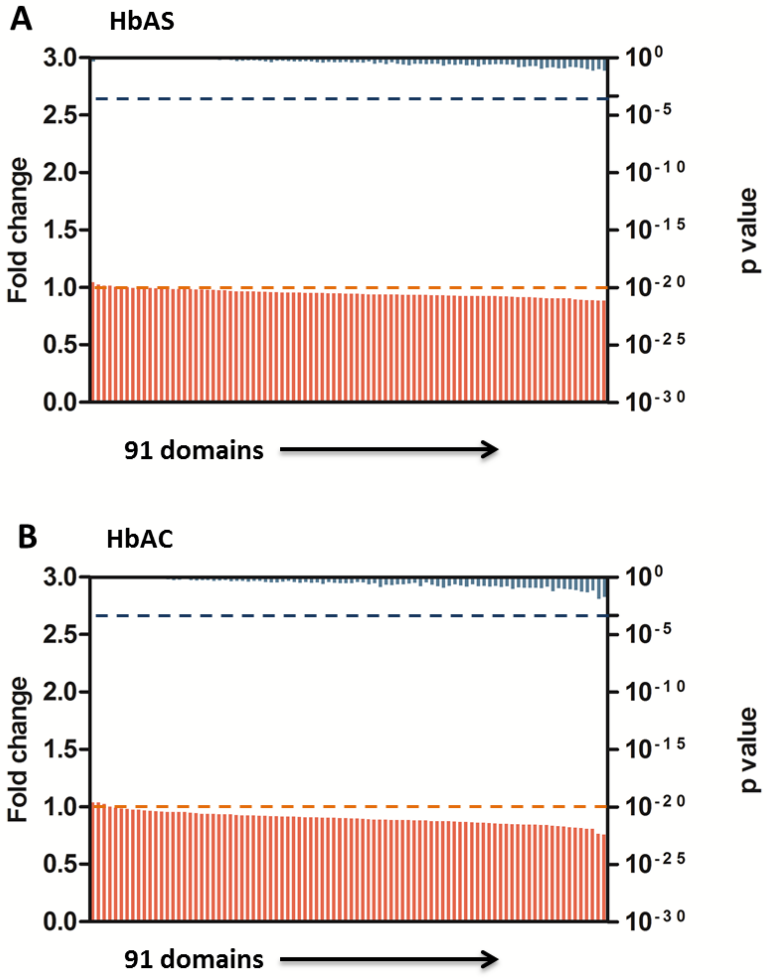



**Figure 5.9 Fold change ratios in age-dependent boosting of PfEMP1 domain-specific IgG for HbAS and HbAC children compared to HbAA children**

The age-dependent fold change ratios in boosting of PfEMP1 domain-specific IgG per year for **A)** HbAS and **B)** HbAC children compared to HbAA children were calculated for each of the 91 domains. The fold change ratios are displayed in red in descending order. The corresponding p values for each domain are indicated in light blue. The dotted orange lines indicate a fold change value of 1.0 and the dotted blue line indicates the level of significant p values after correction for multiple comparisons.

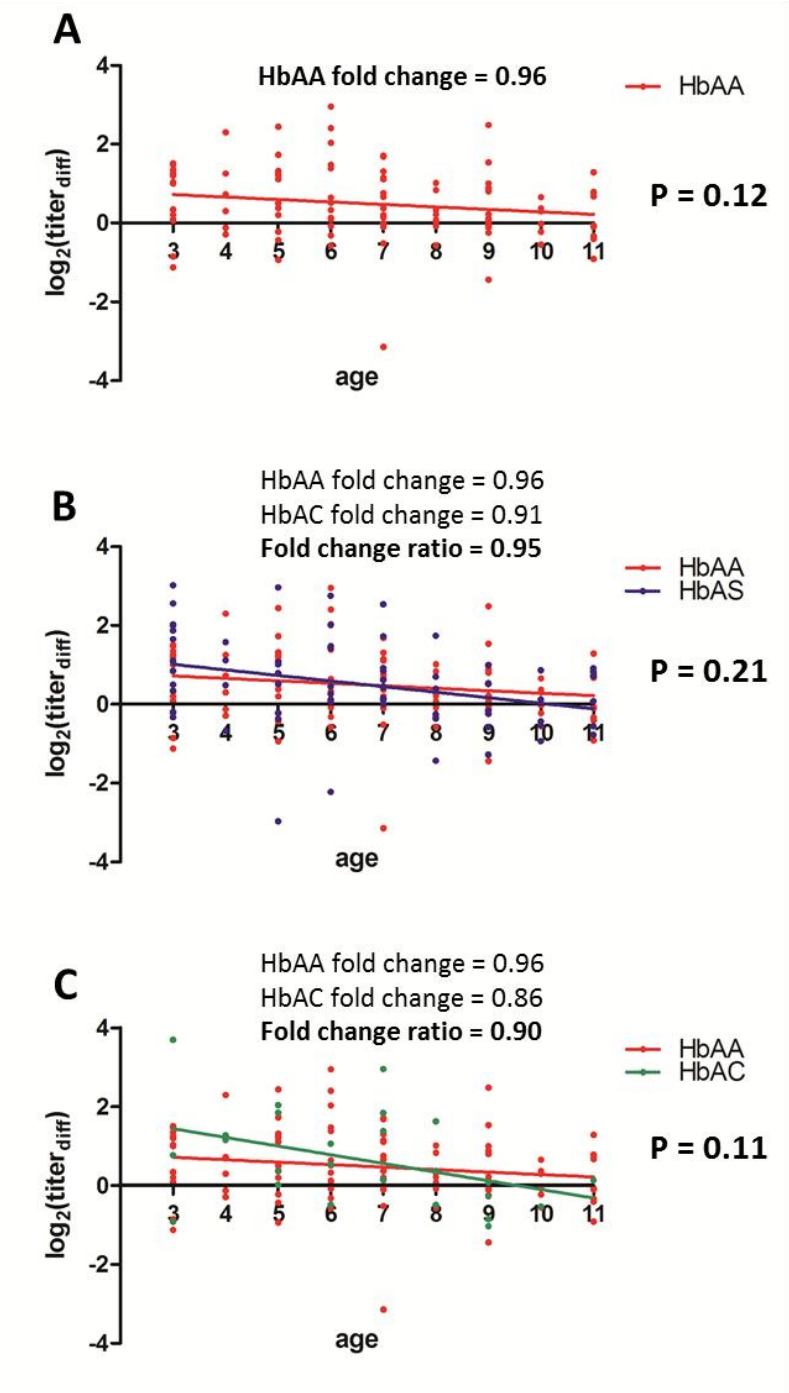



**Figure 5.10 Linear regression analysis for age-dependent boosting of IgG against 'the PfEMP1 repertoire' for HbAA, HbAS, and HbAC children by age**

The fold change in age-dependent boosting of IgG titer against 'the PfEMP1 repertoire' per year for **A)** HbAA children and the fold change ratios for **B)** HbAS and **C)** HbAC children compared to HbAA children were calculated using the difference in IgG titers against 'the PfEMP1 repertoire' in May and December for HbAA.

**Age-related boosting of IgG titers against 'the PfEMP1 repertoire' did not change by Hb type**

By linear regression analysis, there was no difference in the fold change in age-related boosting between HbAA and HbAS (fold change ratio = 0.95, P = 0.21) or HbAC (fold change ratio = 0.90, P =0.11) (**Figure 5.10b, c)**.

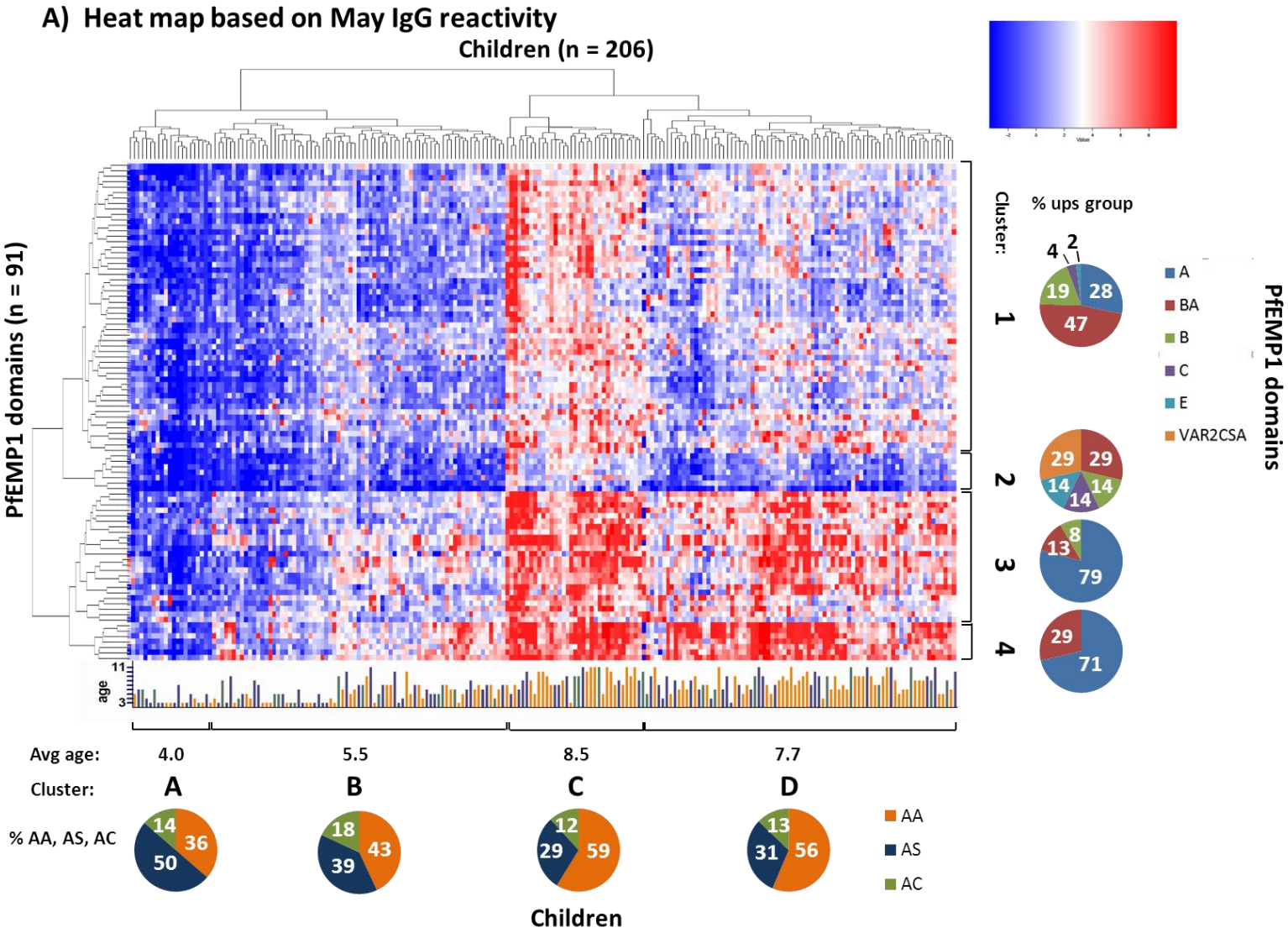
### 5.3.5 Heat map analysis

Heat maps for May and December were constructed using IgG reactivity for 206 HbAA, HbAS, and HbAC children against 91 PfEMP1 domains. Children were clustered based on similarities in their pattern of IgG reactivity towards these domains. Likewise, domains were clustered based on similarities in their IgG recognition by individual children. The boosting of domain-specific IgG titers by the 206 children was also used to construct a heat map.

**Samples cluster by age and Hb type**

The heat map constructed using PfEMP1 domain-specific IgG reactivity in May (**Figure 5.11a**) demonstrates 4 main clusters of children whose IgG reactivity to PfEMP1 domains ranges from poorly reactive towards most domains (Cluster A) to highly reactive towards most domains (Cluster C) with groups that show intermediate-low reactivity (Cluster B) and intermediate-high reactivity (Cluster D) to most domains. Interestingly, individual children showed relatively homogeneous IgG reactivity towards the entire repertoire of PfEMP1 domains. Therefore, a child's IgG reactivity towards one domain predicted their IgG reactivity towards the other domains.

These clusters also seemed to correspond to age and Hb type. The cluster of children that showed the poorest IgG reactivity towards PfEMP1 domains (Cluster A) had the lowest average age out of the four clusters (4.0 years old) followed by the cluster with intermediate-low IgG reactivity (Cluster C) (5.5 years old), the cluster with intermediate-high IgG reactivity


A) Heat map based on May IgG reactivity
Children (n = 206)
PfEMP1 domains (n = 91)
age
11
3
Cluster:
% ups group
1
2
3
4
4
2
19
28
47
29
29
14
14
14
13
8
79
29
71
A
BA
B
C
E
VAR2CSA
PfEMP1 domains
Avg age:
4.0
5.5
8.5
7.7
Cluster:
A
B
C
D
% AA, AS, AC
14
36
50
18
43
39
12
29
59
13
31
56
AA
AS
AC
Children

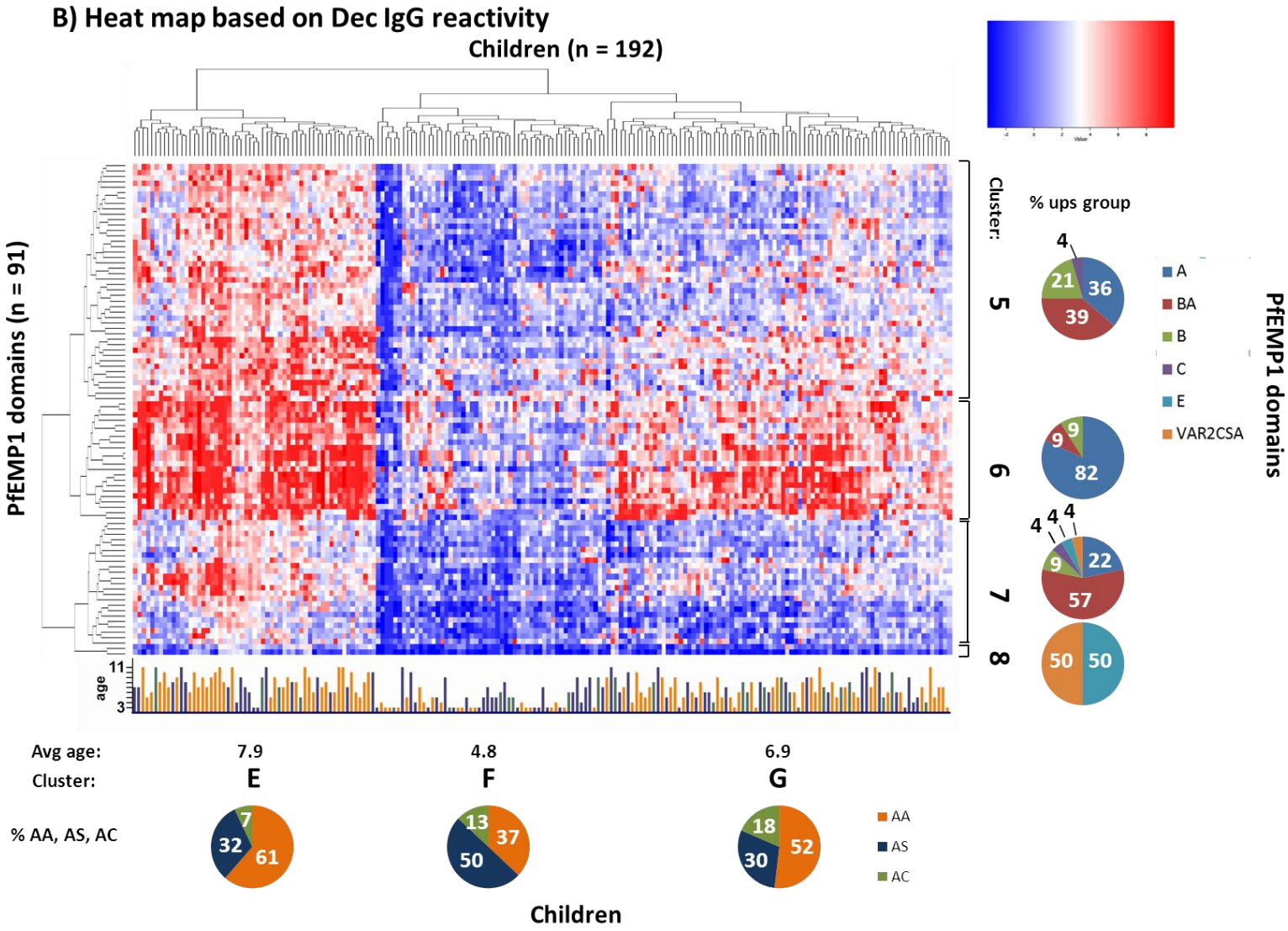

B) Heat map based on Dec IgG reactivity
Children (n = 192)
PfEMP1 domains (n = 91)
Cluster:
% ups group
5
6
7
8
4
21
36
39
9
9
82
4
4
4
9
22
57
50
50
A
BA
B
C
E
VAR2CSA
PfEMP1 domains
age
11
3
Avg age:
7.9
4.8
6.9
Cluster:
E
F
G
% AA, AS, AC
7
32
61
13
37
50
18
52
30
AA
AS
AC
Children

**Figure 5.11 Heat maps for May and December**

Heat maps were constructed using the **A)** May and **B)** December IgG reactivity of 206 and 192 children, respectively, to 91 PfEMP1 domains. The color of each individual square represents the IgG reactivity of a specific child against a specific domain with dark blue as the least reactive and dark red as the most reactive. Children and domains were clustered according to similarities in their patterns of IgG recognition. Children's plasma samples were separated into 7 main clusters (A-G). The age of each child is indicated by the height of the bar below and the average age for each cluster was calculated. The Hb type of each child is indicated by the color of the bar (HbAA – orange, HbAS – blue, HbAC – green) and the % of each Hb type within each cluster is indicated by the pie chart below. Domains were separated into 8 main clusters (1-8). The percentage of each *ups* group represented by the PfEMP1 domains in each cluster is indicated by the pie charts (medium blue = *upsA*, green = *upsBA*, red = *upsB*, purple = *upsC*, light blue = *upsE*, orange = VAR2CSA).

(Cluster D) (7.7 years old), and lastly, the cluster with the highest IgG reactivity (Cluster B) (8.5 years old). The clustering of children based on their pattern of PfEMP1-specific IgG reactivity was significantly associated with age ($P < 0.0001$) (**Figure 5.12a**). In particular, the ages of the children within the following clusters were significantly different: Clusters A and B ($P = 0.001$), A and C ($P < 0.0001$), A and D ($P < 0.0001$), B and C ($P < 0.0001$), and B and D ($P < 0.0001$). Additionally, the percentage of HbAA children in each cluster increased with increasing IgG reactivity (36%, 43%, 56%, 59%) while the percentage of HbAS children decreased (50%, 39%, 31%, 29%). The percentage of HbAC children in each of the clusters was relatively uniform and ranged from 12-18%. However, these differences in Hb type of children clustered by PfEMP1-specific IgG reactivity were not significant ($P = 0.42$) (**Figure 5.12c**).

Similar trends were observed in the heat map constructed from the IgG reactivity of 192 children to PfEMP1 domains in December (**Figure 5.11b**), which were separated into three main clusters of children (Clusters E, F, and G). Children in Cluster F had poor IgG reactivity to most domains, Cluster G had intermediate reactivity, and Cluster E had high reactivity. As with the May heat map, the average age of children within each cluster increased with increasing

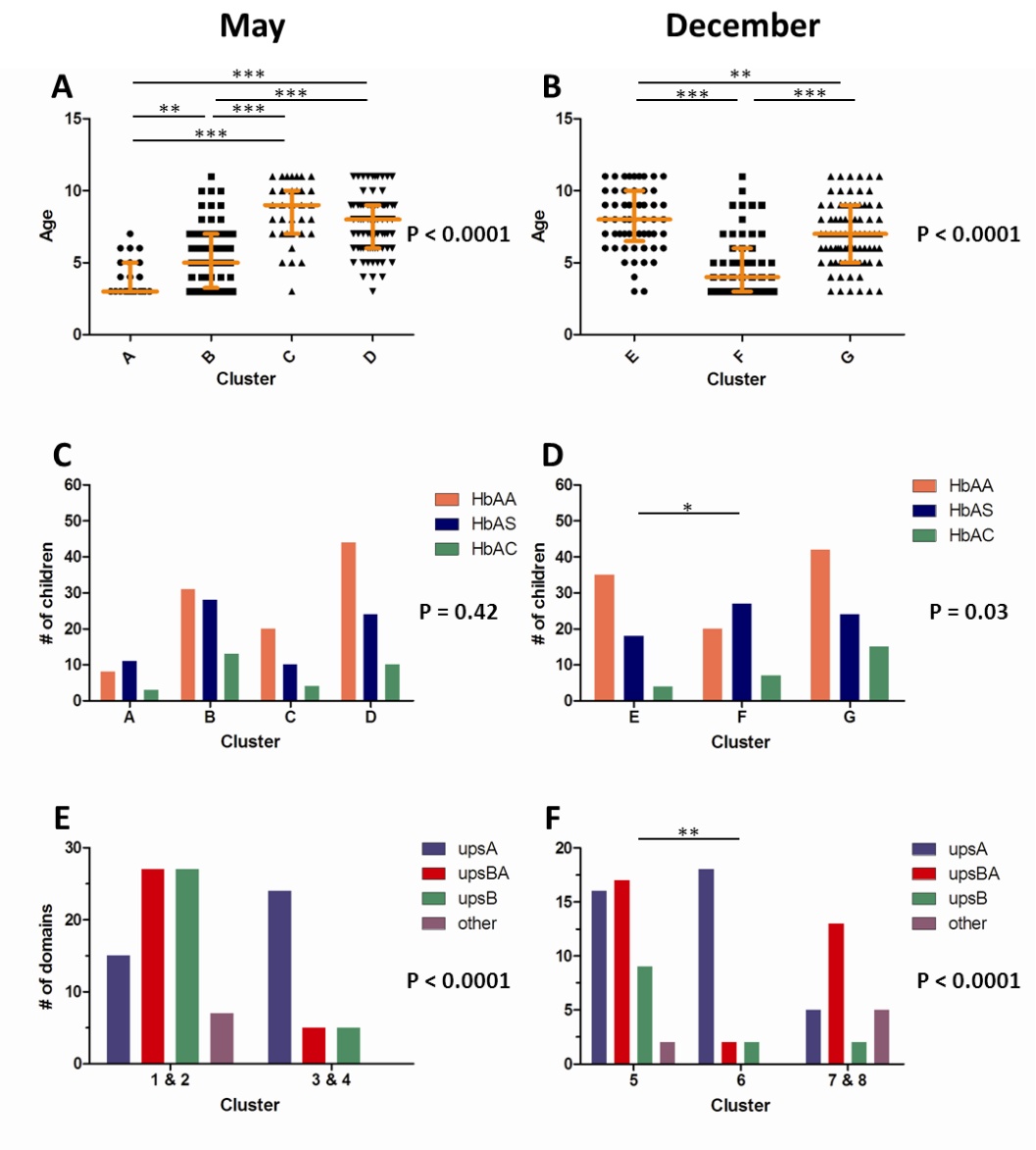



**Figure 5.12 Heat map clusters for May and December**

For the heat map analysis in May and December, children were separated into 7 clusters (A-D May, E-G December) based on their IgG recognition of PfEMP1 domains and domains were separated into 8 clusters (1-4 May, 5-8 December) based on their IgG recogntion by children. **A & B)** The ages of children in Clusters A-D (a) and Clusters E-G (b) with the median age and interquartile range indicated by the line and error bars and **C & D)** the number of HbAA, HbAS, and HbAC children in Clusters A-D (c) and Clusters E-G (d) are shown. **E & F)** The number of *upsA*, *upsBA*, *upsB*, and domains from other *ups* groups (C, E, and VAR2CSA) are shown for Clusters 1-4 (e) and Clusters 5-8 (f). P values are indicated for the comparisons of all groups together to the right of the graph. If this p value was significant, individual comparisons were performed and the resulting p values are indicated above the 2 groups (*** = P value < 0.001, ** = P value < 0.01, * = P value < 0.05).

IgG reactivity (Cluster F: average age = 4.8 years old, G: 6.9 years old, E: 7.9 years old) ($P < 0.0001$). The differences in age between each of the clusters was also significant (**Figure 5.12b**) (E and F: $P < 0.0001$, F and G: $P < 0.0001$, E and G: $P = 0.008$). The percentage of HbAA children in each cluster increased with increasing IgG reactivity (37%, 52%, 61%). The percentage of HbAS children decreased from the 'high reactivity' to the 'intermediate reactivity' group (50% to 30%), but did not decrease further from the 'intermediate reactivity' to 'low reactivity' group (30% to 32%). The percentage of HbAC children for each cluster ranged from 7-18%. In December, the association between Hb type and clustering based on PfEMP1-specific IgG reactivity was significant ($P = 0.03$) (**Figure 5.12d**). Specifically, the difference between the cluster with the highest reactivity (Cluster G) and the lowest reactivity (Cluster F) was significant ($P = 0.03$).

***UpsA* PfEMP1 variants are highly recognized by IgG from Malian children**

The 91 PfEMP1 domains that we tested could be separated into four clusters according to their IgG recognition by individual children in May. Cluster 2 had domains that were poorly recognized and Cluster 4 had domains that were highly recognized by domain-specific IgG. Clusters 1 and 3 showed intermediate-low and intermediate-high reactivity, respectively. Clusters 3 and 4 were primarily made up of domains based on *upsA var* genes (Cluster 3: 79%, 4: 71%) with minor contributions from those based on *upsBA* (Cluster 3: 13%, 4: 29%) and for Cluster 3, *upsB* (8%) *var* genes. The percentage of domains based on *upsA* var genes was lower in the cluster of domains with intermediate-low recognition by children (Cluster 1) (28%) and were not found in Cluster 2. On the other hand, the percentage of domains based on *upsBA var* genes was increased in Cluster 1 (47%). The percentage of domains based on *upsB var* genes was 19% in Cluster 1 and 14% in Cluster 2. For statistical analysis, all domains that were based

on *upsC, upsE,* or VAR2CSA *var* genes were categorized together as "other." The clusters were categorized into two groups: Clusters 1 and 2 and Clusters 3 and 4 based on their similarities in IgG recognition due to the small numbers of domains in Clusters 2 and 4. The clustering of domains based on their IgG recognition by individual children in May was significantly associated with *ups* group ($P < 0.0001$) (**Figure 5.12e**).

Likewise, the domains tested could be separated into four clusters according to their IgG recognition by individual children in December. Cluster 8 was comprised of only two domains that were poorly recognized (one *upsE* and one VAR2CSA) and Cluster 6 had domains that were highly recognized. Clusters 7 and 5 showed intermediate-low and intermediate-high recognition, respectively. These samples showed a similar pattern in which the cluster of domains with highest recognition (Cluster 6) was primarily comprised of domains based on *upsA var* genes (*upsA*: 82%, *upsBA*: 9%, *upsB*: 9%). The percentage of domains based on *upsA var* genes decreased according to IgG recognition (Cluster 5: 36%, Cluster 7 22%, Cluster 8: 0%). For statistical analysis, Clusters 7 and 8 were combined since Cluster 8 only contained 2 domains. The clustering of domains based on their IgG recognition by individual children in December was also significantly associated with *ups* group ($P < 0.0001$) (**Figure 5.12f**).

## 5.4 Discussion

Here, we have reported the first study of acquisition of IgG specific for an entire repertoire of PfEMP1 DBL and CIDR domains by HbAA, HbAS, and HbAC children. In our study, the recognition of PfEMP1 domains by IgG was highly associated with age. Older children demonstrated higher PfEMP1 domain-specific IgG titers than younger children for almost every domain we tested in both May and December. By heat map analysis, age is a strong predictor of a child's pattern of PfEMP1 domain-specific IgG reactivity. Older children are more likely to belong to Cluster C, D, E, or G, which have higher IgG reactivity to PfEMP1 domains, and younger children are more likely to belong to Cluster A, B, or F, which have lower IgG reactivity. These findings suggest that children acquire PfEMP1-specific IgGs with age, likely as the result of repeated infections with heterogeneous strains of *P. falciparum* and some level of cross-reactivity between PfEMP1 variants.

Older children also boosted their IgG titers against 'the PfEMP1 repertoire' less than younger children, which likely reflects that some of the older children in our cohort (ages 9-11 years old) already had levels of IgGs against 'the PfEMP1 repertoire' that were similar to hyperimmune adults as they entered the transmission season. Therefore, any *P. falciparum* strains they would have encountered over the transmission season would have relatively small effects on their IgG titers against 'the PfEMP1 repertoire' compared to younger children who lacked recognition of many domains and had encountered fewer PfEMP1 variants over their lifetime. The leveling off of IgG titers against 'the PfEMP1 repertoire' in older children also correlates with our epidemiological findings from our cohort (see **2.3.2**). Within our cohort, younger children experience significant reductions in their relative risk of developing malaria with each successive year of age. However, this effect seems to plateau in older children and

children > 14 years old do not experience further decreases in their relative risk suggesting that they have reached adult levels of protection against disease.

In contrast with the strong age effect on IgG recognition of PfEMP1 domains, Hb type seems to influence PfEMP1-specific IgG acquisition to a much lesser extent. Contrary to our initial hypothesis, neither HbAS nor HbAC had a significant effect on the rate of acquisition of PfEMP1 domain-specific IgGs. However, we also found that HbAS children may acquire IgGs against 'the PfEMP1 repertoire' at a slower rate particularly when comparing IgG titers after a transmission season. It is worth noting that the magnitude of this difference is relatively small and is only significant for the December time point (May: FC ratio = 0.93, P = 0.21 and Dec: FC ratio = 0.89, P = 0.01). However, in heat maps based on PfEMP1 domain-specific IgG reactivity in May and in December, the percentage of children found in each cluster also seems follow a trend of increasing HbAA percentage and decreasing HbAS percentage with increasing IgG reactivity, Hb type may be working *in vivo* to shape a child's IgG recognition of 'the PfEMP1 repertoire,' although these differences were not significant. Most domains showed a trend of decreased age-related boosting of IgG titers in HbAS children compared to HbAA children. This result, determined by comparing a child's IgG titers in May against the same child's IgG titers in December, suggests that HbAS children might not boost their IgG titers against PfEMP1 domains over a transmission season as much as an HbAA child of the same age. Although this difference is not significant, it is consistent with the age-dependent trend we observe within the entire population of HbAA and HbAS children.

Taken together, these results imply that while carrying HbAS affects a child's acquisition of IgGs against PfEMP1, age is the dominating factor in IgG acquisition to PfEMP1 with Hb type playing relatively minor role. Although HbAC did not demonstrate a significant

effect in any of the tests we performed, we cannot entirely rule out the possibility that HbAC might also exert an effect on the acquisition of IgGs against PfEMP1 since only 30 HbAC children were used in our analysis. However, we would expect that even with a greater sample size, any statistically significant effect HbAC had on IgG recognition of PfEMP1 would likely be relatively small compared to the age-dependent effect since the fold change values for HbAC are of similar magnitude as HbAS.

Interestingly, IgG titers against 'the PfEMP1 repertoire' were similar between HbAA and HbAS children at younger ages, when anti-disease immunity is generally thought to be acquired. However, HbAA children acquire IgG against 'the PfEMP1 repertoire' at an accelerated rate compared to HbAS children so that the differences in their IgG titers are more pronounced for the older children on our study, when anti-parasite immunity is likely developed. The age-dependence of the differences between IgG titers in HbAA and HbAS children is likely driven by a trend towards increased boosting of PfEMP1 domain-specific IgG in HbAA compared to HbAS children over a transmission season. Although these differences in boosting over the 2009 transmission season were not significant in our study, they may explain the trend that emerges in older children towards higher IgG titers against 'the PfEMP1 repertoire' in HbAA compared to HbAS children. Therefore, when considered together, one possible explanation for our data is that very young HbAA and HbAS children start out with similarly low IgG titers against PfEMP1 since they have not been exposed to many variants, but that slightly reduced boosting of PfEMP1-specific IgG titers in HbAS compared to HbAA children over each individual transmission season leads to increasing differences between HbAA and HbAS PfEMP1-specific IgG titers with age.

This discrepancy between our initial hypothesis and the relative IgG recognition of PfEMP1 domains by HbAS children compared to HbAA children in our study may be due to our finding, after conducting this study, that within our population, we do not see the epidemiological interaction between age and Hb type reported by Williams *et al.* in Kilifi, Kenya [342]. The difference in the interaction between age and Hb type in the two populations both epidemiologically as well as potentially in the recognition of PfEMP1 domains may be due to differing genetic backgrounds within the two human populations or differing endemicities within each site. The findings from Verra *et al.* [344] argue for the latter possibility. They found significant differences in recognition of VSAs expressed by a pool of wild parasite isolates by HbAS or HbAC children compared to HbAA children in urban low-transmission areas (EIR: 1-10 annual infective bites per person) by Hb type, but not in rural high-transmission areas (EIR > 100 annual infective bites per person). Likewise, the transmission intensities in Kilifi and Kenieroba, where our own study was performed, are vastly different. In a study done in Kilifi, 9 sites throughout the district were evaluated for their estimated EIR. 7 of 9 sites had EIRs of less than 4 annual infective bites per person and the remaining 2 sites had EIRs of 18 and 59.6. In contrast, children in our field site experience over an estimated 200 infective bites annually. The intensity of transmission within our site and the resulting higher endemicity within our population may explain our findings. Repeating our study in a lower endemicity population where HbAS children are epidemiologically protected against malaria differentially during a specific age range might confirm our initial hypothesis.

The slower acquisition of IgGs and lower IgG titers against ‘the PfEMP1 repertoire’ by HbAS children compared to HbAA children might be explained by several potential mechanisms. First, HbAS children might experience a regulatory defect in their immune system which prevents them from producing wildtype amounts of IgG against PfEMP1. Second, the

altered display on the surface of parasitized RBCs might affect the conformation of PfEMP1 presentation on knobs to B cell receptors (BCRs) and the abnormal distribution of PfEMP1 might affect their ability to cluster BCR and stimulate B cell activation. Decreased B cell activation by PfEMP1 would result in decreased production of PfEMP1-specific IgG. Third, reduced levels of surface PfEMP1 in HbS-containing RBCs might lead to reduced production of PfEMP1-specific IgG. This explanation seems unlikely since the level of surface PfEMP1 expressed on parasitized HbAS RBCs is only reduced 10-15% compared to parasitized HbAA RBCs, a difference that seems negligible in stimulating production of antibodies considering children with malaria often carry parasite burdens on the order of billions of parasites. Lastly, the mere exposure of PfEMP1 variants on the surface of RBCs might be insufficient to activate B cells to produce PfEMP1 variant-specific IgG. Activation of B cells may require the inflammation experienced during an episode of malaria alongside PfEMP1 expression by parasitized RBCs to boost IgG titers to a particular variant. This possibility is supported by the observation by Ofori *et al.* [445] that asymptomatic parasitemia did not result in boosting of IgG response to the surface of that particular parasite isolate. We would expect that more PfEMP1 variants would be expressed in HbAS children over time as a result of increased PfEMP1 switching as well as accumulation of *P. falciparum* isolates from decreased frequency of parasite-clearing treatment due to less frequent malaria episodes. However, since the majority of these variants are expressed while the child is asymptomatically parasitized, the child might not produce IgGs against these variants. In contrast, HbAA children experience malaria episodes with greater frequency, are treated with parasite-clearing drugs more often, and therefore, are less likely to accumulate parasite strains in comparison with HbAS children. However, their increased frequency of malaria episodes might result in higher IgG titers towards the PfEMP1 variants expressed by the parasite during infection.

The question remains: if IgG titers towards PfEMP1 variants are slightly reduced in HbAS children, then how does HbAS result in increased protection against falciparum malaria? While the answer to this question is yet unclear, one possibility is that the enhanced immunity conferred by HbAS children may not actually be mediated by differences in acquired immunity. Other mechanisms of protection such as reduced cytoadherence or differences in innate immunity might work in concert to provide protection against malaria in these children until HbAA children acquire enough PfEMP1-specific IgG to achieve functional immunity against disease. In particular, reductions and altered display of surface PfEMP1 lead to impaired binding between host endothelium and parasitized HbAS RBCs compared to HbAA RBCs. PfEMP1-specific IgGs might further weaken cytoadherence interactions leading to reduced inflammation and activation of host endothelial cells. Although HbAS children have lower IgG titers against 'the PfEMP1 repertoire,' the already-reduced avidity of cytoadherence interactions between HbAS and endothelial cell receptors might be disrupted by lower titers of PfEMP1-specific IgGs. Therefore, despite similar or slightly reduced PfEMP1-specific IgG titers in HbAS children compared to HbAA children, these titers would be more effective in preventing malaria episodes in HbAS children compared to HbAA children at younger ages. Once HbAA children acquired PfEMP1-specific IgG titers sufficient to prevent high-avidity interactions between PfEMP1 and host receptors in HbAA children, they would be protected against disease. At that point, HbAA and HbAS children would experience similar levels of protection against malaria.

One of the major concerns about techniques which use baculovirus-expressed protein domains is that these domains may not be properly folded into their native conformations. Several of our findings provide reassurance that despite these concerns, our data reflects IgG recognition of PfEMP1 domains *in vivo.* First, age-dependent protection against malaria and the

greater magnitude and breadth of VSA recognition in older children has been well established by several groups [231, 440-443, 446, 447] and is likely, at least partially, responsible for the protection older children experience against falciparum malaria in endemic areas. Since PfEMP1 has recently been identified as a main VSA target of the acquired immune response [341], the association between age and IgG titers towards PfEMP1 variants in our study is consistent with these previous studies. Second, other studies have shown that a specific subset of PfEMP1 variants (*upsA)* is associated with SM and with *P. falciparum* infections in younger children [419]. Additional studies examining the acquisition of PfEMP1-specific IgG have shown that children acquire IgGs to *upsA* PfMEP1 variants at a younger age than they do to other PfEMP1 variants. Therefore, the observation that Cluster 3, which contained domains that were highly recognized by PfEMP1 domain-specific IgG, was composed of a disproportionately high percentage of domains from *upsA* PfEMP1 variants also corroborates the validity of our findings. Lastly, in our study, over half of the children showed significant boosting of their IgG titers against PfEMP1 domains over the transmission season. This result suggests that PfEMP1 domain-specific IgGs that a child naturally acquired against circulating strains over the transmission season cross-react with the domains tested in our assay.

Most children on our study seem to recognize multiple DBL domains using this platform. This finding is consistent with previous studies, which have shown discordance between cross-reactivity using recombinant PfEMP1 domains by ELISA and surface cross-reactivity by flow cytometry [230].One possible reason for the increased cross-reactivity by ELISA-based platforms, such as the Luminex platform, may be that the display of recombinant PfEMP1 on Luminex beads exposes semi-conserved regions of the protein, which are concealed from IgG detection when naturally displayed on the parasitized RBC surface. Ideally, we would like to test the reactivity of each child's plasma against multiple naturally-circulating isolates from our

site by flow cytometry, which would enable us to examine the recognition of natively-expressed PfEMP1 and other surface-expressed proteins. However, unpublished data has shown that Malian children's IgG responses to two Malian parasite strains correlates with their IgG response to two Cambodian parasite strains. These data suggest that some degree of surface cross-reactivity exists between parasite strains from locations as geographically distant as Mali and Cambodia, and that *P. falciparum* infection may lead to the development of both strain-specific and strain-transcending IgGs against PfEMP1 variants.

Nevertheless, our study has several significant limitations. The first two have been considered previously in this discussion. First, our study does not demonstrate the same epidemiological interaction between HbAS and age as seen in a previous study. Second, the number of HbAC children included in this study was limited by the total number of HbAC children in the two villages where we conducted this study. Third, 'the PfEMP1 repertoire' we tested was primarily based off of the 3D7 genome. Although there is evidence that the PfEMP1 domains used in this assay show cross-reactivity with children's PfEMP1 domain-specific IgGs against parasite isolates circulating within our field site, 'the PfEMP1 repertoire' tested in our assay still may not have been representative of these isolates. Furthermore, certain types of domains such as DBLα and ups groups such as Group C *var* genes were underrepresented within the domains we tested. Further studies would ideally increase the number of domains tested and the diversity of domains included. Studies examining the recognition by HbAA and HbAS children of autologous as well as heterologous isolates circulating in the study population are needed to more fully understand the acquisition of immunity towards naturally occurring strains by children carrying different Hb types. One attractive possibility would be to create a library of domains from naturally circulating isolates from our site to test for recognition by children carrying wildtype and variant Hb types.

In conclusion, our initial hypothesis was not confirmed by our findings. The contribution of Hb type to the acquisition of PfEMP1-specific IgGs seems to be much smaller in magnitude and level of significance compared to age. Despite these results, Hb type may play a larger role in PfEMP1-specific IgG acquisition in lower endemicity populations, in which Hb type and age interact in epidemiological protection against malaria. Future studies performed in such areas testing for recognition of strains circulating within that particular human population are needed to better understand the role of Hb type in shaping the repertoire of PfEMP1 variants recognized by IgG.

# 6 Discussion

The mortality attributable to *P. falciparum* has exerted significant influence on the human genome and led to the evolutionary selection of malaria-protective RBC polymorphisms such as HbS and HbC. Elucidating the mechanisms underlying this protection may provide critical insights applicable to the development of new therapeutics or to the discovery of novel vaccine candidates. Recent *in vitro* findings have suggested that reductions in cytoadherence ligand PfEMP1's expression and alterations in its display on the surface of parasitized RBCs may explain malaria protection in these children. Based on these observations as well as the malaria protection we have reported in our Malian cohort for HbAS, but not HbAC children (**Chapter 2**), we sought to test whether this protective mechanism might be working *in vivo* to shape the parasite's binding phenotype, its expressed PfEMP1 repertoire, and its recognition by the host's PfEMP1-specific IgG repertoire in HbAS children (**Figure 6.1**).

We hypothesized that to overcome altered PfEMP1 display in HbAS RBCs, parasites from HbAS children with malaria express PfEMP1 variants with an increased binding phenotype to MVECs compared to parasites from HbAA children. Furthermore, we hypothesized that parasites from HbAS children with malaria express a restricted set of multi-domain PfEMP1 variants that might bind multiple host receptors simultaneously to increase the overall avidity of the parasite-MVEC interaction. Lastly, we hypothesized that parasites in HbAS children would have to undergo multiple rounds of antigenic switching between PfEMP1 variants to eventually express a variant that binds host receptors with sufficient avidity to overcome altered PfEMP1 display. Therefore, we expected that HbAS children would acquire PfEMP1-specific IgG at an accelerated rate compared to HbAA children resulting from increased exposure to a more diverse set of PfEMP1 variants.

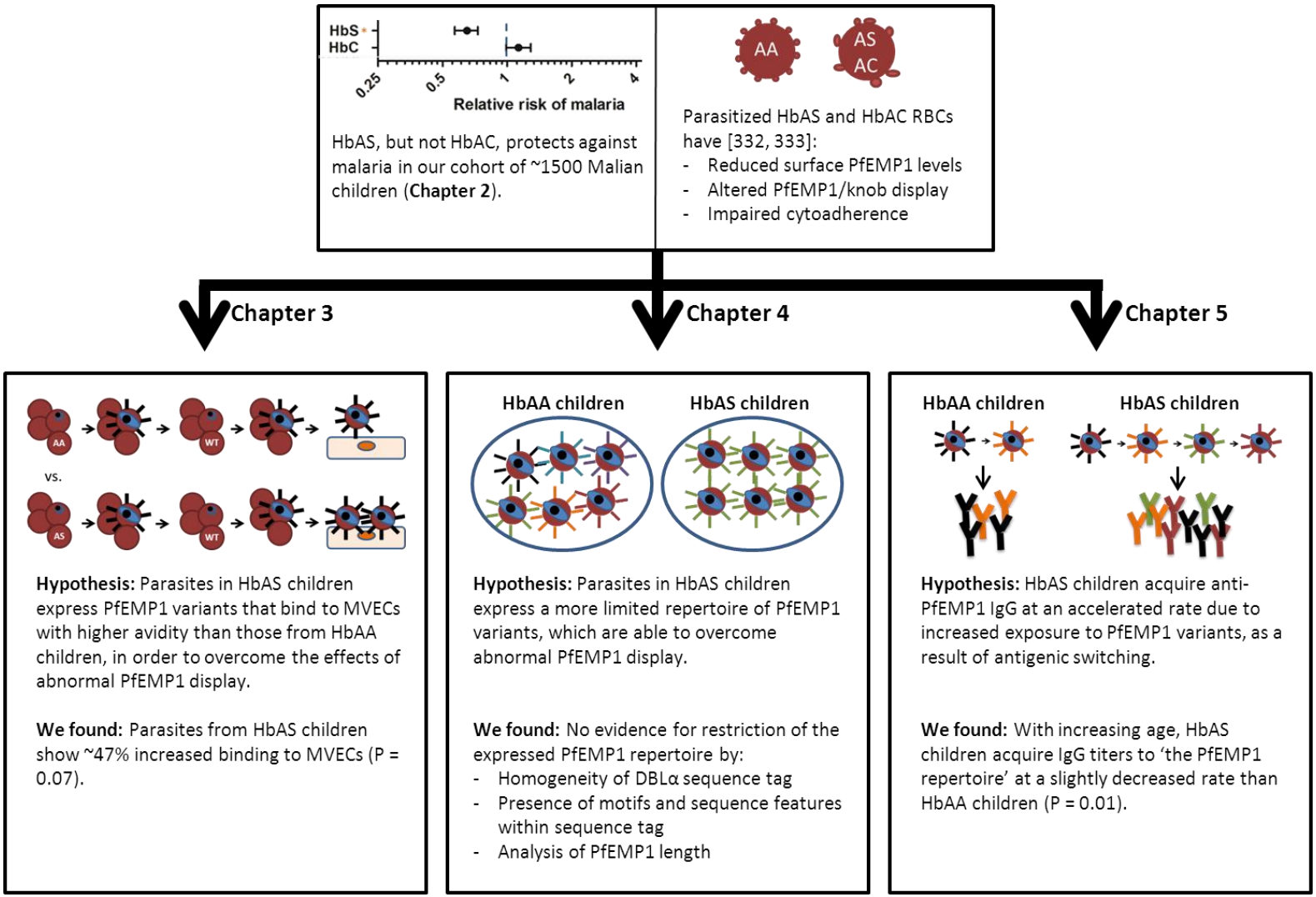



**Figure 6.1 Summary of major findings**

In 31 total comparisons of the binding to MVECs by parasites from HbAS or HbAC children to parasites from HbAA children, we saw a nearly significant ~47% increased binding by parasites from HbAS children to MVECs (P = 0.07), but clearly no difference in binding by parasites from HbAC children (**Chapter 3**). Next, by three approaches, we attempted to determine whether the expressed PfEMP1 repertoire is restricted in HbAS children with malaria (**Chapter 4**). Comparison of DBLα sequence tags expressed by parasites from HbAA, HbAS, and HbAC children showed no differences by Hb type in the homogeneity of the expressed PfEMP1 repertoire either in an individual isolate's repertoire or in the repertoires of all isolates from HbAA, HbAS, or HbAC children. In addition, we found no differences in the percentage of *cys2* variants or PoLV motifs in the DBLα sequence tag expressed by HbAA, HbAS, and HbAC children. We also were unable to detect any differences in length of expressed PfEMP1 variants by Northern blot for HbAA, HbAS, and HbAC children. Lastly, we examined IgG titers against 91 PfEMP1 domains in a cohort of 206 matched HbAA, HbAS, and HbAC children (3- to 11-year-olds) (**Chapter 5**). We observed a slightly diminished rate of PfEMP1-specific IgG acquisition in HbAS children compared to HbAA children. However, Hb type seems to play a relatively minor role in the acquisition of PfEMP1-specific IgG compared to the strong age-dependent effect seen in our study and in others [441, 442, 446, 447].

Using these approaches, we have not found robust evidence to support our originally hypothesized effects of HbAS on PfEMP1 expression. Our findings that parasites from HbAS children bind to MVECs with increased avidity, but that the repertoire of expressed PfEMP1s in HbAS children is not restricted seem, at first glance, difficult to reconcile. However, caution must be exercised in the interpretation of these results due to the limited number of cytoadherence comparisons we were able to perform, the non-physiological conditions of our cytoadherence assay, the limited methods we were able to use in comparing the expressed

PfEMP1 repertoires, and the presence of potential confounders such as co-inherited RBC polymorphisms, differences in age-associated acquired immunity, selection bias for parasites from infections with high parasitemias, and differences in disease severity. Furthermore, although parasites from HbAS children demonstrate an almost 50% increase in binding to MVECs, this effect does not reach significance, which suggests that other factors apart from PfEMP1-mediated mechanisms of protection may be working simultaneously *in vivo* to protect HbAS children against malaria. Additional experiments may be necessary to more fully understand the role of Hb type in shaping a parasite's expressed *var* gene repertoire and resulting binding phenotype. Experiments examining variant-specific PfEMP1 expression have been hindered by the inability to sequence full-length *var* genes due to their highly polymorphic nature and long length. However, a potentially informative experiment could take advantage of new high-throughput sequencing technologies and assembly methods to compare full-length PfEMP1 sequences expressed in HbAA and HbAS children. This approach would provide insight into not only the domain architecture and composition of expressed PfEMP1 variants, but would also reveal any mutations or sequence features differentially found in HbAS children, which would not be detected by DBLα sequence tag analysis.

Our finding that HbAS children exhibit a diminished rate of PfEMP1-specific IgG acquisition compared to HbAA children was also contrary to our original hypothesis. Since HbAS children experience fewer episodes of malaria as a result of their protection, they may lack a necessary 'second signal' for stimulation of IgG production against PfEMP1 variants. In such a scenario, HbAS children would acquire PfEMP1-specific IgG at a reduced rate compared to HbAA children, despite being exposed to a greater number of PfEMP1 variants as a result of increased antigenic switching in HbAS children. Additionally, HbAS children may acquire PfEMP1-specific IgG titers at an accelerated rate compared to HbAA children in an area of lower

transmission where Hb type and age show a significant interaction in their epidemiological protection. A significant limitation of our study was that the domains we used in our assay for detection of IgG reactivity in HbAA, HbAS, and HbAC children were mostly based on *var* gene sequences from 3D7 with small contributions from sequences based on *P. falciparum* HB3 and FCR3 lines. Although a certain level of cross-reactivity exists between the PfEMP1-specific IgGs in our Malian cohort and these heterologous parasite lines, testing the IgG reactivity of HbAA, HbAS, and HbAC children against PfEMP1 variants circulating in their geographic area would more closely mimic a child's IgG response against PfEMP1 variants that they may naturally encounter. The emerging ability to sequence and assemble full-length *var* gene variants as discussed in the previous paragraph might enable us to create a library of clinically-relevant PfEMP1 domains. We could then test a child's IgG reactivity against recombinant domains based on the sequences of PfEMP1 variants actually circulating within our field site.

In conclusion, our investigation into the effect of Hb type on PfEMP1 variant expression and IgG recognition has revealed some evidence that parasites in HbAS children demonstrate an increased binding phenotype to MVECs, and that HbAS children acquire PfEMP1-specific IgG at a diminished rate compared to HbAA children. However, the relationship between Hb type and these effects are complicated by the lack of detected *var* gene restriction by the approaches used in this thesis as well as the absence of statistical significance for the trend towards increased binding in parasites from HbAS children, and a relatively small magnitude of difference in the acquisition rates of PfEMP1-specific IgGs in HbAA and HbAS children. These inconclusive findings may be due to a number of confounding factors and limitations in both the number of comparisons we were able to perform and the techniques available to us. Furthermore, since the transmission intensity, acquired immunity, and genetic background of African children varies tremendously, it is unclear how widely applicable these findings may be.

In fact, some of the results presented in this thesis contradict previously reported findings from an area of Kenya with lower transmission intensity compared to our field site in Mali.

Despite these limitations, our results indicate that although PfEMP1-mediated mechanisms might partially explain the epidemiologically observed protection by HbAS against malaria in Malian children, it seems likely that other mechanisms are likely working in concert to protect these children. Future studies that further elucidate both the relationship between Hb type and expression of PfEMP1 variants, as well as the interaction between the various proposed mechanisms of malaria protection by HbS, are needed to translate nature's protective mechanism against malaria into potential therapeutic targets or vaccine candidates for genetically unprotected individuals.

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# 8 Supplemental tables and figures

**A.**

| Slide # | Year | ID | α-thal | G6PD | ABO | age | parasite density | severity | Well iRBC | Well EC | Well iRBC/EC | Total iRBC | Total EC | Total iRBC/EC | Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2008 | AS1 | WT | A+ | A+ | 6 | 90825 | NPO, RV | 745 | 600 | 1.24 | 1462 | 1216 | 1.20 | 1.41 |
| | | | | | | | | | 717 | 616 | 1.16 | | | | |
| | | AA1 | WT | A+ | B+ | 3 | 19200 | | 522 | 627 | 0.83 | 1054 | 1236 | 0.85 | |
| | | | | | | | | | 532 | 609 | 0.87 | | | | |
| 2 | 2008 | AS2 | WT | A+/- | B- | 3 | 9525 | | 396 | 309 | 1.28 | 758 | 627 | 1.21 | 1.76 |
| | | | | | | | | | 362 | 318 | 1.14 | | | | |
| | | AA2 | WT | A- | A+ | 2 | 16275 | | 185 | 317 | 0.58 | 427 | 620 | 0.69 | |
| | | | | | | | | | 242 | 303 | 0.80 | | | | |
| 3 | 2009 | AS3 | WT | A+ | B+ | 0.5 | 35100 | | 6344 | 350 | 18.13 | 12279 | 709 | 17.32 | 1.68 |
| | | | | | | | | | 5935 | 359 | 16.53 | | | | |
| | | AA3 | WT | A+ | A- | 4 | 76650 | | 3543 | 350 | 10.12 | 7353 | 712 | 10.33 | |
| | | | | | | | | | 3810 | 362 | 10.52 | | | | |
| 4 | 2009 | AS3 | WT | A+ | B+ | 0.5 | 35100 | | 3564 | 354 | 10.07 | 7714 | 704 | 10.96 | 6.73 |
| | | | | | | | | | 4150 | 350 | 11.86 | | | | |
| | | AA4 | WT | A+ | O+ | 4 | 26550 | | 691 | 350 | 1.97 | 1147 | 704 | 1.63 | |
| | | | | | | | | | 456 | 354 | 1.29 | | | | |
| 5 | 2009 | AS4 | WT | A+ | O+ | 5 | 22050 | | 847 | 353 | 2.40 | 1687 | 707 | 2.39 | 0.63 |
| | | | | | | | | | 840 | 354 | 2.37 | | | | |
| | | AA5 | WT | A+ | A+ | 16 | 9150 | | 1257 | 353 | 3.56 | 2672 | 705 | 3.79 | |
| | | | | | | | | | 1415 | 352 | 4.02 | | | | |
| 6 | 2009 | AS5 | HE | A+ | A+ | 8 | 12750 | | 737 | 351 | 2.10 | 1180 | 729 | 1.62 | 1.26 |
| | | | | | | | | | 443 | 378 | 1.17 | | | | |
| | | AA6 | WT | A+ | O+ | 6 | 36925 | | 627 | 357 | 1.76 | 930 | 725 | 1.28 | |
| | | | | | | | | | 303 | 368 | 0.82 | | | | |
| 6 | 2009 | AS6 | HE | A+ | AB+ | 12 | 18600 | | 2146 | 350 | 6.13 | 3773 | 703 | 5.37 | 4.18 |
| | | | | | | | | | 1627 | 353 | 4.61 | | | | |
| | | AA6 | WT | A+ | O+ | 6 | 36925 | | 627 | 357 | 1.76 | 930 | 725 | 1.28 | |
| | | | | | | | | | 303 | 368 | 0.82 | | | | |
| 6 | 2009 | AS7 | HE | A+ | A+ | 3 | 51375 | | 1627 | 351 | 4.64 | 2589 | 703 | 3.68 | 2.87 |
| | | | | | | | | | 962 | 352 | 2.73 | | | | |
| | | AA6 | WT | A+ | O+ | 6 | 36925 | | 627 | 357 | 1.76 | 930 | 725 | 1.28 | |
| | | | | | | | | | 303 | 368 | 0.82 | | | | |
| 7 | 2009 | AS8 | WT | A+ | A- | 6 | 49750 | | 2360 | 356 | 6.63 | 4392 | 708 | 6.20 | 1.18 |
| | | | | | | | | | 2032 | 352 | 5.77 | | | | |
| | | AA7 | WT | A+ | O+ | 7 | 51600 | | 1830 | 357 | 5.13 | 3864 | 733 | 5.27 | |
| | | | | | | | | | 2034 | 376 | 5.41 | | | | |
| 8 | 2009 | AS9 | WT | A+ | O+ | 6 | 31050 | | 460 | 362 | 1.27 | 696 | 727 | 0.96 | 0.33 |
| | | | | | | | | | 236 | 365 | 0.65 | | | | |
| | | AA8 | WT | A+ | O+ | 3 | 72600 | | 1309 | 366 | 3.58 | 2125 | 740 | 2.87 | |
| | | | | | | | | | 816 | 374 | 2.18 | | | | |
| 9 | 2009 | AS10 | WT | A+ | A+ | 8 | 10650 | | 1652 | 354 | 4.67 | 3003 | 711 | 4.22 | 3.78 |
| | | | | | | | | | 1351 | 357 | 3.78 | | | | |
| | | AA9 | WT | A+ | A+ | 4 | 25200 | | 415 | 354 | 1.17 | 795 | 711 | 1.12 | |
| | | | | | | | | | 380 | 357 | 1.06 | | | | |
| 10 | 2010 | AS11 | WT | A+ | O+ | 4 | 22200 | | 2250 | 405 | 5.56 | 4870 | 730 | 6.67 | 1.46 |
| | | | | | | | | | 2620 | 325 | 8.06 | | | | |
| | | AA10 | WT | A+ | A+ | 6 | 8750 | | 1944 | 425 | 4.57 | 3661 | 799 | 4.58 | |
| | | | | | | | | | 1717 | 374 | 4.59 | | | | |
| 11 | 2010 | AS12 | HE | A+ | B+ | 8 | 24625 | | 1800 | 428 | 4.21 | 3818 | 871 | 4.38 | 0.98 |
| | | | | | | | | | 2018 | 443 | 4.56 | | | | |
| | | AA11 | HE | A+ | O+ | 4 | 16025 | | 1507 | 308 | 4.89 | 3712 | 830 | 4.47 | |
| | | | | | | | | | 2205 | 522 | 4.22 | | | | |
| 12 | 2010 | AS13 | WT | A+ | A+ | 1.5 | 13750 | | 568 | 361 | 1.57 | 1759 | 712 | 2.47 | 0.66 |
| | | | | | | | | | 1191 | 351 | 3.39 | | | | |
| | | AA12 | WT | A+ | O+ | 1.5 | 9150 | | 1152 | 363 | 3.17 | 2880 | 747 | 3.86 | |
| | | | | | | | | | 1728 | 384 | 4.50 | | | | |
| 13 | 2010 | AS13 | WT | A+ | A+ | 1.5 | 13750 | | 1721 | 361 | 4.77 | 3180 | 727 | 4.37 | |
| | | | | | | | | | 1459 | 366 | 3.99 | | | | |
| | | AA12 | WT | A+ | O+ | 1.5 | 9150 | | 2135 | 365 | 5.85 | 4768 | 728 | 6.55 | |
| | | | | | | | | | 2633 | 363 | 7.25 | | | | |
| 14 | 2010 | AS14 | WT | A+/- | A+ | 3 | 13200 | | 3578 | 351 | 10.19 | 6033 | 727 | 8.30 | 2.43 |
| | | | | | | | | | 2455 | 376 | 6.53 | | | | |
| | | AA13 | WT | A+/- | O+ | 11 | 13500 | | 1368 | 356 | 3.84 | 2412 | 706 | 3.42 | |
| | | | | | | | | | 1044 | 350 | 2.98 | | | | |
| 15 | 2010 | AS15 | ND | A+ | O+ | 4 | 13400 | P | 1318 | 361 | 3.65 | 2929 | 714 | 4.10 | 1.64 |
| | | | | | | | | | 1611 | 353 | 4.56 | | | | |
| | | AA14 | WT | A+ | A+ | 7 | 15775 | | 866 | 396 | 2.19 | 1917 | 767 | 2.50 | |
| | | | | | | | | | 1051 | 371 | 2.83 | | | | |

**B.**

| Slide # | Year | ID | α-thal | G6PD | ABO | age | parasite density | severity | Well iRBC | Well EC | Well iRBC/EC | Total iRBC | Total EC | Total iRBC/EC | Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 2008 | AC1 | WT | A+ | O+ | 5 | 20550 | | 782 | 300 | 2.61 | 1581 | 600 | 2.64 | 2.20 |
| | | | | | | | | | 799 | 300 | 2.66 | | | | |
| | | AA15 | WT | A+ | A+ | 3 | 56850 | | 360 | 300 | 1.20 | 360 | 300 | 1.20 | |
| 17 | 2008 | AC2 | WT | A+ | A- | 5 | 8850 | | 383 | 300 | 1.28 | 712 | 608 | 1.17 | 1.75 |
| | | | | | | | | | 329 | 308 | 1.07 | | | | |
| | | AA16 | WT | A+ | O+ | 3 | 81600 | | 174 | 300 | 0.58 | 402 | 600 | 0.67 | |
| | | | | | | | | | 228 | 300 | 0.76 | | | | |
| 18 | 2008 | AC3 | HE | A+ | A+ | 2 | 46125 | | 307 | 300 | 1.02 | 307 | 300 | 1.02 | 1.72 |
| | | AA17 | WT | A+ | O+ | 6 | 12150 | | 179 | 300 | 0.60 | 179 | 300 | 0.60 | |
| 19 | 2008 | AC4 | HE | A+ | B+ | 3 | 1400 | | 844 | 300 | 2.81 | 844 | 300 | 2.81 | 2.36 |
| | | AA18 | WT | A+ | B+ | 7 | 14925 | | 364 | 300 | 1.21 | 715 | 600 | 1.19 | |
| | | | | | | | | | 351 | 300 | 1.17 | | | | |
| 20 | 2008 | AC5 | WT | A+ | B- | 11 | 2425 | | 244 | 302 | 0.81 | 244 | 302 | 0.81 | 1.58 |
| | | AA19 | WT | A+ | O+ | 12 | 9525 | | 153 | 300 | 0.51 | 153 | 300 | 0.51 | |
| 20 | 2008 | AC6 | WT | A+ | B+ | 11 | 16750 | | 509 | 325 | 1.57 | 922 | 625 | 1.48 | 2.89 |
| | | | | | | | | | 413 | 300 | 1.38 | | | | |
| | | AA19 | WT | A+ | O+ | 12 | 9525 | | 153 | 300 | 0.51 | 153 | 300 | 0.51 | |
| 20 | 2008 | AC7 | WT | A+ | A+ | 6 | 32025 | | 356 | 300 | 1.19 | 738 | 604 | 1.22 | 2.40 |
| | | | | | | | | | 382 | 304 | 1.26 | | | | |
| | | AA19 | WT | A+ | O+ | 12 | 9525 | | 153 | 300 | 0.51 | 153 | 300 | 0.51 | |
| 21 | 2008 | AC8 | HE | A+/- | O+ | 8 | 7650 | | 672 | 300 | 2.24 | 1089 | 600 | 1.82 | 1.04 |
| | | | | | | | | | 417 | 300 | 1.39 | | | | |
| | | AA20 | HE | A+ | O+ | 10 | 5700 | | 521 | 300 | 1.74 | 1048 | 600 | 1.75 | |
| | | | | | | | | | 527 | 300 | 1.76 | | | | |
| 22 | 2009 | AC9 | WT | A+/- | O+ | 6 | 64500 | NPO, RV | 2568 | 364 | 7.05 | 4452 | 735 | 6.06 | 0.82 |
| | | | | | | | | | 1884 | 371 | 5.08 | | | | |
| | | AA3 | WT | A+ | A- | 4 | 76650 | | 3313 | 350 | 9.47 | 5255 | 713 | 7.37 | |
| | | | | | | | | | 1942 | 363 | 5.35 | | | | |
| 4 | 2009 | AC9 | WT | A+/- | O+ | 6 | 64500 | NPO, RV | 2042 | 354 | 5.77 | 2042 | 354 | 5.77 | 3.54 |
| | | AA4 | WT | A+ | O+ | 4 | 26550 | | 691 | 350 | 1.97 | 1147 | 704 | 1.63 | |
| | | | | | | | | | 456 | 354 | 1.29 | | | | |
| 23 | 2009 | AC10 | HE | A+ | B+ | 4 | 59625 | | 4835 | 352 | 13.74 | 9197 | 706 | 13.03 | 5.00 |
| | | | | | | | | | 4362 | 354 | 12.32 | | | | |
| | | AA21 | WT | A- | B+ | 6 | 25650 | | 800 | 351 | 2.28 | 1825 | 701 | 2.60 | |
| | | | | | | | | | 1025 | 350 | 2.93 | | | | |
| 23 | 2009 | AC10 | HE | A+ | B+ | 4 | 59625 | | 4835 | 352 | 13.74 | 9197 | 706 | 13.03 | 3.37 |
| | | | | | | | | | 4362 | 354 | 12.32 | | | | |
| | | AA22 | WT | A- | B+ | 5 | 48825 | | 1308 | 350 | 3.74 | 2329 | 704 | 3.31 | |
| | | | | | | | | | 1021 | 354 | 2.88 | | | | |
| 24 | 2009 | AC10 | HE | A+ | B+ | 4 | 59625 | | 6353 | 353 | 18.00 | 12512 | 707 | 17.70 | |
| | | | | | | | | | 6159 | 354 | 17.40 | | | | |
| | | AA22 | WT | A- | B+ | 5 | 48825 | | 2323 | 376 | 6.18 | 4255 | 732 | 5.81 | |
| | | | | | | | | | 1932 | 356 | 5.43 | | | | |
| 25 | 2009 | AC11 | WT | A+ | B+ | 8 | 36075 | | 826 | 362 | 2.28 | 1603 | 720 | 2.23 | 0.46 |
| | | | | | | | | | 777 | 358 | 2.17 | | | | |
| | | AA23 | ND | A+ | A+ | 8 | 29700 | | 1940 | 362 | 5.36 | 3460 | 715 | 4.84 | |
| | | | | | | | | | 1520 | 353 | 4.31 | | | | |
| 26 | 2009 | AC12 | HE | A+ | O+ | 1.5 | 79500 | | 1025 | 350 | 2.93 | 2139 | 700 | 3.06 | 0.60 |
| | | | | | | | | | 1114 | 350 | 3.18 | | | | |
| | | AA24 | HE | A+ | A+ | 5 | 26550 | | 1789 | 350 | 5.11 | 1789 | 350 | 5.11 | |
| 27 | 2010 | AC12 | WT | A+ | A+ | 8 | 26500 | | 1292 | 370 | 3.49 | 1970 | 747 | 2.64 | 0.35 |
| | | | | | | | | | 678 | 377 | 1.80 | | | | |
| | | AA25 | WT | A+ | A+ | 6 | 16725 | | 2225 | 350 | 6.36 | 5328 | 707 | 7.54 | |
| | | | | | | | | | 3103 | 357 | 8.69 | | | | |

**Supplemental Table 8.1 HbAS and HbAC-derived *P. falciparum* -infected RBC to MVEC**

Parasites from HbAA and **A)** HbAS or **B)** HbAC children were inoculated into the same wildtype donor RBC then compared in parallel for their binding to MVECs. The slide that the comparison was performed on as well as the year, sample ID #, α-thalassemia genotype, G6PD deficiency genotype, ABO blood group, age, parasite density, severity criteria met (if severe) and all indicated for each sample. As indicated, most parasite comparisons were performed in duplicate wells, counting the number of parasites bound to at least 350 ECs. The ratio of iRBC to EC was then calculated for each well and then sample. The binding of parasites from HbAS or HbAC children were then normalized to the binding of parasites from HbAA children.

| ID # | Hb type | α-thal | G6PD | ABO | Age | Parasite/μL | severity | severity criteria |
|---|---|---|---|---|---|---|---|---|
| AA1 | AA | WT | A+ | O | 15 | 29100 | | |
| AA2 | AA | WT | A+ | A | 7 | 14325 | | |
| AA3 | AA | WT | A+ | O | 3 | 26550 | | |
| AC1 | AC | WT | A+/- | O | 5 | 64500 | S | RV, NPO |
| AA4 | AA | WT | A+/- | B | 2 | 176250 | | |
| AS1 | AS | ND | A+ | B | 0.5 | 35100 | | |
| AA5 | AA | WT | A+ | A | 3 | 76650 | | |
| AS2 | AS | WT | A+ | A | 3 | 74475 | | |
| AC2 | AC | HE | A+ | B | 3 | 59625 | | |
| AA6 | AA | WT | A+ | B | 4 | 47550 | | |
| AA7 | AA | WT | A+ | O | 3 | 86550 | | |
| AA8 | AA | WT | A- | B | 5 | 25680 | | |
| AA9 | AA | HE | A- | B | 2 | 47325 | | |
| AA10 | AA | WT | A+ | O | 5 | 16200 | | |
| AS3 | AS | ND | A+ | O | 2 | 7200 | | |
| AA11 | AA | WT | A+ | B | 2 | 68250 | | |
| AA12 | AA | WT | A+ | A | 8 | 14000 | | |
| AA13 | AA | WT | A+ | A | 1 | 51450 | | |
| AS4 | AS | WT | A+ | B | 10 | 32100 | | |
| AA14 | AA | WT | A+ | A | 3 | 51450 | | |
| CC1 | CC | WT | A+ | A | 4 | 6300 | | |
| AS5 | AS | WT | A+ | O | 4 | 75000 | | |
| AS6 | AS | WT | A+ | O | 4 | 22050 | | |
| AA15 | AA | HE | A+ | B | 9 | 23100 | | |
| AA16 | AA | WT | A+ | O | 6 | 51600 | | |
| AS7 | AS | WT | A- | A | 5 | 48750 | | |
| AS8 | AS | HE | A+ | AB | 11 | 18600 | | |
| AS9 | AS | HE | A+ | A | 2 | 51375 | | |
| AS10 | AS | HE | A+ | A | 7 | 12750 | | |
| AA17 | AA | WT | A+ | O | 5 | 36925 | | |
| AC3 | AC | WT | A+ | O | 1 | 98250 | | |
| AS11 | AS | WT | A+ | B | 2 | 273450 | | |
| AC4 | AC | WT | A+ | B | 7 | 36075 | | |
| AC5 | AC | WT | A+ | O | 5 | 13500 | | |
| AC6 | AC | WT | A+ | O | 2 | 71850 | | |
| AA18 | AA | WT | A+ | AB | 4 | 32625 | S | PR |
| AA19 | AA | WT | A+ | O | 3 | 29550 | | |
| AC7 | AC | HE | A+ | O | 0.5 | 79500 | | |
| AA20 | AA | HE | A+ | A | 4 | 26550 | | |
| AA21 | AA | WT | A+ | O | 2 | 72600 | | |
| AS12 | AS | WT | A+ | O | 5 | 31050 | | |
| AC8 | AC | WT | A+ | B | 2 | 54600 | | |
| SC1 | SC | WT | A+ | B | 7 | 4350 | S | RV, PR |
| AA22 | AA | WT | A+ | O | 15 | 18675 | S | RV |
| AA23 | AA | WT | A+ | AB | 10 | 21600 | | |
| AA24 | AA | WT | A+ | A | 3 | 25200 | | |
| AA25 | AA | WT | A+ | B | 7 | 27450 | S | PR |
| AS13 | AS | WT | A+ | A | 7 | 10650 | | |
| AA26 | AA | WT | A- | B | 5 | 17850 | S | RV |
| AC9 | AC | WT | A+ | O | 6 | 19800 | | |

**Supplemental table 8.2 RNA samples for sequencing**

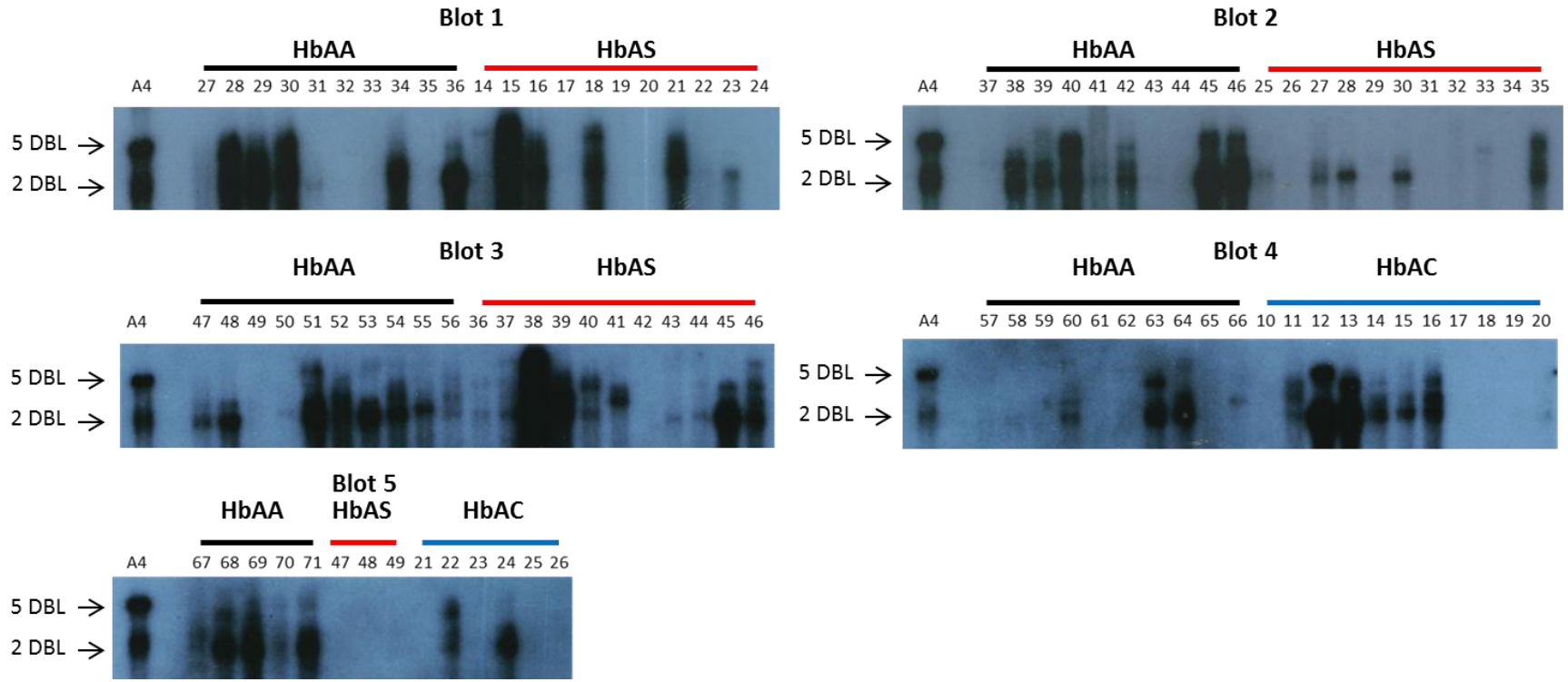
ID # indicates unique identifier for each sample. Although some identifiers are shared with samples from Chapter 3, these samples do not overlap with the samples used in those experiments. The Hb type, α-thalassemia genotype, G6PD deficiency genotype, ABO blood group, age at episode, parasite density (parasites/μL), severity (severe episodes indicated by "S"), and the criteria that severe patients met are indicated. Two samples were not successfully typed for α-thalassemia genotype and are indicated with 'ND' in that column. Samples that were characterized as 'severe' have the severe criteria that the child met indicated under 'severity criteria'). RV = repetitive vomitting, PR = prostration, NPO = unable to eat or drink.

| ID# | Blot # | Position | HbType | α-thal | G6PD | ABO | Age | Parasite/µL | Severity | Severe criteria |
|---|---|---|---|---|---|---|---|---|---|---|
| AA27 | 1 | 3 | AA | WT | A+ | A | 1 | 24550 | Mild | |
| AA28 | 1 | 4 | AA | WT | A+ | B | 3 | 28500 | Mild | |
| AA29 | 1 | 5 | AA | WT | A+ | O | 3 | 57925 | Severe | PR |
| AA30 | 1 | 6 | AA | WT | A+ | B | 4 | 38350 | Mild | |
| AA31 | 1 | 7 | AA | WT | A+ | B | 4 | 21700 | Mild | |
| AA32 | 1 | 8 | AA | WT | A+ | B | 4 | 16525 | Mild | |
| AA33 | 1 | 9 | AA | WT | A+ | A | 4 | 36225 | Mild | |
| AA34 | 1 | 10 | AA | WT | A+ | A | 5 | 15625 | Mild | |
| AA35 | 1 | 11 | AA | WT | A+ | A | 5 | 35800 | Mild | |
| AA36 | 1 | 12 | AA | WT | A+ | A | 5 | 31200 | Mild | |
| AS14 | 1 | 13 | AS | WT | A+ | O | 1 | 6375 | Mild | |
| AS15 | 1 | 14 | AS | WT | A+ | O | 1 | 85000 | Severe | RV |
| AS16 | 1 | 15 | AS | WT | A+ | B | 4 | 27375 | Mild | |
| AS17 | 1 | 16 | AS | WT | A+ | O | 4 | 22200 | Mild | |
| AS18 | 1 | 17 | AS | WT | A+ | B | 4 | 27725 | Mild | |
| AS19 | 1 | 18 | AS | WT | A+ | O | 6 | 26550 | Mild | |
| AS20 | 1 | 19 | AS | WT | A+ | O | 6 | 31650 | Mild | |
| AS21 | 1 | 20 | AS | WT | A+ | B | 6 | 23925 | Mild | |
| AS22 | 1 | 21 | AS | WT | A+ | O | 6 | 43250 | Mild | |
| AS23 | 1 | 22 | AS | WT | A+ | B | 6 | 76800 | Mild | |
| AS24 | 1 | 23 | AS | WT | A+ | A | 6 | 8925 | Mild | |
| AA37 | 2 | 3 | AA | WT | A+ | A | 5 | 5650 | Mild | |
| AA38 | 2 | 4 | AA | WT | A+ | O | 6 | 42900 | Mild | |
| AA39 | 2 | 5 | AA | WT | A+ | AB | 6 | 40900 | Severe | RV |
| AA40 | 2 | 6 | AA | WT | A+ | A | 6 | 22000 | Mild | |
| AA41 | 2 | 7 | AA | WT | A+ | AB | 11 | 13225 | Mild | |
| AA42 | 2 | 8 | AA | WT | A+ | AB | 5 | 32850 | Mild | |
| AA43 | 2 | 9 | AA | WT | A+ | O | 4 | 15600 | Mild | |
| AA44 | 2 | 10 | AA | WT | A+ | O | 12 | 14225 | Mild | |
| AA45 | 2 | 11 | AA | WT | A+ | O | 5 | 18250 | Mild | |
| AA46 | 2 | 12 | AA | WT | A+ | B | 4 | 28250 | Mild | |
| AS25 | 2 | 13 | AS | WT | A+ | O | 7 | 14125 | Mild | |
| AS26 | 2 | 14 | AS | WT | A+ | A | 8 | 5000 | Mild | |
| AS27 | 2 | 15 | AS | WT | A+ | AB | 8 | 32700 | Mild | |
| AS28 | 2 | 16 | AS | WT | A+ | A | 8 | 23675 | Mild | |
| AS29 | 2 | 17 | AS | WT | A+ | O | 11 | 12825 | Mild | |
| AS30 | 2 | 18 | AS | WT | A+ | A | 17 | 8800 | Mild | |
| AS31 | 2 | 19 | AS | WT | A+ | B | 11 | 17050 | Mild | |
| AS32 | 2 | 20 | AS | WT | A+ | A | 9 | 27750 | Mild | |
| AS33 | 2 | 21 | AS | WT | A+ | A | 9 | 7950 | Mild | |
| AS34 | 2 | 22 | AS | WT | A+ | A | 10 | 100 | Mild | |
| AS35 | 2 | 23 | AS | WT | A+/- | A | 3 | 13200 | Mild | |
| AA47 | 3 | 3 | AA | WT | A+/- | O | 6 | 18575 | Mild | |
| AA48 | 3 | 4 | AA | HE | A+ | A | 6 | 24050 | Mild | |
| AA49 | 3 | 5 | AA | HE | A+ | B | 4 | 5000 | Mild | |
| AA50 | 3 | 6 | AA | HE | A+ | O | 6 | 32400 | Mild | |
| AA51 | 3 | 7 | AA | HE | A+ | O | 6 | 12300 | Mild | |
| AA52 | 3 | 8 | AA | HE | A+ | O | 4 | 13550 | Mild | |
| AA53 | 3 | 9 | AA | HE | A+ | O | 3 | 11050 | Mild | |
| AA54 | 3 | 10 | AA | ND | A+ | A | 9 | 12500 | Mild | |
| AA55 | 3 | 11 | AA | WT | A+ | O | 3 | 13250 | Mild | |
| AA56 | 3 | 12 | AA | WT | A+ | A | 3 | 15100 | Mild | |
| AS36 | 3 | 13 | AS | HE | A+ | A | 6 | 15325 | Mild | |
| AS37 | 3 | 14 | AS | HE | A+ | B | 8 | 24350 | Mild | |
| AS38 | 3 | 15 | AS | HE | A+ | B | 1 | 56000 | Mild | |
| AS39 | 3 | 16 | AS | HE | A+ | B | 8 | 24625 | Mild | |
| AS40 | 3 | 17 | AS | HE | A+ | A | 2 | 9500 | Mild | |
| AS41 | 3 | 18 | AS | HE | A+ | A | 4 | 10150 | Mild | |
| AS42 | 3 | 19 | AS | HE | A+ | B | 8 | 12375 | Mild | |
| AS43 | 3 | 20 | AS | HE | A+ | O | 13 | 8625 | Mild | |
| AS44 | 3 | 21 | AS | ND | A+ | O | 4 | 30975 | Mild | |
| AS45 | 3 | 22 | AS | HO | A+ | O | 14 | 6750 | Mild | |
| AS46 | 3 | 23 | AS | ND | A+ | O | 4 | 13400 | Severe | PR |
| AA57 | 4 | 3 | AA | WT | A+/- | AB | 10 | 7725 | Mild | |
| AA58 | 4 | 4 | AA | HE | A+ | B | 5 | 14025 | Mild | |
| AA59 | 4 | 5 | AA | WT | A+/- | O | 6 | 32800 | Mild | |
| AA60 | 4 | 6 | AA | WT | ND | B | 1 | 12200 | Mild | |
| AA61 | 4 | 7 | AA | HE | A+ | A | 5 | 7650 | Mild | |
| AA62 | 4 | 8 | AA | WT | A+ | O | 5 | 58600 | Mild | |
| AA63 | 4 | 9 | AA | WT | A+ | A | 4 | 131050 | Mild | |
| AA64 | 4 | 10 | AA | WT | A+ | B | 3 | 32975 | Mild | |
| AA65 | 4 | 11 | AA | WT | A+ | A | 3 | 36050 | Severe | PR |
| AA66 | 4 | 12 | AA | WT | A+ | O | 3 | 11075 | Mild | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| AC10 | 4 | 13 | AC | WT | A+ | B | 9 | 38000 | Mild | |
| AC11 | 4 | 14 | AC | WT | A+ | A | 7 | 14025 | Mild | |
| AC12 | 4 | 15 | AC | WT | A+ | B | 4 | 22800 | Mild | |
| AC13 | 4 | 16 | AC | WT | A+ | AB | 1 | 91675 | Mild | |
| AC14 | 4 | 17 | AC | WT | A+ | O | 6 | 19725 | Mild | |
| AC15 | 4 | 18 | AC | WT | A+ | B | 9 | 31150 | Mild | |
| AC16 | 4 | 19 | AC | WT | A+ | A | 4 | 68800 | Mild | |
| AC17 | 4 | 20 | AC | WT | A+ | B | 8 | 14250 | Mild | |
| AC18 | 4 | 21 | AC | WT | A+ | B | 15 | 20375 | Mild | |
| AC19 | 4 | 22 | AC | WT | A+ | O | 3 | 10825 | Mild | |
| AC20 | 4 | 23 | AC | HE | A+ | O | 7 | 13200 | Mild | |
| AA67 | 5 | 3 | AA | HE | A+ | O | 4 | 37000 | Mild | |
| AA68 | 5 | 4 | AA | HE | A+ | O | 3 | 13500 | Mild | |
| AA69 | 5 | 5 | AA | HE | A+ | B | 2 | 55400 | Mild | |
| AA70 | 5 | 6 | AA | WT | A- | O | 4 | 33350 | Mild | |
| AA71 | 5 | 7 | AA | HE | A+ | B | 2 | 42300 | Mild | |
| AS47 | 5 | 8 | AS | WT | A+/- | AB | 8 | 6975 | Mild | |
| AS48 | 5 | 9 | AS | WT | ND | A | 1 | 11800 | Mild | |
| AS49 | 5 | 10 | AS | WT | A- | A | 14 | 4350 | Mild | |
| AC21 | 5 | 11 | AC | HO | A+/- | A | 6 | 7000 | Mild | |
| AC22 | 5 | 12 | AC | HE | A+ | O | 2 | 14900 | Mild | |
| AC23 | 5 | 13 | AC | WT | A- | A | 6 | 3700 | Mild | |
| AC24 | 5 | 14 | AC | HO | A+/- | A | 6 | 24375 | Mild | |
| AC25 | 5 | 15 | AC | HE | A+ | O | 1 | 17125 | Mild | |
| AC26 | 5 | 16 | AC | HO | A+/- | A | 6 | 26975 | Mild | |

**Supplemental Table 8.3 RNA samples for Northern blot**

ID # indicates unique identifier for each sample. Although some identifiers are shared with samples from Chapter 3, these samples do not overlap with the samples used in those experiments. The blot and lane on which we ran our sample is indicated under 'blot #' and 'position.' The Hb type, α-thalassemia genotype, G6PD deficiency genotype, ABO blood group, age at episode, parasite density (parasites/µL), severity (severe episodes indicated by "S"), and the criteria that severe patients met are indicated. Three samples were not successfully typed for α-thalassemia genotype and two samples were not successfully types for G6PD deficiency genotype. These are indicated with 'ND' in their respective column. Samples that were characterized as 'severe' have the severe criteria that the child met indicated under 'severity criteria'). RV = repetitive vomitting, PR = prostration.




**Supplemental Figure 8.1 Northern blots for HbAA, HbAS, and HbAC samples**