THE ROLE OF PFEMP1 EXPRESSION AND IMMUNITY IN UGANDAN CHILDREN WITH SEVERE MALARIA

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Elizabeth M. Fernander

THE ROLE OF PFEMP1 EXPRESSION AND IMMUNITY IN UGANDAN CHILDREN WITH SEVERE MALARIA

Severe malaria, primarily caused by *Plasmodium falciparum* infection, is among the leading causes of childhood mortality globally. A key virulence factor and source of antigenic variation and immune evasion during infection is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Encoded for by approximately 60 *var* genes, this complex protein mediates cytoadherence of infected erythrocytes to the host endothelium and is a prominent immune target for the anti-malarial immune response in children. During severe malaria, specific domains of PfEMP1 that bind to endothelial protein C receptor (EPCR) and intercellular adhesion molecule-1 (ICAM-1) on host endothelial cells, are more prevalently expressed. The interaction of these proteins and infected erythrocytes mediates the sequestration of infected erythrocytes and plays a role in severe malaria pathogenesis. Antibodies to these domains develop over time with exposure to the parasite and are thought to contribute to immunity against severe malaria in children.

In this study, whole blood samples from children with different forms of severe malaria, enrolled in two observational prospective cohort studies were used to quantify the expression of PfEMP1 domains using RT-qPCR and to measure the antibody response to PfEMP1 domains via a bead-based multiplex immunoassay. Using these samples, we demonstrated that although the expression of *var* transcripts encoding PfEMP1 domains was generally similar across children with different forms of severe malaria, the expression of variants encoding specific EPCR-binding domains was associated with thrombocytopenia and severe anemia. The antibody response to PfEMP1 domains in children with severe malaria was highest in children with SMA and

children with asymptomatic parasitemia, but not associated with decreased risk of additional malaria episodes. Overall, the results of this study suggest that PfEMP1 is acting similarly across different forms of severe malaria but that it can be related to pathogenesis and severe malaria immunity.

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AKI: acute kidney injury

AMA-1: apical membrane antigen

Angpt-1: angiopoietin-1

Angpt-2: angiopoietin-2

AP: asymptomatic parasitemia

APC: activated protein C

ATS: intracellular acidic terminal segment

BCS: Blantyre coma score

CC: community control children

CI: confidence interval

CIDR: cysteine-rich interdomain region

CM: cerebral malaria

CNS: central nervous system

CSF: cerebrospinal fluid

CSP: circumsporozoite protein

DBL: Duffy binding like

DC: domain cassette

EBA: erythrocyte binding protein

ECM: experimental cerebral malaria

EPCR: endothelial protein C receptor

FDR: false discovery rate

GLURP: glutamate rich protein

ICAM-1: intercellular adhesion molecule-1

IL-33: interleukin-33

IQR: interquartile range

IRR: incidence rate ratio

IV: intravenous

M/S: malaria with repeated seizures

MSP: merozoite surface protein

NCI: neurocognitive impairments

NDI: neurodevelopmental impairments

NP: no parasitemia at enrollment

OR: odds ratio

PAR1: protease activated receptor 1

PDGF: platelet derived growth factor

PECAM-1: platelet endothelial call adhesion molecule-1

PfEMP1: *P. falciparum* erythrocyte membrane protein 1

*Pf*HRP-2*: P. falciparum* histidine-rich protein

Pro: prostration

PV: parasitophorous vacuole

Ratio Angpt 1: Angpt 2: ratio of angiopoietin-1 to angiopoietin-2

RBC: red blood cell

RDS: respiratory distress

RDT: rapid diagnostic test

RIFIN: repetitive interspersed family

SERA: serine repeat antigen

SM: severe malaria

SMA: severe malarial anemia

ß: beta coefficient

STEVOR: subtelomeric open reading frame

TM: transmembrane domain

Tu: transcriptional units

UM: uncomplicated malaria

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

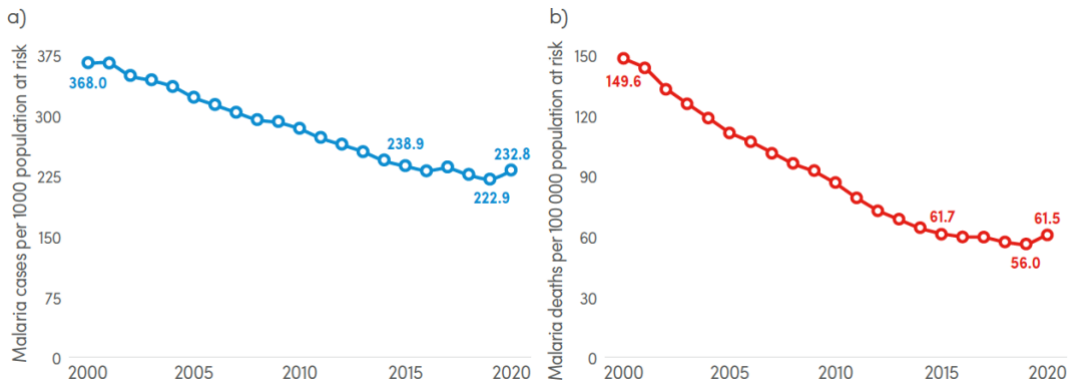
vWF: von Willebrand factor

Chapter 1: Introduction

1.1 Malaria epidemiology and life cycle

Malaria is among the leading causes of disease and death globally, with nearly half of the global population at risk of infection. In 2020, there were an estimated 241,000,000 cases of malaria globally[6], up 19 million cases from 2019 numbers. In 2020, malaria caused 627,000 deaths globally, approximately 96% of which occurred in Africa[6]. In Africa, 80% of malaria deaths are among children under 5 years, who are at the greatest risk of severe disease[6], representing a significant cause of mortality in this population. Although efforts to decrease the burden of malaria in the last 20 years have been successful, using prevention techniques such as bed nets and indoor residual spraying (Figure 1.1[6]), the recent plateau and increases in cases and deaths show that continued research to expand our knowledge of the disease is needed before elimination can be achieved.

Figure 1.1: Trends in malaria case incidence and mortality rate in the WHO Africa region.

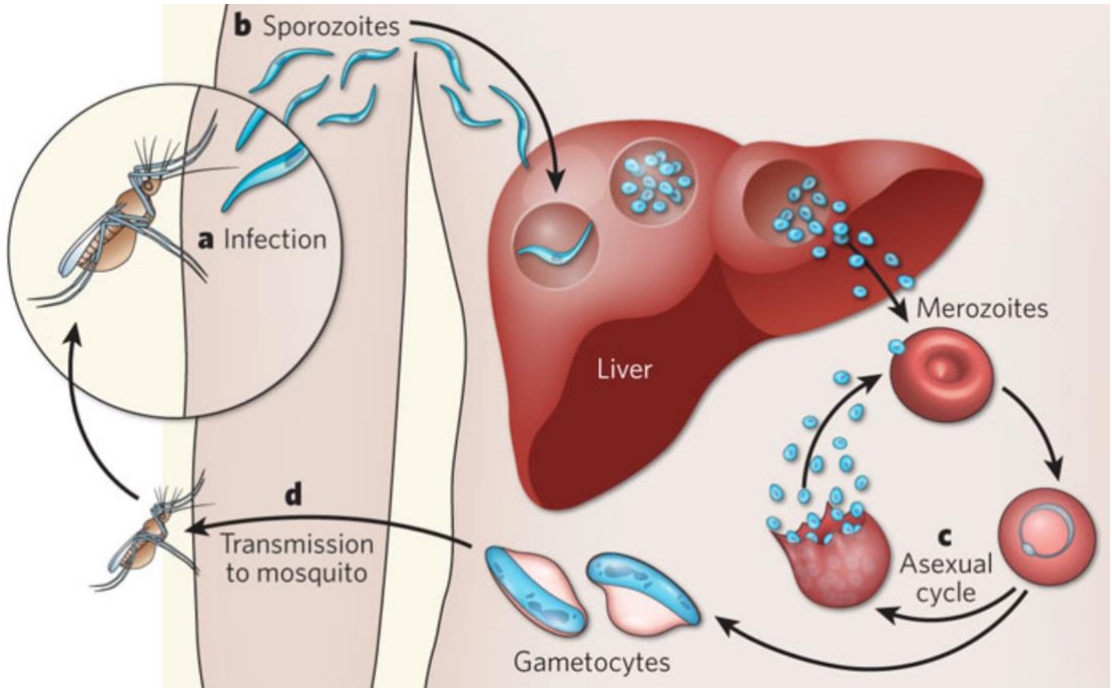


Source: WHO Estimates, World Malaria Report 2021

(A) Malaria case incidence in cases per 1000 population at risk and (B) mortality in deaths per 100,000 population at risk in African countries in 2020.

Malaria is caused by protozoa parasites of the *Plasmodium* family. There are five *Plasmodium* species that can infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi.* Most severe malaria cases are caused by *P. falciparum*, which is also the most common *Plasmodium* species in Africa. The life cycle of *P. falciparum* is complex and involves two hosts: humans and female *Anopheles* mosquitoes. When an infected mosquito bites the host (Figure 1.2a), sporozoites travel through the bloodstream to the liver (Figure 1.2b). There, the sporozoites infect hepatocytes and replicate, eventually releasing thousands of merozoites into the blood. The release of merozoites into the blood is the beginning of the asexual or erythrocytic cycle (Figure 1.2c). During the asexual stage, merozoites rapidly invade erythrocytes and undergo cell division, forming ring stage parasites, then trophozoites, and finally schizonts. After 48 hours, for *P. falciparum*, the schizonts rupture, releasing merozoites into the bloodstream, continuing the asexual cycle. The cyclic rupture of erythrocytes and release of merozoites is related to the cyclic fever associated with uncomplicated malaria and many symptoms associated with malarial disease. Eventually, a portion of parasites in the asexual cycle will differentiate into the sexual form of the parasite, male and female gametocytes. Once matured, these gametocytes travel to the peripheral circulation and can be ingested by a female *Anopheles* mosquito to complete the human stage of the *Plasmodium* life cycle (Figure 1.2d). In the mosquito, the parasite travels through the midgut to the salivary glands where it can be transmitted to the next human host during the mosquito's next blood meal.

Figure 1.2 *P. falciparum* life cycle.



Source: Michalakis, 2009[1]

1.2 Spectrum of malaria symptoms: asymptomatic parasitemia to severe malaria

Plasmodium falciparum infections can range from asymptomatic to uncomplicated illness to severe disease and death. Most *P. falciparum* infections result in uncomplicated malaria (UM), characterized by nonspecific symptoms, such as fever, headache, nausea, chills, and muscle pains. With proper treatment, these symptoms generally resolve within a few days. Clinical immunity to *P. falciparum* develops over time with exposure to the parasite[7] and with repeated exposure, *P. falciparum* infections can also be asymptomatic, resulting in asymptomatic parasitemia (AP). Quantifying the burden of AP in a population is difficult, and the prevalence rates depend on the molecular or clinical tests used to detect parasitemia. However, understanding the burden and biological relevance of AP in a population is important as we take steps towards elimination of malaria and reducing transmission in a population.

At the opposite end of the disease spectrum from AP is severe malaria, which is commonly caused by *P. falciparum* compared to the other *Plasmodium* species. Severe malaria can occur following an episode of uncomplicated malaria that worsened or independent of UM. It is a rare but deadly manifestation of malaria. The symptoms of severe malaria vary based on transmission setting and age. Most malaria deaths occur in sub-Saharan Africa, where transmission intensity can be high. In this region severe malaria is primarily a pediatric disease. In this population, the primary manifestations of severe malaria include cerebral malaria (CM), severe malarial anemia (SMA) and respiratory distress syndrome (RDS)[8,9]. According to the WHO guidelines of severe malaria, CM describes patients who are unarousable, generally with a Blantyre coma score (BCS) of less than 3 out of 5 in children, and no other cause for coma[10]. The BCS is a pediatric measure of impaired consciousness, with fully conscious child receiving a score of 5. This score consists of three categories: eye, verbal and motor responses, scored from 0-2, with a score of 0 given when the patient has an inappropriate or lack of response within each category. SMA is defined as a hemoglobin of less than 5 g/dL in children with a positive blood smear for *Plasmodium* species[10]. RDS is characterized by deep, labored and rapid breathing with increased chest excursion. In children, RDS presents as sustained nasal flaring and recession of bony structures of the lower chest wall on inspiration[10]. There are additional manifestations and symptoms of SM, including but not limited to acidosis, hypoglycemia, acute kidney injury, jaundice, multiple seizures, and hyperparasitemia[10].

With proper treatment and access to medical care, the mortality rates of severe malaria vary with manifestation, as CM and RDS are far more fatal than SMA[9]. CM is considered the most severe form of severe malaria with mortality rate around 15-20%, depending on study parameters. Children who recover from CM are at risk of developing acute and long-term neurodevelopmental (NDI)[11,12] and neurocognitive impairments

(NCI)[13-16], with up to 25% of children showing signs of neurological deficits up to 24 months post-infection[16]. Recently, NCI have been related to SMA[17], which lacks the obvious central nervous system (CNS) involvement seen in CM. The significant long-tern burden of severe malaria warrants further study into the mechanisms leading to neurocognitive impairment and long-term sequela.

1.3 Severe malaria pathogenesis

Severe malaria pathogenesis is multi-faceted and involves numerous processes, but is driven by rapid parasite multiplication, resulting in infected erythrocyte sequestration, endothelial cell activation and dysfunction, and excessive inflammation. Together, these processes contribute to tissue hypoxia and ischemia throughout the host, resulting in a variety of symptoms and multi-organ involvement.

A key component of pathogenesis of *P. falciparum* infection is the sequestration of infected erythrocytes in microvascular beds. Sequestration helps the parasite to avoid splenic clearance and contributes to impairment of blood flow in the microvasculature, which can result in metabolic acidosis and end organ damage, particularly in the brain[18]. Infected erythrocytes can also bind uninfected erythrocytes, forming rosettes[19], or activated platelets, forming clumps[20]; both processes contribute to microvascular obstruction and severe malaria pathogenesis.

During severe malaria pathogenesis, excessive host immune response exacerbated by the parasite in the bloodstream, perpetuates pathogenesis. Pro-inflammatory cytokines TNF-α[21,22] and IFN-gamma[23,24] are elevated during *P. falciparum* infection. The production of TNF-a, IL-6 and IL-10 are elevated in children with severe malaria compared to children with uncomplicated malaria[24], with concentrations of IL-6 and IL-10 in children with CM compared to other types of severe malaria,[24] demonstrating the correlation of cytokine levels with disease severity. Elevated pro-

inflammatory cytokines during disease enhance the inflammatory response, resulting in increased leukocyte adhesion, and release of cytotoxic mediators, which causes the endothelial damage and increased permeability.

Endothelial activation and dysfunction are key components that contribute to the pathogenesis of severe malaria, whereby parasite interactions with the endothelium and excessive inflammatory responses result in endothelial barrier damage. As a result of inflammation-induced endothelial activation, the cell adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin are upregulated on the surface of host endothelial cells, mediating the cytoadherence of infected erythrocytes to the microvasculature. Soluble versions of these cell adhesion molecules are shed by the activated endothelium as sICAM-1, sVCAM-1, and sE-selectin. Severe malaria is associated with elevated surface-bound and soluble forms of these cell adhesion molecules[25]. When activated, the endothelium also releases storage granules, Weibel-Palade bodies, containing von Willebrand factor (vWF), P-Selectin, and angiopoietin-2. Previous studies have shown elevated vWF in severe malaria[26-28]. Angiopoietin-1 and angiopoietin-2 work in opposition with their shared endothelial receptor, Tie-2, to maintain endothelial homeostasis. Angiopoietin-2 released during endothelial activation promotes endothelial permeability and inflammation. During severe malaria, angiopoietin-1 decreases and there is evidence of an increase in angiopoietin-2 and the angiopoietin-2: angiopoietin-1 ratio[29-32]. In short, the activation of endothelial cells increases the endothelial receptors required for the binding of infected erythrocyte and leukocytes, which further promotes sequestration of parasites in the microvasculature thereby exacerbating inflammation.

There are several clinical risk factors that can be assessed during an episode of severe malaria that reflect aspects of severe malaria pathogenesis, including thrombocytopenia and severe anemia. Thrombocytopenia, or platelets counts <150,000

platelets, is common severe malaria and has been associated with disease severity[33]. Activated platelets form clumps in the microvasculature during cerebral malaria[34], forming obstructions and exacerbating pathogenesis. Platelets can also directly inhibit parasite growth[35,36] as a protective mechanism during infection. Severe anemia, commonly defined as hemoglobin levels <5 g/dL, is thought to be caused by a combination of the increased destruction of erythrocytes and decreased erythropoiesis[37]. These represent only two of numerous clinical markers associated with severe disease or worse clinical outcomes in children with severe malaria.

Overall, severe malaria pathogenesis is a complex process that impacts numerous host pathways throughout the body, including the host immune and inflammatory response, alterations in coagulation, and the hemolysis pathway. The key to pathogenesis is thought to be an imbalance in parasite growth and immune reaction, where both are exaggerated to produce worse symptoms and outcomes. Here, we focus on the impact of *var* gene usage among parasite-infected erythrocytes in the bloodstream on the host inflammatory response and endothelial activation.

1.4 Antibody response to malaria

The risk of severe malaria and death declines rapidly with exposure and indirectly, age, in high transmission settings, followed by a decreased risk of mild or uncomplicated malaria into adulthood and the persistence of asymptomatic infections. The relationship between decreased disease severity and age contributes to the development of naturally acquired immunity. People living in malaria-endemic regions develop immunity to malaria over time with exposure to the parasite[38]. One of the key mechanisms of this immunity are antibodies, demonstrated in a seminal study in which passive transfer of immunoglobulins from malaria immune adults rapidly controlled and reduced parasitemia in children[39,40].

Antibodies against numerous stages of the parasite life cycle have been studied with the goal of preventing or controlling different aspects of infection. For example, antibodies to the sporozoite in the pre-erythrocytic stage that aim to block the initial infection of the parasite[41,42], against merozoites in the asexual stage to target invasion of the parasite[43,44], antibodies targeting the surface of the infected erythrocyte to block cytoadherence[45], and against sexual stage gametocytes in an attempt to block transmission[46,47], have all been studied to better out understanding of malarial immunity. Antibodies against blood-stage antigens are well characterized and highly immunogenic and a target for vaccines as this stage is responsible for most of the symptoms of clinical disease. Antibodies to these antigens have been associated with protection from clinical malaria. Numerous merozoite antigens have been associated with protection from episodes of clinical malaria, including merozoite surface protein (MSP), erythrocyte binding antigens (EBA) and apical membrane antigen-1 (AMA-1)[48,49], particularly in children. Studies that quantify IgG subclass-specific responses in malaria have demonstrated that protection associated with merozoite antigens is related to IgG3 antibodies, suggesting the importance of functional antibodies in malaria immunity[50,51]. IgG3 antibodies against these antigens are related to opsonic phagocytosis of merozoites or complement fixation[52,53], demonstrating an example of the importance of anti-malarial antibodies.

Beyond antibodies to merozoite antigens, the pre-erythrocytic antigen circumsporozoite protein (CSP) is the primary antigen in the most successful malaria vaccine to date. Targeting pre-erythrocytic antigens is a goal in vaccine development to prevent the initial infection of the parasite and thus the symptoms and transmission of *Plasmodium.* The RTS,S vaccine is the first malaria vaccine to be tested in phase 3 clinical trials[54] and in 2021 was recommended by WHO for use in children over 5 months of age in a four-dose schedule[55]. Despite this advancement in malaria immunity and

disease prevention, further work will be needed to develop more effective malaria vaccines with shorter immunization schedules in order to substantially improve childhood health outcomes.

1.5 PfEMP1 and var *genes*

Antigenic variation is a strategy employed by many pathogens, in which surface antigens are varied and highly diverse, contributing to immune evasion. Families of variant surface antigens are found across *Plasmodium* species. As *P. falciparum* is the primary cause of severe malaria in sub-Saharan Africa, the families of variant surface antigens within *P. falciparum* are highlighted here. During the asexual stage, *P. falciparum* achieves antigenic variation through the expression of variant surface antigens on the surface of infected erythrocytes, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1)[56-58], repetitive interspersed family (RIFIN)[59,60], and subtelomeric open reading frame (STEVOR) proteins[61]. Of the three, PfEMP1 is the most well characterized and an important target of the immune response to the asexual blood stage[62].

Antigenic variation from surface antigens, such as PfEMP1, is driven in part by immune pressure, demonstrated in spenectomized model systems, where surface antigens do not switch surface expression patterns without splenic pressure [63,64]. The variation within PfEMP1 specifically is attributed in part to the approximately 60 *var* genes located throughout the parasite genome, across different chromosomes that encode for the protein[56,65]. *Var* genes are organized into groups A, B and C based on upstream promotors and chromosome location[66].

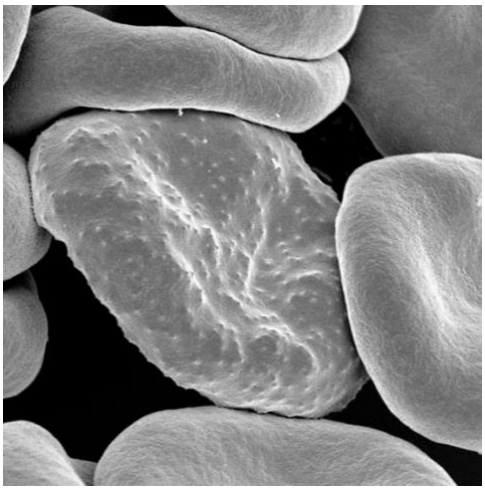
Despite the numerous copies of the gene within the genome, *var* genes are expressed in a mutually exclusive manner, with only one unique PfEMP1 molecule expressed on the surface of an infected erythrocyte[67]. The expression of a single *var*

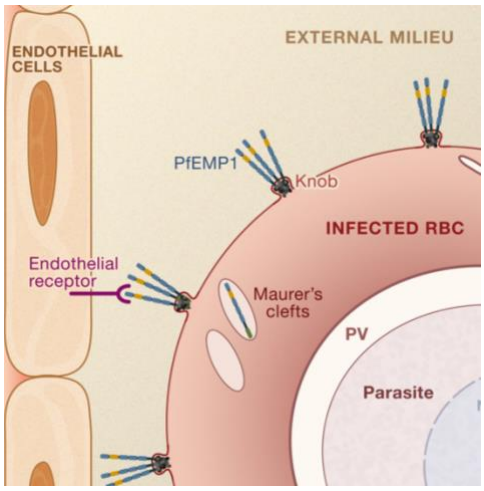
gene involves histone modifications via *P. falciparum* histone methyl transferase and histone deacetylase enzymes[68-70] (reviewed in[71]). This process occurs within an infected erythrocyte to alter PfEMP1 expression for one parasite. However, during malaria infection, there are multiple parasite strains within a single patient. Therefore, even if one specific PfEMP1 molecule is expressed on the surface of an infected erythrocyte, a single infection could contain numerous different PfEMP1 molecules expressed at any time. Thus, the population of parasites could be under selection pressure from antibodies to certain PfEMP1 domains, allowing the whole population to alter *var* gene expression, contributing to antigenic variation.

PfEMP1 is anchored to the infected erythrocyte membrane via the intracellular acidic terminal sequence (ATS) to forms a part of the knob structure, a group of parasite proteins found on the intracellular side of the infected red blood cell that are necessary for PfEMP1 expression[72] (Figure 1.3).

Figure 1.3 PfEMP1 is expressed on the surface of infected erythrocytes.

A. **B.**



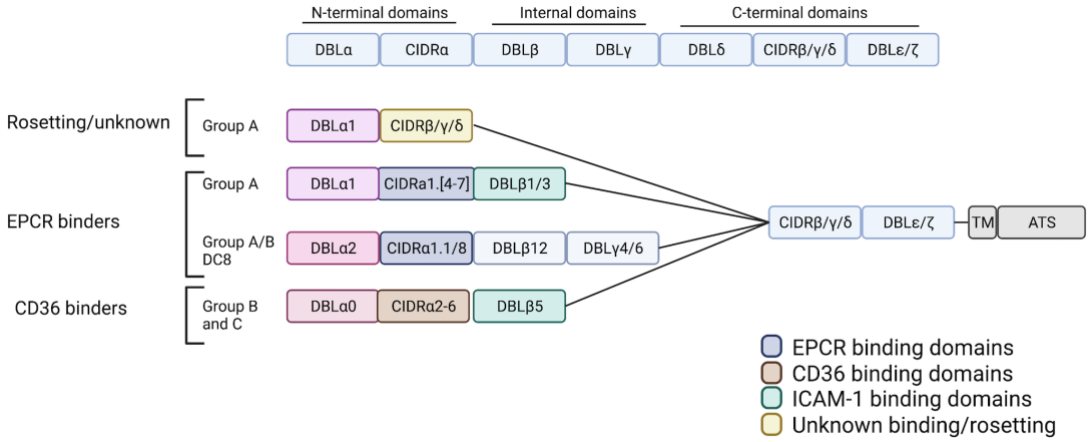


(A) Electron micrograph of an erythrocyte infected with *P. falciparum.* In this micrograph the knobs on the surface of the infected erythrocyte can be seen (center). The infected erythrocyte is surrounded by uninfected erythrocytes, lacking the knob structures. Credit: Rick Fairhurst and Jordan Zuspann, National Institute of Allergy and Infectious Diseases, National Institutes of Health. (B) Schematic of PfEMP1 proteins expressed on the surface of an infected erythrocyte, anchored at the surface via the know structure. Source: Bonnefoy, 2008[4]. PV: parasitophorous vacuole; RBC: red blood cell.

The extracellular portion of PfEMP1 is composed of 2-10 cysteine-rich interdomain region (CIDR) and Duffy-binding like (DBL) domains. These CIDR and DBL domains can be divided into three classes (α, β, γ), and seven classes (α, β, γ, δ, ε, ξ, x), respectively[73]. Nearly all PfEMP1 molecules have an N-terminal head structure composed of a DBLα domain followed by a CIDR domain(Figure 1.4)[65]. The CIDR domain in this N-terminal head structure is generally invoved in the binding of PfEMP1 to the host endothelium. Group A *var* genes encode PfEMP1 proteins with CIDRα1 domains that bind to endothelial protein C receptor (EPCR)[74,75] or a more diverse CIDRβ/γ/δ with unknown binding functions but that have been associated with rosetting[76,77]. Group B and C genes encode proteins that bind CD36 via CIDRα2-6 domains[78,79]. However, domain cassette 8 (DC8), a conserved tandem sequence of domains, represents an exception, and is encoded by group A/B *var* genes. Group A/B

var genes are a hybrid group that shares chromosome location with other group B genes but encode group A-like proteins that bind EPCR, demonstrating that *var* group does not always match binding patterns.

Figure 1.4 Schematic PfEMP1 domain organization.

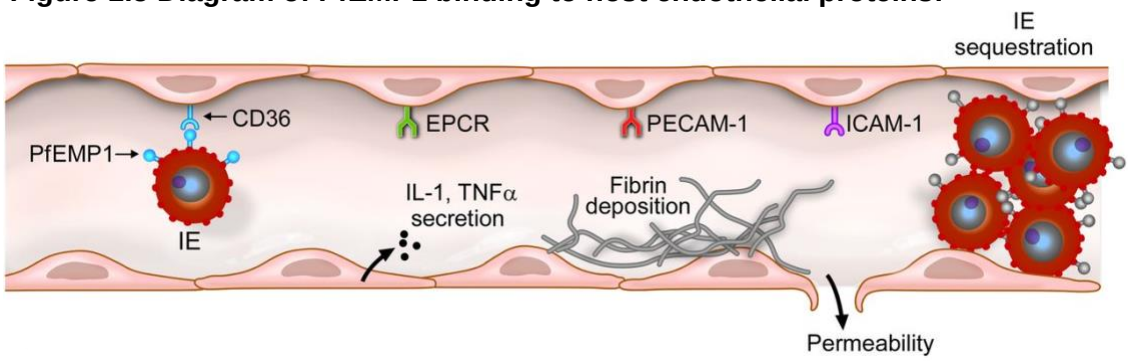


Schematic of PfEMP1 domain structure depicting the possible domain organization (top) and specific domain combinations that define binding phenotypes and their corresponding *var* gene group designations (below). The N-terminal domains include a DBLα domain followed by a CIDR domain with specific host cell binding phenotypes: CIDRα1 for EPCR (Groups A and B, DC8, blue), CD36 (Groups B and C, brown), or unknown targets (Group A, yellow). ICAM-1-binding domains (DBLβ1, 3, or 5, green) can be found in all three *var* genes groups and are located within the internal domains of PfEMP1. A PfEMP1 molecule can have two to six subsequent C-terminal domains that are generally unrelated to prior N-terminal domains and have unknown binding partners. Created with biorender.com. Adapted from Storm, et al. *EMBO Mol Med*, 2019[3].

Moving toward the surface of the infected erythrocyte are internal domains, which can contain DBLβ domains that can bind to intercellular adhesion molecule-1 (ICAM-1) and are found in all three *var* gene groups[80-82]. The structural organization of PfEMP1 molecules is more diverse and less ordered closer to the C-terminal end of the protein, as PfEMP1 molecules can have between 5-8 domains following the N-terminal head. Thus, these domains are less frequently studied and not as well characterized.

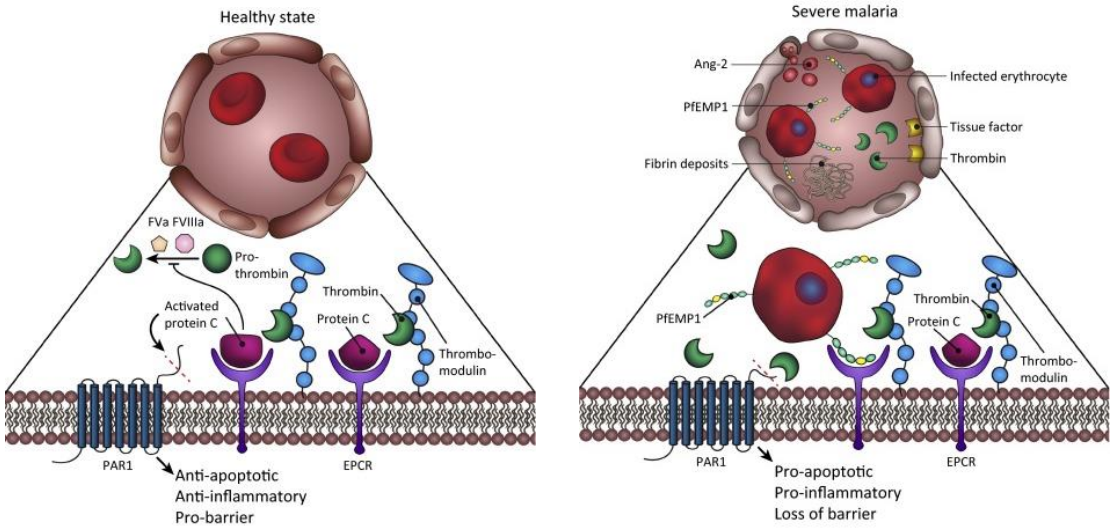
PfEMP1 can interact with a range of vascular surface molecules (Figure 1.5), helping infected erythrocytes sequester in the microvasculature and avoid splenic clearance. Infected erythrocytes adhere to the endothelium during the late trophozoite stage, when PfEMP1 expression on the surface is at its highest. Cytoadherence of infected erythrocytes to the endothelium is mediated by PfEMP1, although other proteins are found on the surface of the infected erythrocyte, including additional variant surface antigens, RIFINs and STEVORS. The binding capabilities of these antigens are less well characterized and believed to bind to host proteins on the surface of erythrocytes and leukocytes[60,83,84]. Different binding patterns of PfEMP1 have been related to disease manifestations in malaria. The binding of CD36 to CIDRα2-6 domains is related to episodes of uncomplicated malaria[85]. Meanwhile, the expression of group A *var* genes, particularly those that bind to EPCR, are elevated in severe disease[75,86]. The binding of PfEMP1 to EPCR is thought to block activated protein C from binding EPCR and inhibiting its cytoprotective signalling[87-89] (Figure 1.6). This loss in signaling could result in pro-inflammatory and pro-apoptotic pathways as well as alterations in the coagulation pathway, resulting in increased barrier permeability and perpetuating severe malaria pathogenesis. The role of PfEMP1 binding ICAM-1 in severe malaria pathogenesis is more complex, with studies showing both an association to severe malaria, particularly CM[90], and other studies demonstrating no relation to CM[91,92]. A recent study demonstrated that PfEMP1 molecules with both EPCR and ICAM-1-binding domains were elevated in children with CM, and *var* transcript levels of these dual binders could differentiate children with CM from SMA[93]. It could be that ICAM-1 is involved in both uncomplicated and severe malaria pathogenesis, depending on its presence in a variant with an EPCR or CD36 binding domain, highlighting the importance of dual-binding PfEMP1 molecules.

Figure 1.5 Diagram of PfEMP1 binding to host endothelial proteins.



PfEMP1 is expressed on the surface of infected erythrocytes (IE) and binds to various host receptors on the endothelium, including CD36, EPCR, PECAM-1 and ICAM-1. Binding of infected erythrocytes to the host endothelium could be exacerbating endothelial activation, resulting in the release of pro-inflammatory cytokines, such as IL-1 and TNF-α, fibrin deposition and alternations in endothelium permeability. PECAM-1: platelet endothelial cell adhesion molecule-1
Source: Aird, et al. *Blood*. 2014[5].

Figure 1.6 Schematic of the role of EPCR in severe malaria.



(A) In the healthy microvasculature, protein C binds to EPCR, where it is activated to form APC. APC is cytoprotective in endothelial cells, cleaving protease activated receptor 1 (PAR1) to induce signaling pathways that promote anti-apoptotic and anti-inflammatory responses and maintenance of the barrier integrity. APC also has anti-coagulation activity by inactivating factor V and VIII, which promote thrombin formation. (B) During severe malaria, PfEMP1 binds to EPCR, blocking APC binding to promote pro-apoptotic, pro-inflammatory signaling and loss of endothelial barrier integrity. Ang-2: angiopoietin-2
Source: Bernabeu, et al. *Trends in Parasitology,* 2017[2].

Another important role of PfEMP1 is its involvement in rosetting, the binding of infected erythrocytes to uninfected erythrocytes. The formation of rosettes has been associated with severe disease[19] and could contribute to the occlusion of the microvasculature. The formation of rosettes requires the interaction between PfEMP1 domains and receptors on uninfected erythrocytes, complement receptor 1, heparan-sulphate-like molecules and A or B blood group antigens[61,76,94,95]. The domains of PfEMP1 that could be involved in rosetting are predominantly group A, which do not bind to EPCR, possibly the CIDRβ/ɣ/δ domains[76,96,97]. Rosetting is a complicated process and could involve additional parasite proteins on the surface of the infected erythrocyte or additional domains of PfEMP1 not yet characterized.

Antibodies to PfEMP1 domains are part of natural immunity and are acquired over time with exposure to the parasite. The development of PfEMP1 immunity occurs in an ordered manner, with antibodies to Group A/EPCR-binding domains developing prior to other PfEMP1 domains and some other blood stage antigens[98,99]. This is in line with the idea that immunity to more virulent variants is acquired first, encouraging the parasite to express more diverse and less virulent variants of PfEMP1, contributing to why severe malaria is seen predominantly in children in Africa as they develop immunity over time. Severe malaria immunity had been related to a subset of conserved PfEMP1 antigens, while protection from clinical or uncomplicated malaria was related to a broad repertoire of antibodies against diverse antigens[100]. Antibodies to specific domains of PfEMP1 have been associated with clinical malaria protection, particularly domains from group A v*ar* genes, CIDRα1.1[101] CIDRβ2[102], and CIDRɣ3[99]. Additional evidence shows the importance of a breadth of response to PfEMP1 antigens[100,103] in protection from malaria, unsurprising based on the significant diversity and variation within PfEMP1.

1.6 Study goals

PfEMP1 is a complex and diverse protein, with numerous domains, binding partners, and functions within severe malaria. Although, much is known about how PfEMP1 is involved in severe malaria pathogenesis and immunity, I wanted to further understand how different variants of this proteins are associated with different phenotypes of severe malaria, including manifestations less commonly studied. We utilized blood samples from children with different forms of severe malaria to assess the expression of PfEMP1 domains and their relationship to clinical outcomes of severe malaria, and the IgG antibody response to PfEMP1 and other *P. falciparum* antigens in children with severe malaria. We hypothesized that PfEMP1 transcripts would correlate with disease severity and differ between children with different manifestations of severe malaria and that antibodies to PfEMP1 would be elevated in children with severe malaria associated higher mortality and be related to protection from additional episodes of severe malaria. To test this hypothesis, we utilized samples from two prospective observational cohort studies enrolling Ugandan children with severe malaria, outlined in Chapter 2. The goals of this dissertation are to improve our understanding of PfEMP1 transcript expression in different manifestations of severe malaria and the relationship to clinical outcomes, and to determine if antibodies to PfEMP1 are related to protection from severe malaria.

Chapter 2: Study populations

2.1 Study population 1 (CM-SMA Study)

Between 2008 and 2013, children with CM (n=269), children with SMA (n=233), and asymptomatic community children (CC, n=216) were enrolled in a prospective observational cohort study in Kampala, Uganda. The study was conducted at Mulago Hospital, the national referral hospital of Uganda and the teaching hospital of Makerere University Medical School, serving as the district hospital for Kampala and the surrounding areas. Children with CM and SMA meeting the inclusion criteria (see below), were recruited, and enrolled from the emergency pediatric unit of the hospital, once stabilized, with written informed consent from parents and guardians of the study participants. Children between 18 months and 12 years of age were included in the study, with more than 75% of children in each group under the age of 5 years, as this population is the most at risk for severe malaria.

2.1.1 Inclusion/exclusion criteria

Cerebral Malaria (CM) was defined as follows:

Inclusion criteria – enrolled if met all criteria

1. Coma (Blantyre coma score$\leq$2, Glasgow coma score $\leq$8)
2. *P. falciparum* on blood smear
3. No response to a glucose bolus 1 hour after administration if hypoglycemic
4. Unarousable coma at least 1 hour after termination of seizure or after administration of first line anticonvulsants

Exclusion criteria

1. Previous history of head trauma
2. Previous history of coma
3. Previous history of hospitalization for malnutrition

4. Previous diagnosis of cerebral palsy

Post lumbar puncture exclusion criteria

1. White blood cell count >10 cells
2. Gram stain or culture positive for infection

Note: A diagnosis of CM required a lumbar puncture to exclude other causes of coma, unless the physician on duty deemed it unsafe to perform a lumbar puncture, in which case the lumbar puncture could be postponed until the patient is clinically stable.

Severe Malarial Anemia (SMA) was defined as follows:

1. *P. falciparum* on peripheral thin blood smear
2. Blood hemoglobin ≤5 g/dL measured by Hemo Control point-of-care hemoglobin analyzer

Exclusion criteria

1. Impaired consciousness to any degree on physical exam, (e.g., Glasgow coma score <15 or Blantyre coma score <5)
2. Seizure activity prior to admission
3. Any other clinical evidence of CNS disease

Note: Children with CM may also have concurrent SMA; the presence of SMA did not exclude them from the study.

Asymptomatic community children (CC)

Inclusion criteria: children from the neighborhood, extended household, or nearby neighborhood of a child with CM, and in the same age group as a child with CM. Only children living within a 50 km radius from Mulago Hospital were recruited. Children were matched by age group, not to an individual child.

Exclusion criteria:

1. Any active illness or illness within the past 4 weeks requiring medical care
2. Chronic illness requiring medical care

3. Major medical abnormalities on screening history or physical exam
4. Known developmental delay
5. Prior history of coma

2.1.2 Clinical treatment

Children with CM and SMA were treated according to the national treatment guidelines in Uganda. At study initiation in 2008, treatment included intravenous quinine until the patient was alert, then oral quinine for hospitalized patients. Artemether combination drugs, usually artemether-lumefantrine, were used for outpatient malaria visits. Initial labs and testing were completed by study physicians and nurses, but clinical care of all patients was given by house staff and attending physicians of Mulago Hospital following the Mulago Hospital guidelines for the treatment of severe malaria, in line with WHO guidelines.

2.1.3 Sample collection and storage

Up to 5.5 mL of blood was collected by venipuncture from all children at enrollment. Drops of the blood were utilized immediately for hemoglobin, glucose, and lactate tests at bedside by point-of-care, handheld devices, as well as for thick and thin blood smears for parasite microscopy quantification and speciation. Blood was placed in separate tubes and transported to the appropriate labs for processing, storage, and testing. Whole blood was also collected by venipuncture into EDTA tubes. Two 0.4 mL aliquots of this EDTA whole blood were added to PAXgene Blood RNA reagent (PreAnalytiX, Hombrechtikon, Switzerland) to stabilize RNA for future testing. The remainder of the blood was separated into plasma and packed blood cell components by centrifugation, with the latter stored for future DNA extraction. Blood and plasma samples were frozen and stored at -80 C. In children with CM, where a lumbar puncture was indicated, CSF (cerebrospinal fluid) was collected and stored at -80 C for future testing.

2.1.4 Laboratory testing

All study participants underwent the following clinical tests: microscopy for *P. falciparum* identification and parasite density, a CBC with differential and platelet count and blood levels of hemoglobin, glucose, and lactate. For microscopy identification of *P. falciparum*, thick and thin blood smears were prepared with Giemsa stain according to standard protocols and read by two experiences technicians. Parasite density was estimated independently by both readers, and if the two readings were >20% different, a third reader counted independently. To account for sequestered parasites in the microvasculature, *P. falciparum* histidine rich protein-2 (PfHRP2) levels were quantified in plasma from enrollment samples, using the Malaria Ag CELISA kit (Cellabs, Brookvale, Australia). Stool was collected at enrollment and examined for the presence of erythrocytes, trophozoites and cysts of protozoa, and helminth ova and larva. Blood cultures were also collected and examined for the growth of bacteria.

At discharge for children that survive CM, a neurological exam was conducted to look for neurologic deficits. At discharge for children with CM or SMA, or at enrollment for CC, age-appropriate cognitive tests were administered to measure cognitive abilities; see Appendix 1 methods for more details. Additional laboratory testing was performed related to Chapters 3, 4 and Appendix 1, see Methods section for each chapter for additional information.

2.1.5 Study follow-up

Study participants were followed for 24 months after enrollment. During scheduled follow-up visits at 6, 12, and 24 months, blood samples were collected and stored or processed into plasma. Additional neurological and cognitive tests were also performed at each follow-up visit. During the 24 months of follow-up, hospital and clinic visits of study participants were monitored and recorded. Children with clinical features of malaria were tested by blood smear or point-of-care rapid diagnostic testing (RDT) for

malaria, and all uncomplicated malaria visits and readmissions for severe malaria were recorded.

2.2 Study population 2 (Five forms of severe malaria (5 SM) study)

Study 2 was designed as a follow-up study to the children from study population 1, examining the immediate pathogenesis and long-term neurodevelopmental impacts of severe malaria. Study 2 enrolled children between 2014 and 2018 with severe malaria as well as asymptomatic community children. Designed as a follow-up for study 1, aspects of the study conduct were carried onto the study 2, including sample collection methods, use of the Mulago Hospital, and some personnel and staff members. The differences between study populations are outlined here. First, study 2 aimed to provide an inclusive perspective of severe malaria by expanding the definitions of severe malaria to five different manifestations: cerebral malaria (CM, n=86), respiratory distress (RDS, n=121), malaria with repeated seizures (M/S, n=162), severe malarial anemia (SMA, n=155), and prostration (Pro, n=76); see below for inclusion and exclusion criteria. Community control children (CC, n=120) were enrolled from the same neighborhoods as children with severe malaria, as in study 1. Unlike the previous study, in which children from 18 months to 12 years were enrolled, this study enrolled children from 6 months to 4 years, as children under 5 years are most at risk of severe malaria. Another key difference in these studies is the use of a positive rapid diagnostic test (RDT) or positive *P. falciparum* blood smear as an inclusion criterion for children with severe malaria. This definition was in line with the WHO definition of severe malaria and permitted faster diagnosis of malaria; but could allow for possible false positives. All children with presumptive *P. falciparum* by RDT had subsequent diagnostic confirmation by examination of blood smears collected at the time of enrollment.

Another difference between the studies is the addition of a second study site at Jinja Regional Referral District Hospital in Jinja, Uganda, which is a major referral hospital located 82 km east of Mulago. This second, more rural site allows for a larger population for recruitment and comparison of severe malaria across two different geographical settings. The follow-up for the second study was shorter than study 1, with follow-up clinic visits occurring at 1-month and 12-months post-discharge. During the 12 months surveillance period, study participants also had additional phone and home follow-up visits. Sample collection at 12 months was limited in study 2, with 12-month blood samples collected in 178 study participants: 147 children who recovered from severe malaria, and 31 CCs. Between the start of study 1 and study 2, the national recommendations for treatment of severe malaria in Uganda changes to the use of intravenous (IV) artesunate followed by artemether-lumefantrine when patient can take oral medication, compared to the use of IV quinine in study 1. Overall, study 2 enrolled patients with an expanded definition of severe malaria, including manifestations of the disease commonly overlooked, allowing us to understand differences in the pathogenesis and immune response to severe malaria, beyond CM and SMA.

2.1.1 Inclusion/exclusion criteria

The hierarchy of group definition: Cerebral Malaria>Respiratory Distress>Malaria with Complicated Seizures>Severe Malarial Anemia>Prostration.

Exclusion criteria for all categories of severe malaria and CC

1. Previous history of head trauma or coma
2. Previous history of hospitalization for malnutrition
3. Cerebral palsy or other severe neurologic disease prior to enrollment
4. Known chronic illness requiring medical care
5. Major medical abnormalities on screening history of past health
6. Known developmental delay prior to episode of CM

Cerebral Malaria (CM)

Inclusion criteria

1. *P. falciparum* on blood smear or positive RDT for malaria
2. Coma (BCS ≤2)
3. Unarousable coma (BCS ≤2) at least 1 hour after: 1) termination of seizure activity; 2) administration of first line anticonvulsants if child has had seizures; or 3) administration of a glucose bolus if child is hypoglycemic

Post lumbar puncture exclusion criteria

1. >5 white blood cells/µL of CSF
2. Positive CSF Gram stain
3. Positive CSF culture for bacteria that cause meningitis in children

Note: A diagnosis of CM required a lumbar puncture to exclude other causes of coma, unless the physician on duty deemed it unsafe to perform a lumbar puncture, in which case the lumbar puncture could be postponed until the patient is clinically stable.

Respiratory Distress (RDS)

Inclusion criteria

1. *P. falciparum* on blood smear or positive RDT for malaria
2. Deep acidotic breathing or lower chest wall retractions
3. No crepitations on pulmonary examination

Exclusion criteria

1. Meeting inclusion criteria for CM (see hierarchy of group definitions)

Malaria with Repeated Seizures (M/S)

Inclusion criteria

1. *P. falciparum* on blood smear or positive RDT for malaria
2. Two or more generalized seizures in 24 hours, or seizure >30 minutes in duration

Exclusion criteria

1. Meeting inclusion criteria for CM or RD (see hierarchy of group definitions)

Severe Malarial Anemia (SMA)

Inclusion criteria

1. *P falciparum* on blood smear or positive RDT for malaria
2. Blood hemoglobin ≤5 g/dL measured by Hemo Control point-of-care hemoglobin analyzer

Exclusion criteria

1. Meeting inclusion criteria for CM, RD, or M/S

Prostration (Pro)

Inclusion criteria

1. *P. falciparum* on blood smear or positive RDT for malaria
2. In children ≥1 year-old, lost ability to sit unsupported or stand; in children <1 year-old, lost ability to drink or breastfeed

Exclusion criteria

1. Meeting inclusion criteria for CM, RD, M/S, or SMA

Community control children (CC)

Inclusion criteria

1. Residency in same or nearby neighborhood as a child with severe malaria
2. Same age group as a child with severe malaria

Exclusion criteria

1. All exclusion criteria of severe malaria noted above
2. Active illness or illness within the past 4 weeks requiring medical care
3. Major medical abnormalities on physical exam
4. Measured axillary temperature ≥37.5C

2.3 Study populations in dissertation

In this dissertation, samples from children enrolled in both study populations were studied to answer different questions pertaining to the role of PfEMP1 and severe malaria pathogenesis and immunity. Below are listed the chapter titles along with sample type and study population, and overview of experiments to address the goal of each chapter.

Chapter 3: Var *transcripts encoding PfEMP1 ICAM-1-binding and C-terminal domains are elevated in children with cerebral malaria compared to severe malarial anemia and related to thrombocytopenia (CM/SMA study)*

RNA was isolated from whole blood collected at enrollment from children in study population 1, with CM and SMA, to quantify the expression of *var* transcripts of PfEMP1 domains that bind ICAM-1 and additional C-terminal domains.

Chapter 4: Var *transcripts of EPCR-binding domains of PfEMP1 are expressed in different manifestations of severe malaria and associated with anemia and thrombocytopenia (5 SM study)*

RNA was extracted from whole blood collected at enrollment in children with severe malaria from study population 2, children with CM, RDS, M/S, SMA and Pro, to examine if the expression of *var* transcripts of PfEMP1 domains that bind EPCR, ICAM-1 and additional C terminal domains is related to these manifestations of severe malaria and markers of disease outcome.

Chapter 5: Antibodies to PfEMP1 and other P. falciparum *antigens are maintained in children with asymptomatic parasitemia but not in children with severe malaria (CM-SMA study)*

The antibody response to PfEMP1 and other *P. falciparum* antigens was measured in the plasma from children enrolled in study 1, with CM, SMA, and from asymptomatic CCs. Plasma from enrollment, 6- and 12-month follow-up was studied to understand the persistence of antibodies after a severe malaria episode and possible associations with risk of additional malaria episode over 12 months.

Chapter 6: The presence of antibodies to PfEMP1 CIDR domains is highest in children with severe malarial anemia compared to other forms of severe malaria (5 SM study)

Antibodies to PfEMP1 domains that bind EPCR and CD36 were measured in the serum of children enrolled in study population 2, children with CM, RDS, M/S, SMA and Pro, to understand how antibodies to PfEMP1 may differ in children with different forms of severe malaria and relate to protection from clinical malaria.

Appendix 1: Elevated plasma ST2 levels are associated with neuronal injury and neurocognitive impairment in children with cerebral malaria (CM-SMA study)

Appendix 1 is included as it includes samples from study population 1 that were studied to elucidate the role a host immune pathway, IL-33/ST2 in severe malaria pathogenesis. Although PfEMP1 is a key virulence factor during severe malaria, Appendix 1 addressed another aspect of pathogenesis and the long-term impacts of severe malaria. In this chapter, plasma from children in study 1, children with CM and SMA, and CSF from children with CM, was utilized to measure levels of sST2, a biomarker of disease severity in other infectious and inflammatory diseases. Levels of sST2 were related to endothelial activation markers, markers of neuronal injury and cognitive outcomes to understand how the sST2/IL-33 pathway could be playing a role in severe malaria in children.

Chapter 3: *Var* transcripts encoding PfEMP1 ICAM-1-binding and C-terminal domains are elevated in children with cerebral malaria compared to severe malarial anemia and related to thrombocytopenia

3.1 Introduction

Severe malaria is among the leading causes of morbidity and mortality in children in sub-Saharan Africa, resulting in 602,000 deaths in Africa in 2020, approximately 75% of which occurred in children[6]. Severe malaria is commonly caused by *Plasmodium falciparum* and can present in a few different forms, including cerebral malaria (CM), defined as Blantyre coma score (BCS) <3 and no other cause for coma, and severe malarial anemia (SMA), characterized by hemoglobin levels <5 g/dL.

The pathogenesis of severe malaria is complex and multifaceted, involving host–parasite interactions on the endothelium, excessive inflammation, and obstructions within the microvasculature[90,104-108]. Endothelial activation is a critical aspect of severe malaria pathogenesis, resulting in disruption of the endothelial barrier[28]. Endothelial activation can be induced by the binding of infected erythrocytes to the surface of the endothelium, or indirectly, by excessive inflammatory factors. Infected erythrocytes bind to the host endothelium in a process known as sequestration, to avoid splenic clearance. Additionally, erythrocyte rosetting is a key component of pathogenesis[109,110], in which infected erythrocytes bind to uninfected erythrocytes, resulting in clumps of erythrocytes that can obstruct the microvasculature and increase invasion of erythrocytes[111]. These processes involve interactions between host receptors and parasite proteins expressed on the surface of infected red blood cells.

One of the most well studied parasite proteins involved in these processes is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed on the surface of infected erythrocytes during the late trophozoite stage of the parasite life

cycle. PfEMP1 is encoded by the *var* gene family, which is made up of approximately 60 diverse genes within the *P. falciparum* genome and acts as a major source of antigenic variation for the parasite[56,57]. Each PfEMP1 molecule is composed of combinations of two to ten Duffy binding-like (DBL) domains and cysteine-rich interdomain region (CIDR) domains that are anchored to the surface of the infected erythrocyte as a part of the knob structure[72]. DBL domains can be divided into 7 classes (α, β, γ, δ, ε, ξ, x) and CIDR domains can be divided into three classes (α, β, γ), respectively, each with further subdivisions[73]. Additionally, *var* genes are characterized into groups A, B, and C based on chromosome location and direction, and upstream sequences[73]. The diversity within PfEMP1 comes from sequence variability and diversity in domains within PfEMP1 and plays a role in host-immune evasion and parasite survival.

Despite the diversity of PfEMP1 domain structures, its role in cytoadhesion of infected erythrocytes of the host endothelium is conserved. The different groups of *var* genes have different characteristics and host binding partners. Group A *var* genes encode PfEMP1 proteins with CIDRα1 domains, which bind to endothelial protein C receptor (EPCR)[74,75], or a more diverse CIDRβ/γ/δ domain with unknown binding functions that is potentially involved in rosetting[76,77]. Group B and C genes encode PfEMP1 proteins that bind host receptor CD36 via CIRDa2-6 domains[78,79]. Located closer to the surface of the infected erythrocyte are DBLβ domains that can bind to intercellular adhesion molecule-1 (ICAM-1) on the surface of the host endothelium[80-82] and are found in both group A, DBLβ3 and group B *var* genes, DBLβ5. Following DBLβ domains are various C-terminal CIDR or DBL domains that have unknown functions. These domains are not as well understood due to difficulty in predicting sequences farther from the beginning of the gene and high recombination events. However, these domains have been identified in some domain cassettes, tandem groups of domains commonly found together, in both group A and B *var* genes[73].

Elevated expression of the PfEMP1 CIDRα1 domain, which binds EPCR, has consistently been seen in children with cerebral malaria[112]. Binding of infected erythrocytes to the endothelium is thought to reduce the production and cytoprotective effects of activated protein C due to functional blocking of EPCR by PfEMP1[87-89]. This could impact sequestration of infected erythrocytes, coagulation defects, endothelial activation and permeability which could alter the outcomes of severe malaria. The expression of EPCR-binding domains in children with CM and SMA enrolled in the same cohort utilized in this study, were measured previously[113]. In that study, EPCR-binding domains were elevated in children with CM compared to SMA, higher in children with retinopathy positive CM versus retinopathy negative CM and elevated in children that survived the episode of CM compared to those that died[113]. Although, *var* transcripts in the previous study were not related to markers of endothelial activation or sequestration, we showed that EPCR-binding domain transcripts are related to disease severity and potentially play a role in both CM and SMA pathogenesis[113].

However, EPCR-binding domains represent a small subset of the domains within PfEMP1, about less than 20% of all *var* genes[114]. The expression of ICAM-1-binding domains of PfEMP1 has been related to CM pathogenesis[85,90,115], suggesting that the binding of infected erythrocytes to ICAM-1 in the brain is palying a role of sequestration of parasites in the brain and could be impacting endothelial activation in the brain. Children with CM had elevated percentage of *var* transcripts with dual group A EPCR and ICAM-1-binding capabilities compared to children with SMA and uncomplicated malaria[93], highlighting the importance of PfEMP1 proteins that contain both EPCR and ICAM-1-binding domains with respect to cerebral malaria pathogenesis.

In this study, we used samples from a cohort of Ugandan children with CM or SMA that were used previously to measure the expression of *var* transcripts encoding domains previously shown to be EPCR-binding[113] or ICAM-1-binding, as well as

additional C-terminal domains to expand our understanding of PfEMP1 in severe malaria. Primers designed based on 226 *var* gene sequences were used to amplify var regions encoding five DBLβ domains, four DBLε domains, and seven DBLζ domains that are predicted to bind ICAM-1. These primers have been used previously to compare expression of *var* transcripts in children with cerebral malaria and uncomplicated malaria[3] and hospitalized children with and without severe malaria symptoms[116]. To expand our knowledge of how these PfEMP1 domains are impacting pathogenesis, we assessed associations between transcript levels and markers of endothelial activation disease severity, such as mortality, malaria retinopathy, seizures, anemia, thrombocytopenia and parasite burden. In our previous study, transcript levels of EPCR-binding domains of PfEMP1 were different between children with severe malaria that died and survived and elevated in those with malaria retinopathy[113]. Additionally, transcript levels of EPCR-binding domains have been associated with low platelet counts, or the presence of thrombocytopenia in children and adults with CM[117,118]. Together these data suggest, that not only are transcript levels of EPCR-binding domains elevated in children with severe malaria, but they are related to clinical markers of disease severity. In this study, we included primer sets to additional DBL domains of PfEMP1 to expand our knowledge on how these domains could be playing a role in the pathogenesis of severe disease through endothelial activation or clinical outcomes.

3.2 Methods

3.2.1 Study population

This prospective study was performed at Mulago Hospital, Kampala, Uganda, from 2008 to 2015, as previously described[17]. Children with cerebral malaria (CM) and severe malarial anemia (SMA) were enrolled if they were between 18 months and 12 years of age. CM was defined as (1) coma (Blantyre Coma Score ≤2); (2) *P. falciparum*

on blood smear; and (3) no other known cause of coma (e.g., meningitis, a prolonged postictal state, or hypoglycemia-associated coma reversed by glucose infusion). Exclusion criteria for CM included prior history of coma, head trauma, hospitalization for malnutrition, or cerebral palsy. SMA was defined as a positive *P. falciparum* blood smear and serum hemoglobin ≤5 g/dL. Exclusion criteria for SMA included impaired consciousness, seizures prior to admission, or other clinical evidence of CNS involvement. All children underwent a medical history and physical examination at enrollment. Children with severe malaria were treated at Mulago Hospital according to Ugandan national treatment guidelines. Asymptomatic children aged 18 months to 12 years were enrolled from the neighborhoods and extended households of those enrolled with severe malaria to act as community control children (CC). Children with active or recent illness, chronic illness requiring medical care, or prior coma were excluded as community controls. Peripheral blood smears were assessed for *Plasmodium* species by microscopy with Giemsa staining using standard protocols.

3.2.2 Clinical and demographic assessments

All children underwent a medical history and physical examination at enrollment. Peripheral blood smears were assessed for *Plasmodium* species by microscopy with Giemsa staining using standard protocols. Nutritional status was assessed by height-for-age and weight-for-age z scores (WHO Child Growth Standards). Thrombocytopenia was defined as platelet count <150,000 platelets/μL. Children with CM were assessed for malarial retinopathy by indirect ophthalmoscopy, by trained medical officers at admission and every 24 hours while comatose. Children with retinopathy on any exam were classified as retinopathy positive.

3.2.3 Blood sample collection and detection of *P. falciparum* parasitemia

Blood samples were collected from all participants at enrollment and stored at –80°C. *Plasmodium falciparum* parasitemia was confirmed with both thick and thin blood

smears that were read independently by two experienced laboratory technicians, as previously described[17]. Parallel testing for histidine-rich protein 2 (*Pf*HRP-2) was performed on all samples with available plasma using the Malaria Ag CELISA kit (Cellabs, Brookvale, Australia) as previously described[119]. Samples from CC were tested for *P. falciparum* infection by nested PCR to determine the prevalence of submicroscopic infection using DNA extracted from whole blood, as previously described[120].

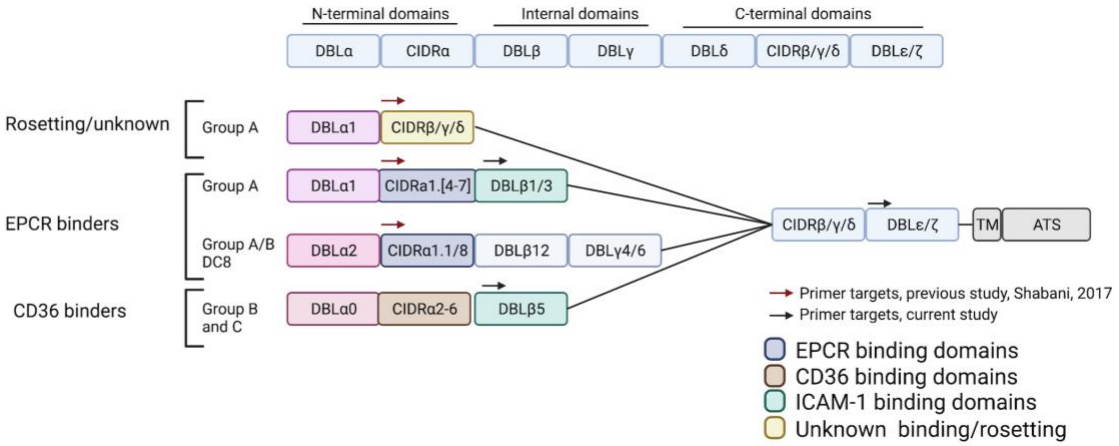
3.2.4 RNA isolation

Whole blood was collected at enrollment in PAXgene Blood RNA preservative solution (PreAnalytiX, Hombrechtikon, Switzerland) at a 2.76:1 ratio of PAXgene additive to whole blood. Samples were stored long term at –80°C. RNA was isolated using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland).

3.2.5 Primer design

Primers were designed and optimized as previously described[116]. Briefly, the primers used in this study were based on full-length DBL and CIDR domain encoding sequences from seven *P. falciparum* genomes and 226 Illumina whole-genome-sequenced *P. falciparum* field isolates[116]. Good coverage was achieved for primers targeting C-terminal DBLε and DBLζ domains based on in silico analysis of specificity and coverage against full-length domains[116]. Primer sets against ICAM-1–binding domains DBLβ1/3 and DBLβ5 had limited coverage but good specificity. The primer sets targeted five DBLβ domains found in PfEMP1 encoded by *var* group A and B genes that have been predicted to bind to host cell ICAM-1, DBLβ1/3, and DBLβ5 domains (Figure 3.1). The PfEMP1 DC5 domain, which binds PECAM-1 (platelet endothelial cell adhesion molecule-1) also contains a DBLβ domain, target by these sets of primers. Primers were additionally validated using genomic DNA from lab strains of *P. falciparum* parasites to ensure appropriate amplification.

Figure 3.1. Schematic of PfEMP1 domain organization.



Schematic of PfEMP1 domain structure depicting the possible domain organization (top) and specific domain combinations that define binding phenotypes and their corresponding *var* gene group designations (below). The N-terminal domains include a DBLα domain followed by a CIDR domain with specific host cell binding phenotypes: CIDRα1 for EPCR (Groups A and B, DC8, blue), CD36 (Groups B and C, brown), or unknown targets (Group A, yellow). ICAM-1-binding domains (DBLβ1, 3, or 5, green) can be found in all three *var* genes groups and are located within the internal domains of PfEMP1. A PfEMP1 molecule can have two to six subsequent C-terminal domains that are generally unrelated to prior N-terminal domains and have unknown binding partners. Domains studied by qRT-PCR in the previous study, Shabani, 2017 are denoted by a red arrow. Domains examined in this study are highlighted with a black arrow and include DBLβ domains that bind ICAM-1, as well as C-terminal DBLε and DBLζ domains. Created with biorender.com. Adapted from Storm, et al. *EMBO Mol Med*, 2019[3].

3.2.6 Quantification of *var* transcript levels by qRT-PCR

Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using random hexamers and the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed in 20-µL reactions using KiCqStart® SYBR® Green qPCR ReadyMixTM (Sigma-Aldrich, St. Louis, MO, USA) with the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification was performed following the previously published conditions[113,121], and data were collected at the final elongation step. Controls containing no reverse transcriptase or no template for were included to rule out DNA contamination

in the RNA samples or any nucleic acid contamination in reagents, respectively. Gene expression was normalized to the average of two housekeeping genes: seryl-tRNA synthetase and fructose-bisphosphate aldolase ($\Delta C_{t\ var\_primer} = C_{t\ var\_primer} - C_{t\ average\_control\ primers}$). $\Delta C_{t\ var\_primer}$ was transformed into arbitrary transcript units using $Tu = 2^{(5-\Delta Ct)}$. Only samples that had an average C_t of the two housekeeping genes <25 were included in the analysis. Melting temperature analysis was performed for each target, and only samples with T_m close to median T_m were analyzed. If only primer dimers were detected, Tu for that target was assigned as 1. Data were excluded for melt curves with multiple peaks or lacking a primary peak at the appropriate T_m or T_m indicating primer dimers.

3.2.7 Biomarker assessments

Plasma levels of soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), sE-selectin, and sP-selectin were measured by magnetic cytometric bead assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Plasma angiopoietin-1 (Angpt-1) and angiopoietin-2 (Angpt-2) levels were measured using the human angiopoietin 2 DuoSet ELISA kit (R&D Systems; Minneapolis, MN, USA). Plasma levels of Von Willebrand Factor (vWF) antigen were measured via ELISA (Corgenix; Bloomfield, Colorado, USA).

3.3.8 Statistical analysis

Data were analyzed using Stata/SE 14.1 (StataCorp, College Station, TX, USA). Transcript abundance of *var* genes was compared between disease groups using the Kruskal-Wallis and Wilcoxon rank sum tests. Clinical and laboratory findings for children in the different disease groups were compared using the chi-squared test for categorical data and Kruskal-Wallis tests for continuous measures. Univariate logistic regression

was utilized to identify associations between log-transformed transcript units of identified DBL domains and presence of mortality, retinopathy and seizures in children with cerebral malaria. Associations between *var* types and markers of endothelial activation were determined by univariate linear regression of log-transformed values and adjusted for multiple comparisons using false discovery rate thresholds. Associations between *var* types and the presence of thrombocytopenia were measured by univariate logistic regression of log-transformed *var* transcriptional units.

3.2.9 Ethics review

Written informed consent was obtained from parents or guardians of study participants. Ethical approval was granted by the institutional review boards for human studies at the Makerere University School of Medicine, the Uganda National Council for Science and Technology, and the University of Minnesota Medical School.

3.3 Results

3.3.1 Study population characteristics

A total of 718 children aged 18 months to 12 years were enrolled between 2008 and 2015 in Kampala, Uganda. However, due to the large amount of extracted RNA required for this study, only a subset of participants was included for analysis: 47 of the 269 children enrolled with CM and 51 of the 233 children with SMA had sufficient RNA volumes and quality remaining to be included in this study. Differences in study characteristics between participants with CM or SMA for which the expression DBL domains were tested compared to enrolled participants not tested are outlined in Table 3.1. There were no significant differences between those included in this study and not included, except the white blood cell count was higher in the included children for both CM and SMA, and parasite density was higher in included children with CM. By nested

PCR, 73/216 (33.8%) enrolled community children (CC) had asymptomatic parasitemia, six of which were included in this study. Additional CC samples were tested but excluded due to low amplification of parasite genes. Samples from this cohort study were tested previously for EPCR-binding domains[26] using the same methods and different primer sets. Of those included in this study, 44 children with CM, 34 with SMA, and five CCs were also included in our prior study[113].

Table 3.1. Demographic and clinical characteristics for children with CM or SMA included in this study compared with those not included.

Cerebral Malaria					
	Tested (n=47)[a]		**Not Tested (n= 222)[a]**		**P-value[b]**
Sex, male; n (%)	27 (57.45)		132 (59.46)		0.80
Age, years	3.33 [2.26, 4.06]		3.51 [2.57, 5.01]		0.16
Weight for age z-score	-0.78 [-1.65, -0.30]		-0.99 [-1.74, -0.38]	220	0.80
Height for age z-score	-1.14 [-2.12, -0.37]		-1.06 [-1.79, -0.13]		0.48
Hemoglobin, g/dL	7.30 [5.80, 8.50]		6.55 [5.10, 8.70]		0.30
Platelet count	62 [34, 123]	46	59 [35, 105]	218	0.44
White blood cell count	12.15 [8.90, 16.50]	46	8.95 [6.50, 12.90]	218	**0.0008**
Parasite density, parasites/μL	189680 [3440, 435540]		37080 [9650, 188340]	220	**0.003**
Parasite burden, *Pf*HRP2; ng/mL	2421.60 [883.20, 4394.40]		2906.99 [1097.26, 5659.20]		0.23
Severe Malarial Anemia					
	Tested (n=51)		**Not Tested (n= 182)**		**P-value**
Sex, male; n (%)	35 (68.63)		105 (57.69)		0.16
Age, years	2.44 [1.99, 4.15]		2.90 [2.08, 4.49]		0.41
Weight for age z-score	-1.20 [-1.92, -0.51]	50	-1.32 [-2.10, -0.41]	181	0.49
Height for age z-score	-1.49 [-2.26, -0.72]		-1.35 [-2.40, -0.53]	181	0.74
Hemoglobin, g/dL	3.90 [3.30, 4.30]		3.90 [3.10, 4.50]		0.78
Platelet count	158.50 [102, 226]	50	144 [90, 225]	181	0.44
White blood cell count	14.15 [10.10, 23.10]	50	10.90 [7.95, 15.25]	180	**0.002**
Parasite density, parasites/μL	47020 [7820, 156040]		34272.50 [10100, 137580]		0.50
Parasite burden, *Pf*HRP2; ng/mL	733.80 [273.60, 2101.20]	48	1005.60 [418.27, 2790]		0.09

For continuous variables, data are presented as median [IQR].

[a]For factors missing values, n's are listed to the right.

[b]For binary outcomes, group, mortality, and sex, Chi-squared was used. For all other continuous variables, Wilcoxon rank sum test was used.

Demographic and clinical characteristics for the children included in this study are outlined in Table 3.2. There were no differences between children with CM or SMA and community children with respect to demographic factors such as age, sex, or weight and height age-adjusted z-scores. As expected, children with CM had significantly lower platelet counts, higher parasite density, and higher plasma *Pf*HRP-2 (*P. falciparum* histidine-rich protein-2) levels (a measure of parasite burden), compared with children with SMA or asymptomatic CC.

Table 3.2. Demographic and clinical characteristics of children included in this study.

	CM (n=47)[a]	SMA (n=51)[b]	CC (n=6)[c]	P-value[d]
Sex; male n(%)	27 (57.45)	35 (68.63)	4 (66.67)	0.51
Age, years	3.33 [2.26, 4.06]	2.44 [1.99, 4.15]	3.02 [2.58, 5.91]	0.30
Weight for age z-score	-0.78 [-1.65, -0.30]	-1.20 [-1.92, -0.51]	-0.95 [-0.97, 0.15]	0.68
Height for age z-score	-1.14 [-2.12, -0.37]	-1.49 [-2.26, -0.72]	-1.15 [-1.68, -0.86]	0.43
Hemoglobin, g/dL	7.30 [5.80-8.50]	3.90 [3.30, 4.30]	10.60 [8.90, 11.70]	**<0.0001[e]**
Platelet count	62 [34, 123]	158.50 [102, 226]	226.00 [152, 395]	**<0.0001[f]**
White blood cell count	12.15 [8.90, 16.50]	14.15 [10.10, 23.10]	9.30 [8.60, 9.90]	0.052
Parasite density, parasites/μL	189680 [3440, 435540]	47020 [7820, 156040]	3400 [1280, 11880]	**0.001[e]**
Parasite burden, *Pf*HRP2; ng/mL	2421.6 [883.2, 4394.4]	733.8 [273.6, 2101.2]	48.0 [4.8, 98.4]	**<0.0001[e]**

For continuous variables, data are presented as median [interquartile range, IQR]

[a]n=46 for platelet count and sequestered parasite biomass.

[b]n=50 for weight for age z-score, platelet count, and white blood cell count; n=48 for parasite burden; n=47 for sequestered parasite biomass.

[c]n=5 for weight for age z-score, hemoglobin, platelet count, white blood cell count, parasite density, and sequestered parasite biomass.

[d]For dichotomous mortality and sex, Chi-squared was used. For all other continuous variables, Kruskal-Wallis test was used. Wilcoxon rank sum test was used for post-hoc, between-group comparisons.

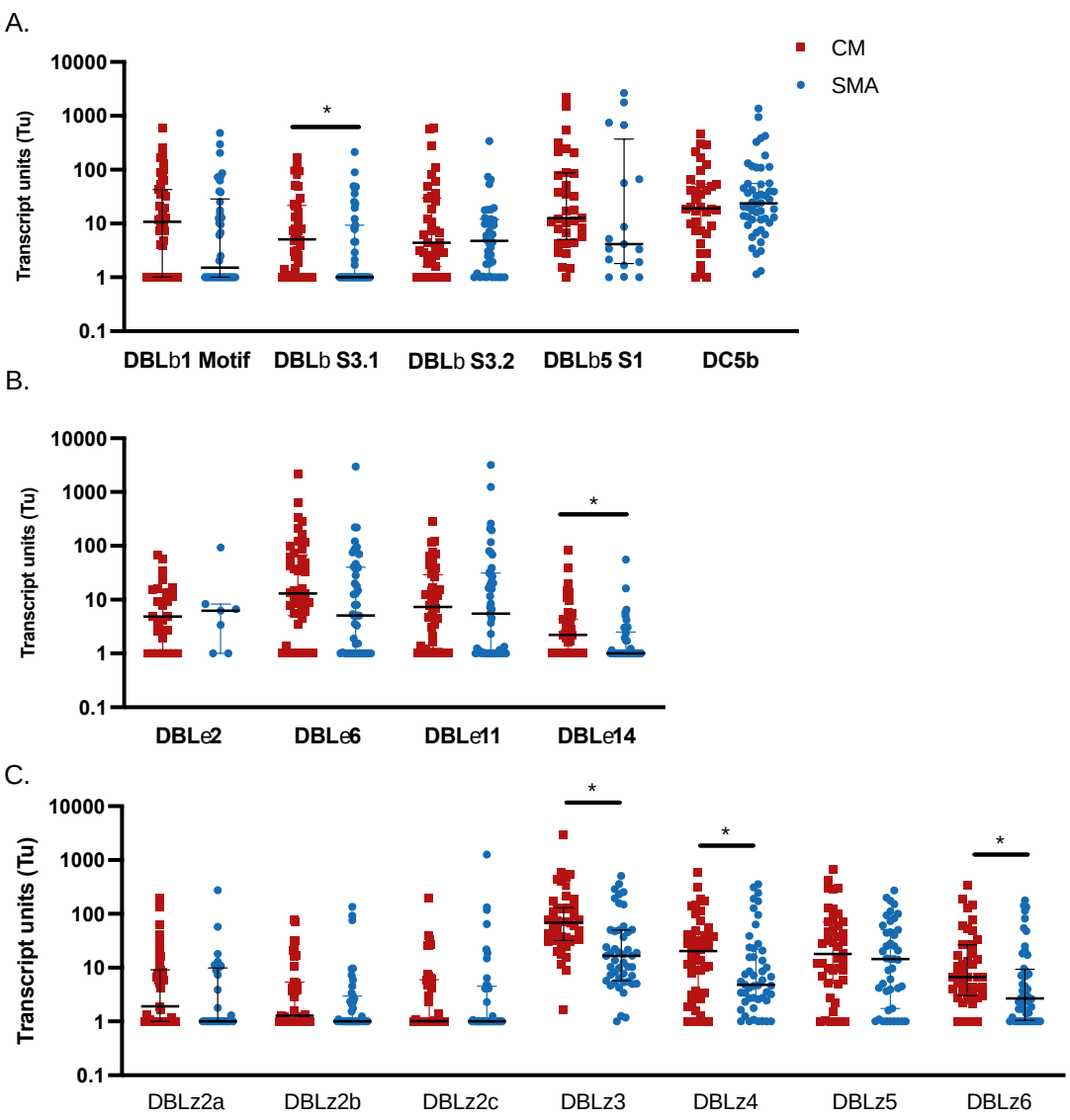
[e]In post-hoc testing, all three groups were significantly different.

[f]In post-hoc testing, CM was significantly different from SMA and CC

3.3.2 Expression of DBLβS3.1 transcripts is elevated children with CM compared to SMA

Primer set DBLβS3.1, which is predicted to amplify group A ICAM-1-binding domains, produced higher levels of transcripts in children with CM compared to those with SMA (Figure 3.2A). The other four primer sets had similar transcriptional units (Tu) in children with CM compared to SMA, including DBLβ S3.2 (another group A ICAM-1-binding domain), DBLβ5 S1 (a group B ICAM-1-binding domain), and a primer set that amplifies the DBLβ domain found within DC5, which binds to PECAM-1. The primer set for DBLβ motif 1 and DBLβ S3.1 had elevated transcript units in children with CM compared to CC, but they were not significantly higher than in children with SMA (Table 3.3).

Figure 3.2. Transcript levels of some DBL domains are elevated in children with CM compared to SMA.



Median [IQR] of transcript units (Tu) of DBL domains in children with CM (n=47, red) or SMA (n=51, blue) *p<0.05 based on Wilcoxon rank sum test. Different n's for each primer set are listed in Table 3.3, based on the number of samples that amplified with correct Tm; see chapter 3 methods.

Table 3.3. Transcript levels of DBL domains in children with CM or SMA and CCs.

Domain	Cerebral Malaria (n=47)		Community Children (n=6)		P-value
DBLβ motif 1	10.70 [1.00, 42.68]	43	1.00 [1.00, 1.00]	4	**0.02**
DBLβs3.1	5.09 [1.09, 21.29]	42	1.00 [1.00, 1.00]	4	**0.01**
DBLβs3.2	4.40 [1.59, 29.60]	39	3.49 [1.54, 8.19]	5	0.78
DBLβ5s1	12.59 [5.64, 86.41]	33	22.35 [19.52, 25.18]	2	0.72
DC5b	19.15 [7.28, 53.72]	36	21.34 [10.91, 302.65]	6	0.35
DBLε6	12.98 [5.60, 62.92]	45	3.47 [2.91, 4.25]	5	0.11
DBLε11	7.35 [1.29, 28.51]	44	1.90 [1.49, 4.01]	5	0.49
DBLε14	2.20 [1.00, 4.34]	44	2.10 [1.00, 4.32]	5	0.92
DBLζ2a	1.91 [1.00, 9.16]	39	1.00 [1.00, 15.90]	5	0.67
DBLζ2b	1.28 [1.00, 5.25]	41	2.06 [1.00, 4.45]	3	0.94
DBLζ2c	1.01 [1.00, 5.93]	40	1.00 [1.00, 1.00]	5	0.31
DBLζ3	69.05 [32.48, 132.0]	44	18.61 [14.08, 55.26]	3	0.09
DBLζ4	20.36 [3.37, 41.35]	46	11.42 [2.37, 19.49]	5	0.36
DBLζ5	18.06 [5.08, 70.70]	43	7.54 [1.00, 12.27]	5	0.24
DBLζ6	6.63 [3.05, 26.38]	45	3.85 [1.02, 8.06]	5	0.23
	Severe Malarial Anemia (n=51)		**Community Children (n=6)**		**P-value**
DBLβ motif 1	1.52 [1.00, 25.21]	42	1.00 [1.00, 1.00]	4	0.07
DBLβs3.1	1.00 [1.00, 9.34]	43	1.00 [1.00, 1.00]	4	0.12
DBLβs3.2	4.73 [1.11, 12.05]	40	3.49 [1.54, 8.19]	5	0.83
DBLβ5s1	4.15 [1.92, 66.69]	17	22.35 [19.52, 25.18]	2	0.51
DC5b	23.58 [11.76, 54.97]	50	21.34 [10.91, 302.65]	6	0.75
DBLε6	5.09 [1.00, 39.15]	45	3.47 [2.91, 4.25]	5	0.83
DBLε11	5.48 [1.00, 31.17]	47	1.90 [1.49, 4.01]	5	0.84
DBLε14	1.00 [1.00, 2.39]	42	2.10 [1.00, 4.32]	5	0.24
DBLζ2a	1.00 [1.00, 9.54]	32	1.00 [1.00, 15.90]	5	0.75
DBLζ2b	1.00 [1.00, 2.98]	39	2.06 [1.00, 4.45]	3	0.67
DBLζ2c	1.00 [1.00, 4.41]	40	1.00 [1.00, 1.00]	5	0.47
DBLζ3	16.53 [5.73, 50.09]	47	18.61 [14.08, 55.26]	3	0.50
DBLζ4	4.80 [2.57, 20.79]	49	11.42 [2.37, 19.49]	5	0.98
DBLζ5	14.41 [1.73, 50.41]	43	7.54 [1.00, 12.27]	5	0.43
DBLζ6	2.68 [1.10, 9.05]	48	3.85 [1.02, 8.06]	5	0.95

Data presented as median [IQR]; n's listed to the right denote the number of samples per primer set that were included in the analysis. P-values determined using the Wilcoxon rank sum test, comparing the SM group and CC.

3.3.3 Transcript units of specific C-terminal DBL domains are elevated in children with CM compared to SMA

In additional to DBLβ domains, we also measured the transcript levels of DBLε and DBLζ domains that do not have identified binding partners but are located closer to the C terminus of the PfEMP1 protein and may play a role in rosetting. In this study,

children with CM had higher transcript levels of DBLε14 compared to children with SMA (Figure 3.2B; Table 3.3). Additionally, out of seven primer sets against DBLζ domains, three, DBLζ3, DBLζ4, and DBLζ6 had elevated transcript levels in children with CM compared to SMA (Figure 3.2C). The remaining DBLε and DBLζ domains were similar across the three groups (Figure 3.2B, C and Table 3.3). Although transcript levels were not significantly different between children with CM or SMA and CC children, the levels of a number of these domains were lower in CC children, and the number of CC children tested was low, making it difficult to make adequately powered comparisons.

3.3.4 DBLβ transcripts are not associated with mortality, retinopathy, or seizures in children with CM

To understand the role of these specific DBL transcripts in severe malaria pathogenesis, associations between these domains and clinical outcomes, such as mortality, malaria retinopathy or presence of seizures in children with CM, were assessed. Mortality, malaria retinopathy, and presence of seizures during admission were only assessed in children with CM because there was no mortality and little seizure activity (2%) in children included in this study with SMA, and retinopathy was assessed only in children with CM. There were no significant associations with mortality and malarial retinopathy in children with CM, as seen in the previous study in this cohort in which EPCR-binding domains were assessed (Table 3.4). Additionally, were did not find any significant associations between DBL transcripts and the presence of seizures in children with CM (Table 3.4).

Table 3.4. Associations between DBL transcript units and mortality, retinopathy and presence of seizures during admission in children with CM.

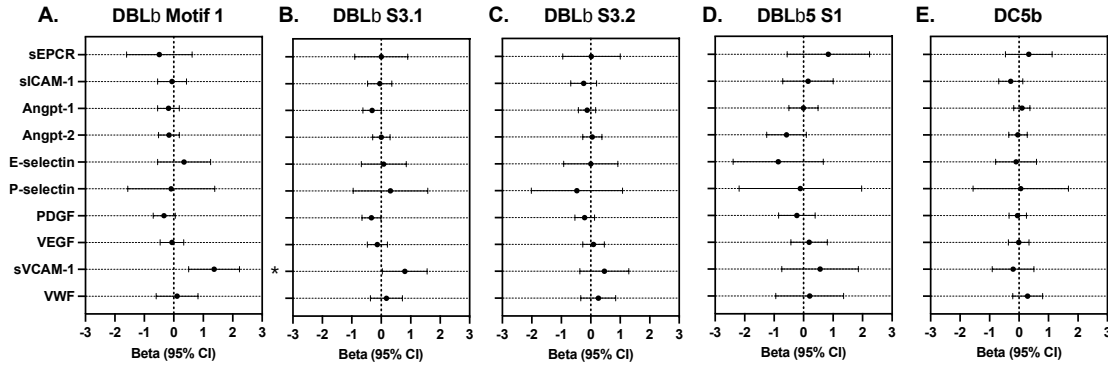
	Mortality			Malaria Retinopathy			Seizure		
	n	OR [95% CI]	P-value	n	OR [95% CI]	P-value	n	OR [95% CI]	P-value
DBLβ motif 1	43	0.73 [0.23, 2.37]	0.60	43	1.44 [0.68, 3.03]	0.34	43	0.89 [0.42, 1.88]	0.76
DBLβs3.1	42	1.35 [0.31, 5.82]	0.69	42	1.64 [0.66, 4.07]	0.29	42	1.22 [0.49, 3.03]	0.67
DBLβs3.2	39	2.11 [0.70, 6.36]	0.18	39	2.01 [0.81, 4.95]	0.13	39	1.09 [0.48, 2.46]	0.84
DBLβ5s1	33	0.44 [0.02, 8.75]	0.59	33	0.68 [0.30, 1.55]	0.36	33	0.59 [0.25, 1.38]	0.23
DC5b	36	2.46 [0.48, 12.58]	0.28	36	1.19 [0.47, 3.02]	0.72	36	1.39 [0.52, 3.69]	0.51
DBLε6	45	0.59 [0.18, 1.94]	0.38	45	1.82 [0.86, 3.86]	0.12	45	0.76 [0.37, 1.56]	0.46
DBLε11	44	2.47 [0.62, 9.79]	0.20	44	1.05 [0.45, 2.45]	0.90	44	0.80 [0.34, 1.89]	0.61
DBLε14	44	4.58 [0.71, 29.39]	0.11	44	9.23 [1.58, 53.92]	0.01	44	1.83 [0.47, 7.06]	0.38
DBLζ2a	39	3.22 [0.82, 12.60]	0.09	39	1.16 [0.44, 3.03]	0.76	39	0.74 [0.28, 1.96]	0.55
DBLζ2b	41	0.52 [0.07, 3.60]	0.51	41	1.15 [0.40, 3.32]	0.79	41	1.05 [0.36, 3.10]	0.93
DBLζ2c	40	1.54 [0.32, 7.41]	0.59	40	2.27 [0.72, 7.22]	0.16	40	1.10 [0.37, 3.23]	0.86
DBLζ3	44	2.41 [0.47, 12.49]	0.29	44	1.55 [0.52, 4.57]	0.43	44	1.39 [0.47, 4.16]	0.55
DBLζ4	46	1.23 [0.34, 4.47]	0.75	46	1.07 [0.48, 2.37]	0.87	46	1.13 [0.50, 2.55]	0.78
DBLζ5	43	1.64 [0.48, 5.61]	0.43	43	1.30 [0.60, 2.80]	0.51	43	0.53 [0.23, 1.21]	0.13
DBLζ6	45	2.22 [0.55, 8.92]	0.26	45	1.07 [0.43, 2.67]	0.88	45	0.81 [0.32, 2.06]	0.66

Logistic regression of log transcript units of indicated DBLs and mortality, presence of malaria retinopathy and presence of a seizure in children with cerebral malaria. No significant associations after correction for multiple comparisons.

3.3.5 Specific DBLβ domains are associated with elevated sVCAM-1 levels and thrombocytopenia in children with CM or SMA

DBLβ domains are predicted to bind to host receptors ICAM-1 and PECAM-1, which are associated with endothelial activation. We looked for correlations between transcript levels of five primer sets to DBLβ domains and levels of soluble molecules found on the host endothelium to better understand how these DBLβ domains are interacting with the endothelium and could be perpetuating endothelial activation. We included 8 markers of endothelial activation and PfEMP1 binding partners, sICAM-1 and sEPCR, which binds to CIDRα1 PfEMP1 domains. Only sVCAM-1 (soluble vascular cell adhesion molecule-1) was associated with increased transcript levels of the DBLβ motif 1 primer set in children with CM or SMA (Figure 3.3).

Figure 3.3. DBLβ domains show limited associations with markers of endothelial activation in children with severe malaria.



Linear regression of log-transformed DBLβ domain transcript levels and log -transformed levels of markers of endothelial activation in children with cerebral malaria and severe malaria anemia. *P<0.05 and significant after adjusting for multiple comparisons using FDR threshold.

To further our understanding of the role of these PfEMP1 transcripts in pathogenesis, associations between *Pf*HRP-2, a marker of parasite burden, and hemoglobin levels in plasma from children with CM or SMA were assessed. There were no associations between DBL transcript levels and PfHRP2 levels in children with CM or SMA (Table 3.5). However, increased transcript units of DBLζ3 were associated with

increased hemoglobin levels, after correction for multiple comparisons (Table 3.5), but no other domains were related to hemoglobin levels.

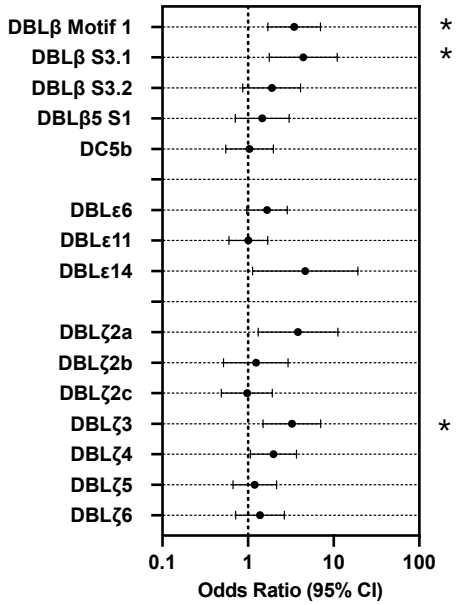
Table 3.5. Associations between transcript units of DBL domains and PfHRP2 and hemoglobin in children with severe malaria.

		*Pf*HRP2			Hemoglobin	
	n	**Beta [95% CI]**	**P-value**	**n**	**Beta [95% CI]**	**P-value**
DBLβ motif 1	82	0.12 [-0.13, 0.37]	0.35	85	0.50 [-0.48, 1.47]	0.32
DBLβs3.1	82	0.17 [-0.03, 0.36]	0.10	85	0.34 [-0.48, 1.16]	0.41
DBLβs3.2	77	0.01 [-0.22, 0.25]	0.93	79	0.09 [-0.82, 0.99]	0.85
DBLβ5s1	49	0.25 [-0.19, 0.70]	0.26	50	1.09 [-0.41, 2.60]	0.15
DC5b	83	-0.08 [-0.25, 0.10]	0.37	86	-0.69 [-1.50, 0.11]	0.09
DBLε6	88	0.14 [-0.10, 0.38]	0.24	90	0.37 [-0.63, 1.37]	0.46
DBLε11	88	0.07 [-0.15, 0.30]	0.52	91	-0.09 [-1.05, 0.87]	0.85
DBLε14	84	0.10 [-0.02, 0.22]	0.11	86	0.30 [-0.20, 0.81]	0.24
DBLζ2a	69	0.17 [-0.04, 0.38]	0.11	71	0.46 [-0.39, 1.30]	0.28
DBLζ2b	78	0.05 [-0.12, 0.22]	0.55	80	0.02 [-0.68, 0.73]	0.95
DBLζ2c	78	0.03 [-0.18, 0.24]	0.78	80	-0.33 [-1.14, 0.49]	0.43
DBLζ3	88	0.19 [0.02, 0.36]	0.03	91	1.24 [0.50, 1.98]	**0.001**
DBLζ4	92	0.17 [-0.02, 0.36]	0.08	95	0.34 [-0.48, 1.16]	0.41
DBLζ5	84	0.10 [-0.11, 0.30]	0.36	86	0.06 [-0.86, 0.98]	0.89
DBLζ6	90	0.06 [-0.12, 0.24]	0.50	93	0.63 [-0.14, 1.39]	0.11

Linear regression of log-transformed DBL domains and plasma levels of *Pf*HRP2, a measure of parasite burden, and hemoglobin levels. Multiple comparisons adjusted for using FDR threshold for the number of domains.

The presence of thrombocytopenia, a marker of disease severity in malaria[33], and previously associated with EPCR-binding domains in children and adults with CM[117,118], was associated with increased transcript levels of numerous DBL domain primer sets in children with CM or SMA, including DBL motif 1, DBLβ S3.1, and DBLζ3 (Figure 3.4).

Figure 3.4. DBL domain are associated with increased risk of thrombocytopenia in children with severe malaria.



Logistic regression of log transcript level of DBL domains and the presence of thrombocytopenia (platelet count less than 150,000 platelets/μL). *P<0.05 and FDR threshold to correct for multiple comparisons.

3.4 Discussion

In this study we explored the expression of PfEMP1 domains beyond the EPCR-binding domains included in our previous study[113] in children with CM or SMA. Understanding the differences in PfEMP1 expression between different manifestations of severe malaria may help to improve our understanding of the pathogenesis of severe malaria. Here we used RT-qPCR to measure the expression of specific *var* transcripts that encode four DBLβ domains that bind ICAM-1 and PECAM-1 along with four DBLε and seven DBLζ domains located towards the C terminal end of PfEMP1. We show that DBLβS3.1, an ICAM-1-binding domain, DBLε14, and multiple DLBζ domains had higher expression in children with CM compared to children with SMA; and that most DBL domain transcript levels were not associated with endothelial activation but that higher levels of the DBLβ motif 1 primer, DBLβ S3.1 domain and DBLζ3 were associated with thrombocytopenia. Together, these findings suggest that non-EPCR-binding DBL domains may differentially affect risk of CM vs. SMA in children, and that the risk of severe malaria associated with specific non-EPCR-binding DBL domains is unlikely to relate to endothelial activation but may relate to thrombocytopenia. The study findings provide further insights into the potential role of PfEMP1 DBL domains that do not bind to EPCR in the development of severe malaria and in development of CM vs. SMA in children.

In our previous study in this cohort of children with CM or SMA, the expression of EPCR-binding domains was assessed using the same methods but a different set of primers. In that study, we showed that the expression of these domains is elevated in children with CM compared to SMA but higher in both than the community children with asymptomatic parasitemia[113]. We also demonstrated that children with retinopathy CM had higher expression of these domains than children with retinopathy negative CM, suggesting that these domains increased with increasing disease severity[113]. In the

present study, we found higher levels of specific DBL domain transcripts in children with CM compared to SMA or CC, but we did not see significant associations with retinopathy in children with CM. There was also no evidence of these domains being associated with mortality in children with CM, as in the previous study[113]. Together, the findings suggest that the non-EPCR-binding PfEMP1 DBL domains may also affect the clinical phenotype of CM vs. SMA but may not play the same role in CNS blood vessel endothelial cell binding, leading to retinopathy, as EPCR-binding PfEMP1 domains.

As in studies of EPCR-binding domains, expression of multiple non-EPCR-binding DBL domains were higher in children with severe malaria (CM or SMA) than in community children with asymptomatic parasitemia. The small number of community children with asymptomatic parasitemia in the present study precluded detection of all but very large differences, so levels in multiple additional domains were numerically but not statistically higher in children with severe malaria compared to community children. The study findings are consistent with those of a prior study in which the same DBL primers were used, which found that children with CM had higher levels of expression of all but one DBLβ domain, as well as of DBLε2, DBLζ2a, DBLζ2c, DBLζ3, and DBLζ5 compared to children with uncomplicated malaria, and suggest these PfEMP1 domains may also contribute to development of severe disease in malaria [3]. In particular, the findings of elevated expression of DBLζ3, DBLζ4 and DBLζ6 in children with CM compared to children with SMA or asymptomatic parasitemia in the present study, in conjunction with the prior study findings, show that some DBLζ domains are consistently higher in children with CM, and suggest a possible role for these DBLζ domains in CM pathogenesis. However, the elevated expression of DBLε and DBLζ domains in children with CM compared to SMA could also occur because they are commonly found in domain cassettes that include EPCR-binding domains[73], which are elevated in children with CM vs SMA in this population[113]. Additional sequencing methods may be required to

evaluate whether these DBL domains confer an independent risk of CM compared to SMA.

The role of ICAM-1-binding domains of PfEMP1 in severe malaria pathogenesis, particularly CM, is complex. Parasites that bind to ICAM-1 are elevated in cerebral malaria[3], particularly those that express PfEMP1 proteins with both ICAM-1 and EPCR-binding domains[93]. Although we did not assess the presence of dual binding PfEMP1 molecules in this study, we saw that DBLβ S3.1 expression was elevated in CM compared to SMA, suggesting that it may be playing a role in pathogenesis specific to CM. Parasite binding to ICAM-1 in the brain could be exacerbating endothelial activation, with EPCR-binding at the brain endothelium. Although correlations between PfEMP1 domains that bind to EPCR and endothelial activation markers have been seen previously[93,122], limited evidence is available for ICAM-1-binding domains. We did not find any associations between the expression of DBLβ domains that bind to ICAM-1 and PECAM-1 and soluble endothelial activation markers or the soluble binding partners of PfEMP1, including ICAM-1 and EPCR. However, transcript units of DBLβ motif 1 were significantly associated with increased concentrations of sVCAM-1. VCAM-1 is a cell adhesion molecule which helps in the recruitment of leukocytes and its expression is increased when the endothelium is activated or stimulated by cytokines, making it a marker of endothelial activation. It could be related to the expression of ICAM-1-binding DBLβ transcripts because these domains could be exacerbating endothelial activation; however no other markers of EA were significantly associated with these ICAM-1 binding domains. This suggests that although parasites in severe malaria express ICAM-1-binding domains, the pathways leading to endothelial activation are more complex and involve additional processes beyond parasite binding to ICAM-1 on the endothelium.

PfEMP-1 variants could also increase disease severity through thrombocytopenia. Thrombocytopenia is well-described as a risk factor for severe

malaria, as compared to uncomplicated malaria[33]. Platelets are thought to play an important role in pathogenesis through multiple mechanisms. Activated platelets aggregate and form microvascular obstructions during CM[34]. It has also been shown that they can bind to endothelial cells and provide receptors for surface antigens of infected red blood cells, such as PfEMP1[123] to assist in parasitized erythrocytes binding to the endothelium. Platelets can also inhibit parasite growth[35,36] and could partially be a part of a protective mechanism during infection. In the present study, increased expression of multiple variants, including DBL motif 1, DBLβ S3.1, and DBLζ3 was associated with thrombocytopenia. Previously, in a pediatric cohort of children with CM the expression of EPCR-binding domains of PfEMP1, CIDRα1.7 was associated with thrombocytopenia[117] and this was shown again in a cohort of adults with CM[118]. In the present study, we demonstrated an increased risk of thrombocytopenia with increased expression of domains of PfEMP1 other than EPCR-binding domains. It is not clear how increased transcript levels of PfEMP1 variants might relate to thrombocytopenia, and whether the relationship is causal or if an underlying process leads to both phenomena. Increased *var* gene transcripts could lead to thrombocytopenia by direct binding of PfEMP1 to platelets, removing them from circulation. Alternatively, higher parasite biomass could be related to both phenomena.

There are a few limitations of this study, related to sample size and availability. Due to the large volume of RNA required for the testing, a small subset of the cohort was tested for these PfEMP1 transcripts. Although many of the demographic and clinical characteristics were similar between those included in this study and the entire cohort, white blood cell count was significantly higher for those included in the study and parasite density was significantly elevated in children with CM included in the study compared to those not tested. It is possible that these differences impacted the results, although parasite density is adjusted for using expression levels of the housekeeping

genes. Overall, this study had numerous strengths including the expansive database of clinical outcomes and markers and data on EPCR-binding transcript levels.

In conclusion, this study measured the transcript levels of PfEMP1 DBL variants that bind ICAM-1 and additional C-terminal domains. We demonstrated that transcript levels of DBLβS3.1, DBLε14, and multiple DLBζ domains were elevated in children with CM and that higher levels of the DBLβ motif 1 primer, DBLβ S3.1 domain and DBLζ3 were associated with thrombocytopenia in children with CM or SMA. By examining domains of PfEMP1 beyond EPCR-binding domains, we demonstrated differences in transcript levels of additional domains between children with different forms of malaria, CM and SMA. Future studies that expand the definition of severe malaria to additional manifestations could identify specific differences in PfEMP1 expression related to severe malaria symptoms, highlighting roles of PfEMP1 in severe malaria pathogenesis.

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Chapter 4: *Var* transcripts of EPCR-binding domains are expressed in different manifestations of severe malaria and are associated with anemia and thrombocytopenia

4.1 Introduction

Severe malaria caused by *Plasmodium falciparum* is among the leading causes of morbidity and mortality in children living in Africa, where 80% of the over 600,000 malaria deaths in 2020 were in children under 5 years[6]. Severe malaria is commonly studied as cerebral malaria, defined as positive blood smear, Blantyre coma score<3 and no other cause of coma; and severe malarial anemia (SMA), defined as positive blood smear and hemoglobin <5 g/dL[10]. However, pediatric severe malaria can manifest with numerous other symptoms, including respiratory distress, seizures, and prostration, that are commonly not included or individually defined in studies of severe malaria in children. Expanding the definition of severe malaria could offer new insights into the pathogenesis of disease and improve clinical outcomes of severe malaria.

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is a key virulence factor during *P. falciparum* malaria, mediating cytoadherence through specific interactions of PfEMP1 domains and host endothelial receptors[56,124]. Encoded for by the approximately 60 *var* genes in the parasite genome[56,57], PfEMP1 is a major source of antigenic variation for the parasite. The *var* genes encoding PfEMP1 are classified into groups A, B, and C based on chromosomal location and sequence similarity[73]. PfEMP1 is expressed on the surface of infected erythrocytes and is composed of two to nine cysteine-rich interdomain regions (CIDR), divided into 3 classes (α, β, γ), and Duffy binding-like (DBL) domains, divided into 7 classes, (α, β, γ, δ, ε, ξ, x)[73]. Specific binding interactions between PfEMP1 and host receptors have been related to different manifestations of malaria; for example, CIDRα2-6 domains bind to CD36 and are more common in uncomplicated malaria[78,125]. Group A *var* genes, particularly the CIDRα1

domain, which binds endothelial protein C receptor (EPCR)[75], are more frequent in children with severe malaria[86,126]. Domain cassette 8 (DC8), a conserved tandem arrangement of PfEMP1 domains, is considered an intermediate group A/B gene, as it contains a CIDRα1 EPCR-binding domain but is recombined into a group B *var* location[73] and is consistently associated with severe malaria[116,121]. Additionally, DBLβ domains of PfEMP1, found in both groups A and B, have been shown to bind intercellular adhesion molecule-1 (ICAM-1)[80-82]. PfEMP1 molecules expressing both ECPR-binding domains and ICAM-1-binding domains can be used to differentiate children with cerebral malaria from children with severe malarial anemia[93].

Cytoadherence of infected erythrocytes to the endothelium through PfEMP1 is believed to be a key player in severe malaria pathogenesis. Specifically, the binding of infected erythrocytes to EPCR on the endothelium occurs throughout the microvasculature[127,128] and is thought to functionally block the production and cytoprotective effects of activated protein C[87-89]. Additionally, the interaction between ICAM-1 and PfEMP1 could affect endothelial activation, a key component of pathogenesis[28]. During severe malaria, the interactions between PfEMP1 and the endothelium could contribute to sequestration, defects in the coagulation pathway, endothelial activation, and endothelium permeability, all processes involved in pathogenesis that could impact patient outcomes and disease severity. In one study of children with CM, elevated expression of EPCR-binding domains, particularly CIDRα1.7, were associated with brain swelling, parasite burden measured by *P. falciparum* histidine-rich protein-2 (*Pf*HRP2) plasma levels and thrombocytopenia[117]. Elevated transcript levels of EPCR-binding domains have also been associated with malarial retinopathy in children with CM but decreased levels in children with CM who died[113].

Most studies to date have focused on the relationship between the expression of PfEMP1 domains and clinical outcomes of severe malaria in children with cerebral

malaria. Therefore, these studies could be missing the role of PfEMP1 in other forms of malaria and if the role of PfEMP1 is different in different types of severe malaria. In the present study, we used samples from a cohort of Ugandan children enrolled with one of or more of the five most common manifestations of severe malaria: cerebral malaria (CM), respiratory distress (RDS), malaria with repeated seizures(M/S), severe malarial anemia (SMA) and prostration (Pro). We used RT-qPCR primer sets with coverage and high specificity[116] for *var* transcripts of PfEMP1 EPCR-binding domains, ICAM-1-binding domains and additional PfEMP1 domains to measure the transcript levels of numerous PfEMP1 domains in five manifestations of severe malaria and how these expression profiles relate to key clinical outcomes, including thrombocytopenia and anemia, which are frequently seen in severe disease, and mortality. These specific predictors and outcomes were selected because we hypothesize that *var* transcript levels would be correlated to disease severity and many of the predictors or outcomes selected are related to severe disease and could inform about the role of PfEMP1 in SM pathogenesis. Additionally, we hypothesized that children with manifestations of severe malaria with greater mortality, such as cerebral malaria and respiratory distress, would have elevated transcript levels of PfEMP1 domains, particularly EPCR-binding domains.

4.2 Methods

4.2.1 Study population

Between 2014 and 2017, 600 children with severe malaria and 120 community children were enrolled in a prospective cohort study at two sites in Uganda: Mulago National Referral and Teaching Hospital in Kampala, Uganda and Jinja Regional Referral Hospital in Jinja, Uganda. This study is a part of a larger study designed to evaluate the impact of the five most common forms of severe malaria on neurocognition at 12-months follow-up in children <5 years of age. Children were enrolled with one of

five forms of severe malaria: cerebral malaria (CM), respiratory distress (RDS), malaria with repeated seizures (M/S), severe malarial anemia (SMA), and prostration (Pro). A hierarchy based on disease severity was used to place children with multiple symptoms into groups, with CM being the most severe, and the hierarchy continuing in the order noted above. Inclusion criteria included children aged 6 months to 4 years of age with malaria (positive rapid diagnostic test for *P. falciparum* histidine-rich protein-2 (*Pf*HRP-2) or direct visualization of parasites by Giemsa microscopy) who required hospitalization for coma, respiratory distress, two or more seizures in 24 hours, severe anemia defined as hemoglobin <5 g/dL or prostration. The definition of malaria using either rapid diagnostic test or microscopy aligns with the Uganda Clinical Guidelines on malaria diagnosis. Exclusion criteria included known chronic illness requiring medical care, known developmental delay, or history of coma, head trauma, and prior hospitalization for malnutrition. Comatose children with lumbar puncture findings suggestive of another cause of coma were excluded. For asymptomatic controls, 120 community children (CC) from the nuclear family, extended family, or household area of the children with severe malaria were enrolled. Additional exclusion criteria for the controls included an illness requiring medical care within the previous four weeks, a major medical or neurologic abnormality at screening physical examination, or active illness. On enrollment, children had a complete history, and physical exam, and venous blood draw. Children were managed according to Ugandan National Treatment guidelines for severe malaria, including intravenous artesunate followed by oral artemisinin-combination therapy.

4.2.2 Clinical and demographic assessments

All children underwent a medical history and physical examination at enrollment. Peripheral blood smears were assessed for *Plasmodium* species by microscopy with Giemsa staining using standard protocols. Nutritional status was assessed by height-for-

age and weight-for-age z-scores (WHO Child Growth Standards). Thrombocytopenia was defined as platelet count <150,000 platelets/μL.

4.2.3 Blood sample collection and detection of *P. falciparum* parasitemia

Blood samples were collected from all participants at enrollment and stored at –80°C. *P. falciparum* parasitemia was confirmed with both thick and thin blood smears that were read independently by two experienced laboratory technicians, as previously described[17]. Parallel testing for histidine-rich protein 2 (*Pf*HRP-2) was performed on all samples with available plasma using the Malaria Ag CELISA kit (Cellabs, Brookvale, Australia) as previously described[119]. Samples from CC were tested for *P. falciparum* infection by nested PCR to determine the prevalence of submicroscopic infection using DNA extracted from whole blood, as previously described[120].

4.2.4 RNA isolation

Whole blood was collected at enrollment in PAXgene Blood RNA preservative solution (PreAnalytiX, Hombrechtikon, Switzerland) in a ratio of 2.76 mL of additive per mL of blood. Samples were stored long term at –80°C. RNA was isolated using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland).

4.2.5 Quantification of *var* transcript levels by qRT-PCR

The number of children with RNA isolated and then tested by qPCR was smaller than the total number enrolled, for the reasons outlined in Table 4.1.

Table 4.1. Reasons for loss of sample size from study enrollment to inclusion in analysis.

	CM	RDS	M/S	SMA	Pro	CC	Total
Blood Collected	85	121	160	155	76	120 (33)[a]	717
RNA Extracted[b]	85	121	44	155	21	33	459
cDNA Generated[c]	80	116	40	147	19	29	431
Sufficient cDNA/RNA[d]	53	90	29	107	10	18	307
qPCR[e]	39	58	22	58	10	5	192
Included in study[f]	38	56	21	52	10	5	182

[a]Only community children with asymptomatic parasitemia (AP) detected by nested PCR were included in this study (n=33).
[b]This study designed to focus on comparisons between CM, RDS and SMA.
[c]cDNA generated based on RNA nanodrop readings. Samples with concentration of RNA greater than 15 ng/μL were included. Samples with A260/280 less than 1.8 or greater than 2.2 were excluded, most had low RNA concentrations.
[d]1.5ug of genetic material required for the study (62.5ng for 24 reactions). Excluded 16 samples that were negative for *P. falciparum* by nested conventional PCR.
[e]Lost number of samples here because poor amplification of housekeeping genes, as they did not have the correct Tm or had multiple melting temperatures.
[f]Samples lost after qPCR due to high Ct for housekeeping genes.

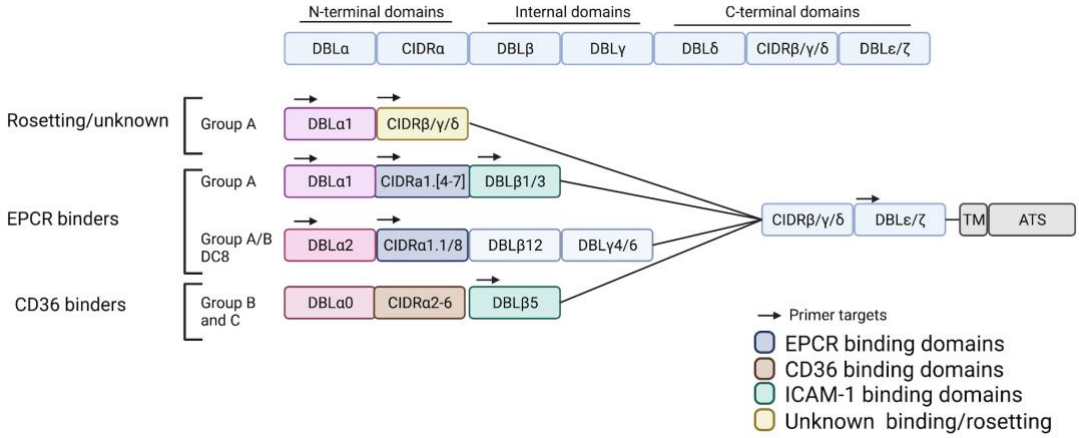
4.2.6 Primer design

Primers were designed and optimized as previously described[116]. Briefly, the primers used in this study were based on full-length DBL and CIDR domain encoding sequences from seven *P. falciparum* genomes and 226 Illumina whole-genome-sequenced *P. falciparum* field isolates[116]. Good coverage was achieved for primers targeting EPCR-binding domains and C-terminal DBLε and DBLζ domains based on in silico analysis of specificity and coverage against full-length domains[116]. Primer sets against ICAM-1–binding domains DBLβ1/3 and DBLβ5 had limited coverage but good specificity.

In this study, we used primer sets to quantify *var* transcript levels of EPCR-binding domains (CIDRα1), ICAM-1-binding domains (DBLβ) and additional N-terminal (DBLα) and C-terminal (DBLε/DBLζ) PfEMP1 domains (Figure 4.1) to understand how different domains of PfEMP1 are expressed during an episode of severe malaria. First, primer sets amplifying eight CIDRα1 domains in both group A and B *var* genes were used to assess EPCR-binding domain expression. An additional primer set to CIDRd1 was used

to amplify non-EPCR group A domains. To quantify different subsets of group A PfEMP1 domains, primers against DBLα domains located at the N-terminal of the protein were used. The primer set “DBLα1ALL” was used to target loci common to all group A *var* genes. “DBLα1.5/6/8” targeted a subset of group A genes typically linked to CIDRβ/ɣ/δ domains, non-EPCR-binding group A genes. “DBLα2/1.1/2/4/7” quantified genes typically linked to EPCR-binding CIDRα1 domains in group A/B *var* genes, such as DC8. Additional primer sets to group A ICAM-1-binding domains, DBLβs3.1 and DBLβs3.2 and group B DBLβ5 S1assessed levels of ICAM-1-binding domains. Finally, primer sets designed to amplify DBLε14, DBLζ2a, DBLζ3, DBLζ4, DBLζ5 and DBLζ6 were tested to examine the expression of these C-terminal domains, located closer to the infected erythrocyte surface.

Figure 4.1. Schematic of PfEMP1 domains.



Schematic of PfEMP1 domain structure depicting the possible domain organization (top) and specific domain combinations that define binding phenotypes and their corresponding *var* gene group designations (below). The N-terminal domains include a DBLα domain followed by a CIDR domain with specific host cell binding phenotypes: CIDRα1 for EPCR (Groups A and B, DC8, blue), CD36 (Groups B and C, brown), or unknown targets (Group A, yellow). ICAM-1-binding domains (DBLβ1, 3, or 5, green) can be found in all three *var* genes groups and are located within the internal domains of PfEMP1. A PfEMP1 molecule can have two to six subsequent C-terminal domains that are generally unrelated to prior N-terminal domains and have unknown binding partners. Domains studied by RT-qPCR are denoted with a black arrow and include DBLα domains of group A, CIDRα1 domains that bind EPCR, DBLβ domains that bind ICAM-1, as well as C-terminal DBLε and DBLζ domains. Created with biorender.com. Adapted from Storm, et al. *EMBO Mol Med*, 2019[3].

Complementary DNA (cDNA) was synthesized using random hexamers and the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed in 20-µL reactions using KiCqStart® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, St. Louis, MO, USA) with the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification was performed following the previously published conditions[113,121], and data were collected at the final elongation step. No template controls for both housekeeping genes were included to rule out any nucleic acid contamination in reagents. Gene expression was normalized to the average of two housekeeping genes: seryl-tRNA synthetase and fructose-bisphosphate aldolase (ΔC_t var_primer = C_t

var_primer − C_t average_control primers). $\Delta C_{t\ var\_primer}$ was transformed into arbitrary transcript units using $Tu = 2^{(5-\Delta Ct)}$. Only samples that had an average C_t of the two housekeeping genes <25 were included in the analysis. Melting temperature analysis was performed for each target, and only samples with T_m close to median Tm were analyzed. If only primer dimers were detected, Tu for that target was assigned as 1. Data were excluded for melt curves with multiple peaks or lacking a primary peak at the appropriate T_m or T_m indicating primer dimers. Tu for group A EPCR-binders was determined at the sum of [CIDRα1.4, CIDRα1.5a, CIDRs1.5b, CIDRα1.6b and CIDRα1.7] Tu-4; Tu for group B ECPR-binders was determine as the sum of [CIDRα1.1, CIDRα1.8a CIDRα1.8b] Tu-2; Tu of the CIDRα1 EPCR-binders was calculated as the sum of all CIDRα1 domains] Tu-7.

4.2.7 Biomarker assessments

Plasma levels of soluble intercellular adhesion molecule-1 (sICAM-1) were measured by magnetic cytometric bead assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Plasma levels of soluble endothelial protein C receptor (sEPCR) were measured by ELISA, diluted 1:19.5 (R&D Systems, DuoSet, Minneapolis, MN, USA), according to manufacturer's instructions.

4.2.8 Statistical analysis

Data were analyzed using Stata/SE 14.1 (StataCorp, College Station, TX, USA). Transcript abundance of *var* genes was compared between disease groups using the Kruskal-Wallis and Wilcoxon rank sum tests. Post-hoc testing for comparing multiple groups was done using the Dunn test.. Clinical and laboratory findings for children in the different disease groups were compared using the chi-squared test for categorical data and Kruskal-Wallis tests for continuous measures. Associations between *var* transcript levels and continuous variables, including parasite density, parasite burden, and

cytokine concentrations and soluble binding partners were determined by linear regression of log-transformed values. Associations between *var* types and dichotomous variable, mortality, the presences of malaria retinopathy, anemia and thrombocytopenia were assessed using logistic regression of log-transformed *var* transcriptional units. Analysis was corrected for multiple comparisons using the Benjamini-Hochberg false discover rate (FDR) approach with a significance level of 0.05[129].

4.2.8 Ethics review

Written informed consent was obtained from the parents or legal guardians of study participants after providing emergency clinical care to stabilize children. Ethical approval was granted by Institutional Review Boards at Makerere University School of Medicine, the University of Minnesota School of Medicine, and the Indiana University School of Medicine. The Uganda National Council for Science and Technology approved the study

4.3 Results

4.3.1 Study population characteristics

P. falciparum var transcript levels were quantified in 177 children with severe malaria, 38 children with cerebral malaria, 56 with respiratory distress syndrome, 21 with malaria with repeated seizures, 52 with severe malarial anemia and 10 with prostration (Table 4.1). Among the 33 asymptomatic community children with *P. falciparum* infection by PCR, only 5 had sufficient quality and quantity of RNA to measure *var* transcript levels. There were no significant differences in demographic characteristics, such as age, sex, and study site between the children with different forms of severe malaria in the study (Table 4.2). However, parasite density, plasma *Pf*HRP2 level, platelet count, hemoglobin level and mortality did differ significantly (Table 4.2). Because we used a subset of samples from the entire cohort, we also compared demographic and clinical

characteristics between children included in this study with severe malaria and those not included (Table 4.3). The lower age and lower hemoglobin level seen among children tested reflected our testing less samples from children with M/S or Pro (who were older and had higher hemoglobin levels than the large group of children with SMA), while the higher parasite density and parasite biomass (PfHRP2 level) were expected, since children with more parasitemia would have a greater amount of parasite RNA to extract. Among CC with AP, the children tested also had higher parasite density and more detectable PfHRP2, consistent with a group that would yield more RNA (Table 4.3).

Table 4.2. Demographic and clinical characteristics for children with five forms of severe malaria and asymptomatic community controls included in this study.

	CM (n=38)	RDS (n=56)	M/S (n=21)[a]	SMA (n=52)	Pro (n=10)	CC (n=5)	P-value[b]
Age, years	2.05 (1.52-3.16)	1.77 (1.14-2.42)	1.85 (1.43-2.78)	1.79 (1.39-2.54)	2.04 (1.22-2.54)	2.04 (2.02-2.48)	0.19
Sex, n (% male)	25 (65.79)	36 (64.29)	11 (52.38)	25 (48.08)	3 (30.00)	1 (20.00)	0.08
Site, n (% Mulago)	21 (55.26)	25 (44.64)	17 (80.95)	34 (65.38)	5 (50.00)	2 (40.00)	0.06
Parasite Density, parasite/μL	53106.2 (13598.4-276624.4)	69824.4 (17383.6-322575.1)	99500 (8428.4-167388.5)	38160.3 (7429.1-174130.2)	195967 (51430-705705)	6224.4 (3445.2-12915)	**0.04[c]**
Parasite Burden, *Pf*HRP2, ng/mL	4062.68 (2194.11-8637.73)	4623.89 (1605.20-6678.39)	1583.31 (670.75-3047.90)	3544.79 (2056.59-5542.02)	3909.76 (2276.25-8636.60)	327.62 (251.87-467.50)	**0.0005[d]**
Hemoglobin, g/dL	6.2 (4.4-7.7)	4.6 (3.1-6.8)	10.2 (7.0-11.2)	4.0 (3.3-4.8)	7.2 (6.5-8.4)	10.1 (9.8-10.8)	**<0.0001[e]**
Platelet count	92.5 (56.0-214.0)	110.0 (55.0-195.5)	96.6 (67.6-223.5)	121.5 (77.5-158.0)	69.5 (35.2-96.6)	192.0 (192.0 -196.0)	0.10
Mortality	12 (31.58)	4 (7.14)	0 (0)	0 (0)	0 (0)	N/A	**<0.0001[f]**

[a]N=20 for parasite burden, hemoglobin, and platelet count.
[b]P-values generated using Kruskal Wallis rank sum test for continuous variables. Pairwise comparisons performed using the Dunn Test. For dichotomous variables, p-values were generated using Chi-squared test. Multiple pairwise comparisons were adjusted for using Bonferroni correction based on the number of comparisons.
[c]Significant pairwise comparisons: CC vs RDS and Pro
[d]Significant pairwise comparisons: M/S vs CM, RDS, SMA, and Pro; CC vs CM, RDS, SMA, and Pro
[e]Significant pairwise comparisons: CM vs SMA; M/S vs CM, RDS, and SMA; Pro vs RDS and SMA; CC vs CM, RDS, and SMA
[f]Significant pairwise comparisons: CM vs RDS and SMA

Table 4.3. Demographic and clinical characteristics of children with severe malaria and community children included in testing compared to those not included.

Severe Malaria			
	Tested(n=177)[a]	**Not Tested (n=423)[b]**	**P-value**
Age, years	1.82 [1.35, 2.75]	2.11 [1.48, 2.94]	**0.01**
Sex, n (% male)	100 (56.50)	238 (56.26)	0.96
Site, n (% Mulago)	102 (57.63)	230 (54.37)	0.46
Parasite Density, parasite/μL	65224.25 [12403.12, 243008.5]	2090.11 [0, 89523.82]	**<0.0001**
Parasite Burden, *Pf*HRP2, ng/mL	3582.17 [1773.84, 6633.14]	1694.14 [64.80, 4913.14]	**<0.0001**
Hemoglobin, g/dL	5.02 [3.49, 7.3]	6.15 [3.9, 9.1]	**0.004**
Platelet count	99.8 [62.0, 194.5]	127.5 [60.0, 229.5]	0.05
Mortality, n (% died)	16 (9.04)	28 (6.62)	0.30
Community Children with Asymptomatic Parasitemia (CC)			
	Tested (n=5)	**Not Tested (n=28)[c]**	**P-value**
Age, years	2.04 [2.02, 2.48]	2.19 [1.16, 3.00]	0.65
Sex, n (% female)	1 (20.00)	14 (50.00)	0.21
Site, n (% Jinja)	2 (40.00)	8 (28.57)	0.61
Parasite Density, parasite/μL	6224.4 [3445.2, 12915]	0 [0, 657.69]	**0.005**
Parasite Burden, PfHRP2, ng/mL	327.62 [251.87, 467.50]	<64.8	**0.003**
Hemoglobin, g/dL	10.1 [9.8, 10.8]	10.9 [10.2, 11.5]	0.16
Platelet count	192.0 [192.0, 196.0]	342.5 [232.0, 484.0]	0.28

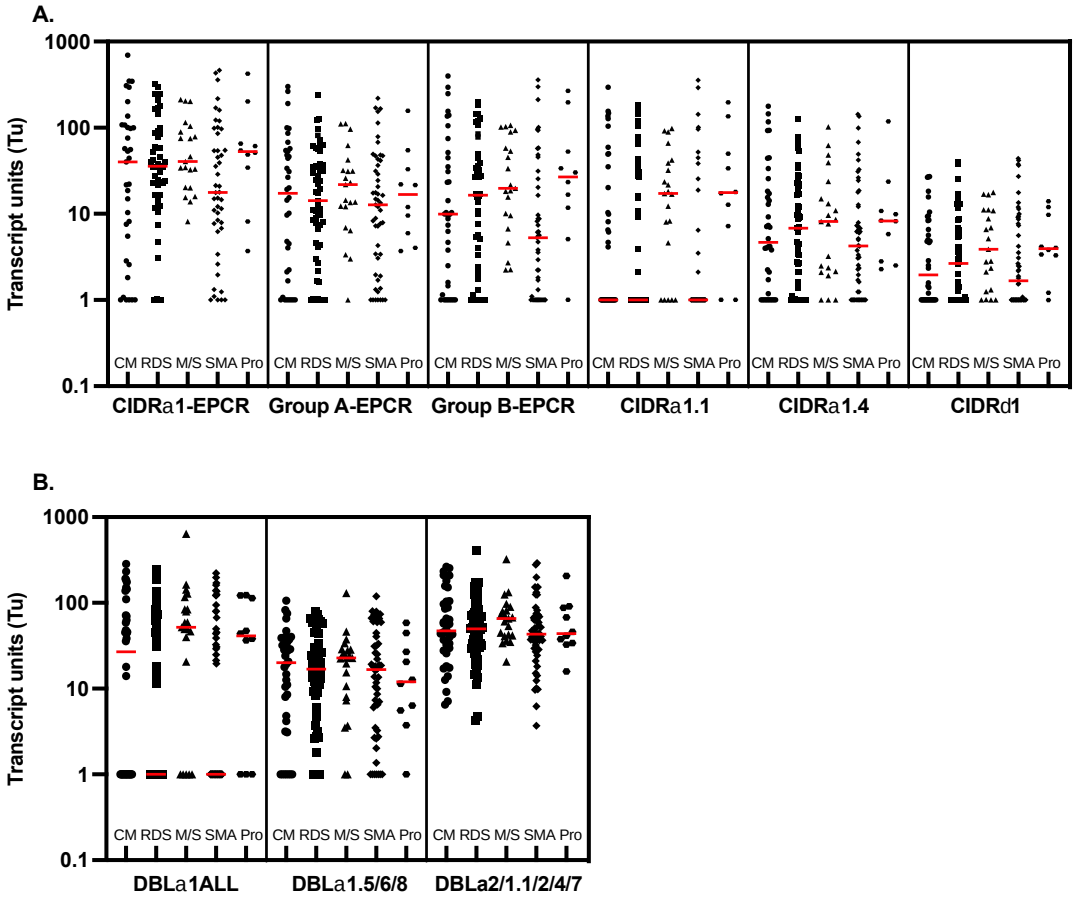
For continuous variables, data presented as median [IQR], and compared using Wilcoxon rank sum test. Dichotomous variables were compared using Chi-squared test.
[a]n=176 for parasite burden, hemoglobin, and platelet count.
[b]n=419 for weight age adjusted z-scores, n=422 for parasite density, n=417 for parasite burden, n=420 for hemoglobin and platelet count
[c]n=26 for hemoglobin and platelet count

4.3.2 Children with severe malaria have similar levels of *var* transcripts

Among children with the five most common forms of severe malaria in Uganda, CM, RDS, M/S, SMA and Pro, only expression of group B EPCR-binders and CIDRα1.1 showed significant differences in expression across the five groups. Children with SMA had significantly lower expression of CIDRα1.1 (median [IQR], n; 1 [1-1.55], 52) than children with M/S (17.2 [4.62-38.74], 21) and Pro (17.63 [7.22-49.77], 10) (Figure 4.2, Table 4.4). Transcript levels for all other domains of PfEMP1 did not differ significantly between the 5 forms of severemalaria (Figure 4.2, Table 4.4). In the 5 community

children with asymptomatic parasitemia and RNA sufficient quantities to test for multiple *var* gene transcript levels, transcript levels were typically lower than in the children with severe malaria, but the difference was statistically significant only for DBLβ S3.1 (Table 4.5). The small number of CC with available RNA provided statistical power for detection of only very large differences between children with SM and CC.

Figure 4.2. Transcript units of EPCR-binding domains of PfEMP1 in children with five different manifestations of severe malaria.



Transcript units (Tu) for EPCR-binding domains are similar amongst children with five forms of severe malaria, cerebral malaria (CM), respiratory distress syndrome (RDS), malaria with repeated seizures (M/S), severe malarial anemia (SMA) and prostration (Pro). (A) Tu of CIDRα1-EPCR, group A-ECPR, group B-EPCR (the sum of certain CIDRα1 domains, see methods), CIDRα1.1, CIDRα1.4 and CIDRδ1 (group A PfEMP1, non-EPCR-binding) in children with severe malaria. (B) Tu of DBLα1ALL (all group A PfEMP1), DBLα1.5/6/8 types (group A PfEMP1, typically non-EPCR-binding) and DBLα2/1.1/2/4/7 (group A PfEMP1, typically EPCR-binding). Tu of expression shown on a logarithmic scale. Horizontal lines in red represent the median Tu of each group.

Table 4.4. Transcript levels of *var* domains in children with five forms of severe malaria.

Domain	Family/ Group	Binding	CM	RDS	M/S	SMA	Pro	P-value[a]
CIDRα1 Domains	Group A and B	EPCR	39.99 (2.84-107.05) (35)	35.60 (16.51-87.23) (50)	40.48 (20.33-89.07) (21)	17.74 (6.77-85.63) (46)	52.77 (34.31-65.45) (10)	0.29
Group A EPCR-binders	Group A	EPCR	17.28 (1.89-53.65) (36)	14.22 (4.36-36.51) (55)	21.85 (12.12-31.84) (21)	12.72 (1.95-41.42) (51)	16.81 (5.97-32.94) (10)	0.81
CIDRα1.4	Group A	EPCR	4.67 (1.00-18.31) (38)	6.80 (1.48-18.88) (56)	8.16 (2.15-14.97) (21)	4.23 (1.12-12.44) (52)	8.27 (2.80-10.80) (10)	0.66
CIDRα1.5a	Group A	EPCR	1.00 (1.00-1.00) (38)	1.00 (1.00-1.78) (55)	1.00 (1.00-2.72) (21)	1.00 (1.00-2.06) (52)	2.13 (1.00-7.67) (10)	0.33
CIDRα1.5b	Group A	EPCR	1.00 (1.00-1.00) (38)	1.00 (1.00-1.00) (55)	1.00 (1.00-2.59) (21)	1.00 (1.00-1.00) (52)	1.00 (1.00-1.00) (10)	0.78
CIDRα1.6b	Group A	EPCR	1.00 (1.00-3.92) (38)	1.00 (1.00-1.85) (55)	2.43 (1.00-3.53) (21)	1.00 (1.00-3.77) (52)	1.00 (1.00-2.59) (10)	0.61
CIDRα1.7	Group A	EPCR	2.40 (1.00-7.44) (36)	2.76 (1.00-8.33) (55)	5.44 (3.54-8.95) (21)	1.88 (1.00-6.92) (51)	3.36 (1.14-9.10) (10)	0.29
Group B EPCR-binders	Group B	EPCR	9.89 (1.46-44.68) (37)	16.45 (2.38-45.01) (50)	19.76 (9.54-57.80) (21)	5.26 (1.00-25.46) (47)	26.73 (11.79-52.65) (10)	**0.04[b]**
CIDRα1.1	Group B	EPCR	1.00 (1.00-35.25) (38)	1.00 (1.00-29.20) (56)	17.20 (4.62-38.74) (21)	1.00 (1.00-1.55) (52)	17.63 (7.22-49.77) (10)	**0.01[c]**
CIDRα1.8a	Group B	EPCR	1.00 (1.00-4.84) (37)	1.38 (1.00-12.10) (50)	2.34 (1.00-8.40) (21)	1.21 (1.00-4.72) (47)	2.67 (1.00-5.43) (10)	0.84
CIDRα1.8b	Group B	EPCR	1.00 (1.00-11.80) (38)	1.16 (1.00-3.24) (55)	3.62 (1.32-5.61) (21)	1.05 (1.00-4.67) (52)	2.70 (1.00-4.13) (10)	0.16
CIDRδ1	Group A	Unknown	1.95 (1.00-8.34) (38)	2.66 (1.00-6.53) (55)	3.88 (1.31-10.86) (21)	1.67 (1.00-8.15) (52)	3.95 (3.32-9.77) (10)	0.35
DBLα1All	Group A		26.86 (1.00-71.47) (38)	1.00 (1.00-62.67) (54)	52.00 (20.68-83.88) (21)	1.00 (1.00-58.60) (52)	41.07 (1.00-113.32) (10)	0.13
DBLα1.5/6/8	Group A		20.00 (4.18-37.36) (38)	16.88 (8.94-29.83) (54)	22.77 (8.02-28.49) (21)	16.63 (3.37-42.48) (52)	12.05 (5.56-26.98) (10)	0.97

DBLα2/1.1/2/4/7	Group A		47.06 (27.78-103.54) (38)	49.85 (30.00-75.82) (56)	65.91 (41.69-81.58) (21)	43.23 (27.99-69.29) (51)	43.73 (34.08-87.68) (10)	0.50
DBLβ Motif 1			6.55 (1.83-30.41) (34)	5.59 (1.99-23.89) (54)	9.77 (1.46-23.26) (20)	7.82 (1.71-22.26) (50)	4.50 (1.00-11.74) (10)	0.77
DBLβs3.1	Group A	ICAM-1	1.30 (1.00-4.18) (38)	1.45 (1.00-7.19) (56)	3.49 (1.00-10.22) (21)	1.76 (1.00-4.35) (52)	2.08 (1.00-8.28) (10)	0.63
DBLβs3.2	Group A	ICAM-1	3.58 (1.18-8.81) (38)	2.94 (1.00-9.40) (55)	4.36 (1.58-14.31) (20)	2.74 (1.00-6.74) (51)	4.99 (1.99-11.94) (10)	0.76
DBLβ5 S1	Group B	ICAM-1	6.55 (1.00-17.55) (37)	2.45 (1.00-8.59) (51)	6.82 (2.23-78.27) (21)	2.09 (1.00-7.97) (50)	2.34 (1.00-5.93) (10)	0.05
DBLε14	C terminal	Unknown	1.00 (1.00-3.15) (37)	1.00 (1.00-1.12) (53)	1.00 (1.00-1.62) (20)	1.00 (1.00-1.92) (51)	1.00 (1.00-1.40) (10)	0.55
DBLζ2a	C terminal	Unknown	3.00 (1.23-6.97) (36)	1.40 (1.00-2.93) (52)	3.33 (1.00-5.90) (17)	2.78 (1.18-5.85) (49)	1.59 (1.00-3.99) (10)	0.08
DBLζ3	C terminal	Unknown	9.71 (3.77-17.11) (38)	7.49 (3.70-16.26) (56)	10.71 (8.47-19.45) (21)	7.65 (3.43-17.38) (52)	7.67 (5.21-12.93) (10)	0.30
DBLζ4	C terminal	Unknown	3.77 (1.85-9.13) (37)	2.65 (1.38-10.06) (55)	4.23 (2.02-8.39) (21)	2.47 (1.00-7.00) (52)	3.66 (1.75-4.41) (10)	0.34
DBLζ5	C terminal	Unknown	5.87 (1.44-12.32) (37)	5.12 (1.54-14.26) (56)	3.06 (1.27-7.80) (21)	4.57 (1.00-11.15) (52)	5.68 (1.00-15.73) (10)	0.83
DBLζ6	C terminal	Unknown	4.09 (1.38-7.18) (38)	2.98 (1.55-5.11) (56)	3.45 (1.64-5.21) (21)	2.05 (1.13-3.59) (52)	2.79 (1.26-5.24) (10)	0.37

Data presented as median Tu [IQR] (n).

[a]P-values based on Kruskal Wallis test to compare the Tu of children with severe malaria.

[b]No groups were significantly different based on post hoc pairwise Dunn test.

[c]SMA is different than Pro and M/S based on post-hoc Dunn test.

Table 4.5. Transcript levels of *var* domains in children any of the five forms of severe malaria and asymptomatic community children.

Domain	Family/ Group	Severe Malaria (n=177)	AP (n=5)	P-value[a]
CIDRα1.1	Group A, EPCR	1.00 (1.00-27.49)	1.00 (1.00-1.00)	0.06
CIDRα1.4	Group A, EPCR	6.01 (1.62-17.23)	3.82 (1.00-13.14) (4)	0.40
DBLα1AII	Group A	18.03 (1.00-73.57) (175)	31.99 (1.00-62.98) (2)	0.92
DBLα1.5/6/8	Group A	18.11 (6.03-33.73) (175)	10.60 (7.94-13.79)	0.52
DBLα2/1.1/2/4/7	Group A	46.75 (30.85-81.53) (176)	45.92 (21.31-58.74)	0.43
DBLβ Motif 1		6.72 (1.77-23.94) (168)	1.00 (1.00-4.69)	0.06
DBLβs3.1	Group A, ICAM-1	1.76 (1.00-6.46)	1.00 (1.00-1.00)	**0.02**
DBLβs3.2	Group A, ICAM-1	3.35 (1.02-9.75) (174)	3.94 (3.73-8.70)	0.51
DBLβ5 S1	Group B, ICAM-1	3.11 (1.00-10.71) (169)	13.58 (8.80-39.40)	0.08
DBLε14	C terminal	1.00 (1.00-1.67) (171)	1.00 (1.00-2.12)	0.77
DBLζ2a	C terminal	2.23 (1.00-5.65) (164)	1.81 (1.00-4.01)	0.50
DBLζ3	C terminal	8.73 (4.34-17.11)	3.78 (2.78-14.51)	0.43
DBLζ4	C terminal	3.41 (1.37-9.04) (175)	3.17 (1.00-7.59)	0.82
DBLζ5	C terminal	5.19 (1.22-12.50) (176)	15.04 (7.32-32.92)	0.16
DBLζ6	C terminal	2.78 (1.26-5.18)	1.16 (1.00-1.22)	0.08

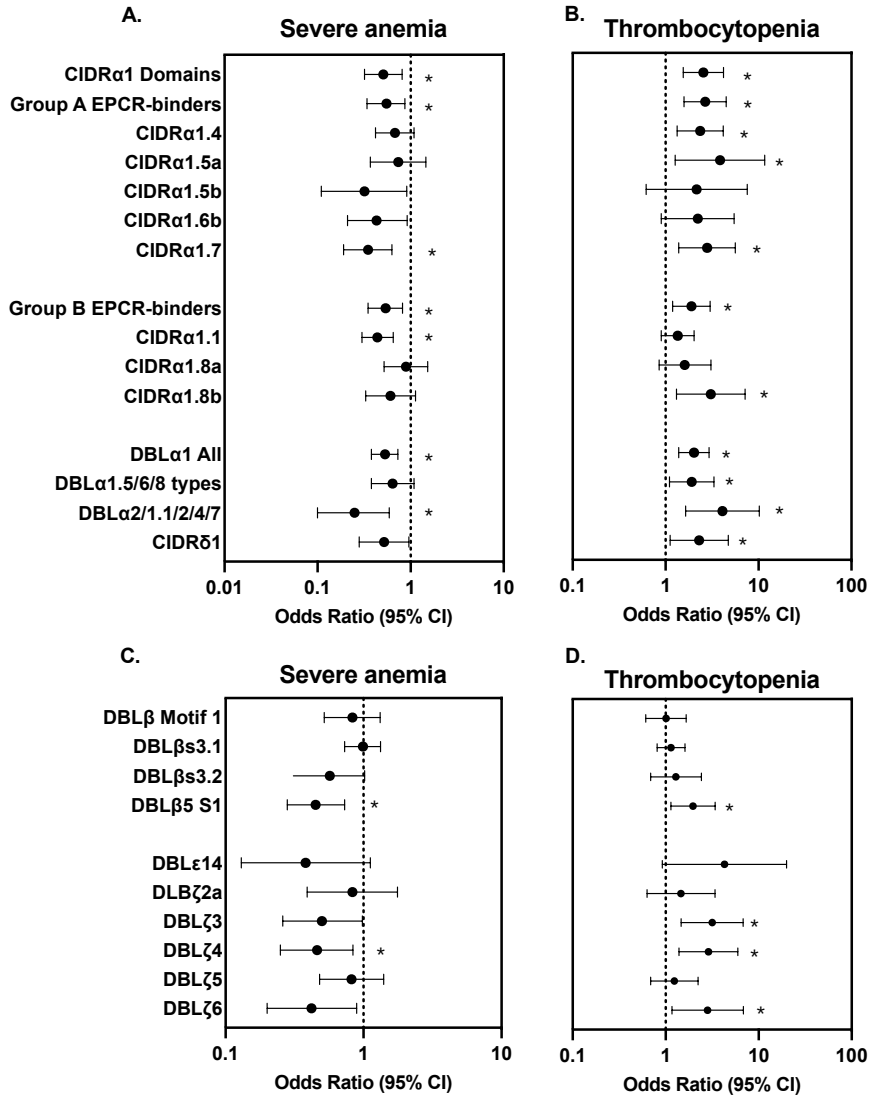
Data presented as median Tu [IQR]. n's are listed following IQR for domains with less than the total number of children tested. Loss of samples due to multiple Tms, see methods.

[a]P-value based on Kruskal Wallis test to compare the Tu of children with any form of severe malaria to children with asymptomatic parasitemia (AP).

4.3.3 Specific *var* transcript levels are associated with a decreased risk of severe anemia and an increased risk of thrombocytopenia, but not with mortality or parasite burden

To understand how expression of *var* transcripts expressing specific PfEMP1 domains could be impacting clinical outcomes, we evaluated the relationships between *var* transcripts of PfEMP1 domains and mortality, retinopathy, parasite density, anemia and thrombocytopenia. In children with severe malaria, transcript levels of numerous EPCR-binding domains and DBLα domains were associated with decreased risk of severe anemia (hemoglobin <5 g/dL) and increased risk of thrombocytopenia (platelet count <150,000 platelets/μL) (Figure 4.3A,B). When looking at ICAM-1-binding domains, DBLβ S5 was associated with decreased risk of severe anemia and increased risk of thrombocytopenia (Figure 4.3C,D). Few of the C-terminal DBLε/z domains showed the same associations; only DBLζ4 was associated with both markers of severe disease (Figure 4.3C,D). Increased transcript levels of CIDRα1.1, CIDRα1.4 and DBLα1ALL were associated with increased parasite density (Table 4.6). There were no significant associations between any *var* transcript and plasma *Pf*HRP2 (a surrogate measure of parasite burden), mortality, or the presence of malarial retinopathy (Tables 4.6, 4.7).

Figure 4.3. *var* transcript levels are associated with increased risk of thrombocytopenia and decreased risk of severe anemia in children with severe malaria.



Var transcript levels of EPCR-binding domains are associated with a decreased risk of severe anemia in children with any form of severe malaria (A) and an increased risk of thrombocytopenia (B). Transcript levels for ICAM-1-binding domains and additional C terminal domains were similarly associated with decreased risk of severe anemia (C) and increased thrombocytopenia (D). Anemia defined as hemoglobin <5 g/dL. Thrombocytopenia defined as platelet count <150,000 platelets/μL Association estimated using logistic regression with $\log_{10}$ transformed transcript levels. *P<0.05 and below FDR threshold to correct for the number of domains.

Table 4.6. Associations between *var* transcript levels and markers of parasitemia.

	Parasite Density			Parasite Burden (*Pf*HRP2)		
	n	Beta (95% CI)	P-value	n	Beta (95% CI)	P-value
CIDRα1 Domains	159	0.28 [0.15, 0.41]	**<0.0001**	161	0.09 [-0.09, 0.28]	0.32
Group A EPCR-binders	170	0.23 [0.11, 0.35]	**0.0001**	172	0.11 [-0.06, 0.28]	0.21
CIDRα1.4	174	0.17 [0.06, 0.28]	**0.002**	176	0.09 [-0.07, 0.25]	0.26
CIDRα1.5a	173	0.03 [-0.05, 0.10]	0.51	175	0 [-0.11, 0.11]	0.96
CIDRα1.5b	173	0.04 [-0.01, 0.10]	0.12	175	-0.02 [-0.10, 0.06]	0.65
CIDRα1.6b	173	0.03 [-0.04, 0.11]	0.36	175	0.05 [-0.06, 0.15]	0.39
CIDRα1.7	170	0.13 [0.04, 0.23]	**0.01**	172	0.02 [-0.12, 0.16]	0.79
Group B EPCR-binders	162	0.31 [0.17, 0.44]	**<0.0001**	164	0.07 [-0.13, 0.26]	0.50
CIDRα1.1	174	0.30 [0.17, 0.44]	**<0.0001**	176	-0.05 [-0.26, 0.16]	0.64
CIDRα1.8a	162	0.13 [0.02, 0.23]	0.02	164	-0.04 [-0.18, 0.11]	0.60
CIDRα1.8b	173	0.07 [-0.02, 0.15]	0.12	175	0.12 [0.00, 0.24]	0.04
CIDRδ1	173	0.05 [-0.03, 0.14]	0.24	175	0.03 [-0.09, 0.15]	0.61
DBLα1All	172	0.44 [0.29, 0.60]	**<0.0001**	174	0.04 [-0.20, 0.28]	0.76
DBLα1.5/6/8	172	0.11 [0.00, 0.21]	0.04	174	0.02 [-0.12, 0.17]	0.75
DBLα2/1.1/2/4/7	173	0.06 [-0.01, 0.13]	0.08	175	0.01 [-0.09, 0.10]	0.84
DBLβ Motif 1	165	-0.03 [-0.14, 0.09]	0.66	167	-0.05 [-0.22, 0.11]	0.52
DBLβs3.1	174	-0.14 [-0.30, 0.02]	0.09	176	0.03 [-0.23, 0.28]	0.84
DBLβs3.2	172	-0.01 [-0.11, 0.08]	0.79	173	-0.02 [-0.15, 0.11]	0.71
DBLβ5 S1	167	0.03 [-0.10, 0.16]	0.61	168	0.10 [-0.08, 0.28]	0.28
DBLε14	168	0.05 [0.00, 0.10]	0.07	170	0 [-0.07, 0.08]	0.95
DBLζ2a	161	-0.06 [-0.13, 0.02]	0.12	163	-0.02 [-0.12, 0.09]	0.75
DBLζ3	174	0.04 [-0.04, 0.12]	0.30	176	0.01 [-0.11, 0.12]	0.89
DBLζ4	172	0.08 [0.00, 0.17]	0.06	174	0.14 [0.01, 0.26]	0.03
DBLζ5	173	-0.05 [-0.14, 0.05]	0.31	175	-0.07 [-0.21, 0.07]	0.32
DBLζ6	174	0.01 [-0.06, 0.08]	0.80	176	0.07 [-0.04, 0.17]	0.20

var transcript levels and markers of parasite density were log10 transformed for linear regression analysis.

Table 4.7. Associations between *var* transcript levels and clinical outcome of severe malaria.

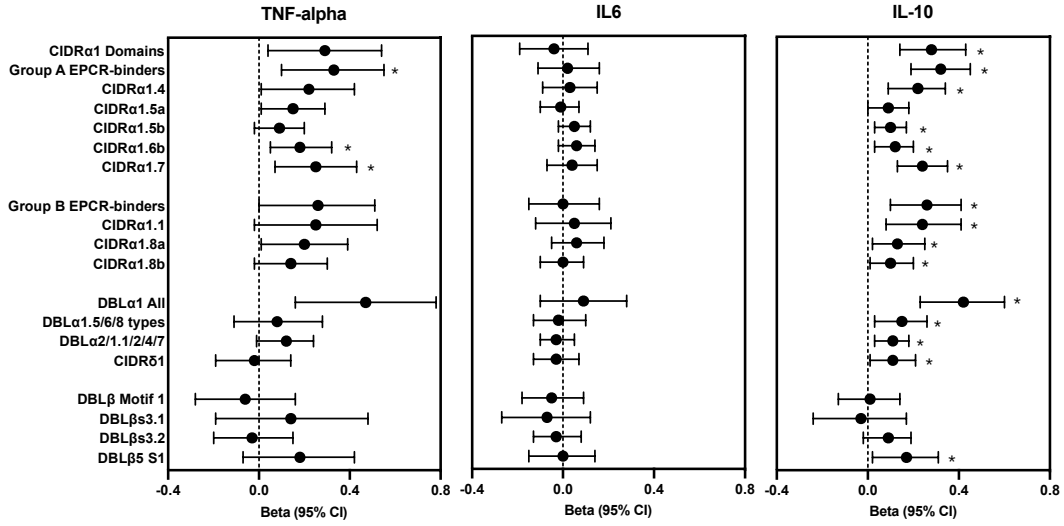
	Mortality			Malarial Retinopathy		
	N obs, N died[a]	Odds Ratio (95% CI)	P-value	N obs, N pos[a]	Odds Ratio (95% CI)	P-value
CIDRα1 Domains	162, 14	0.53 [0.26, 1.11]	0.09	162, 19	0.59 [0.38, 0.92]	0.02
Group A EPCR-binders	173, 15	0.66 [0.30, 1.44]	0.29	173, 21	0.72 [0.46, 1.12]	0.15
CIDRα1.4	177, 16	1.15 [0.52, 2.56]	0.73	177, 22	0.89 [0.55, 1.42]	0.61
CIDRα1.5a	176, 16	0 [0.00, 11.90]	0.17	176, 22	0.92 [0.46, 1.82]	0.80
CIDRα1.5b	176, 16	0.85 [0.17, 4.31]	0.84	176, 22	1.46 [0.61, 3.50]	0.40
CIDRα1.6b	176, 16	1.58 [0.52, 4.78]	0.42	176, 22	1.26 [0.62, 2.58]	0.52
CIDRα1.7	173, 15	0.31 [0.08, 1.10]	0.07	173, 21	0.61 [0.35, 1.07]	0.09
Group B EPCR-binders	165, 15	0.77 [0.38, 1.56]	0.47	165, 20	0.67 [0.45, 1.01]	0.06
CIDRα1.1	177, 16	0.73 [0.37, 1.45]	0.37	177, 22	0.71 [0.49, 1.03]	0.07
CIDRα1.8a	165, 15	1.40 [0.59, 3.30]	0.45	165, 20	0.76 [0.44, 1.32]	0.33
CIDRα1.8b	176, 16	0.92 [0.31, 2.74]	0.88	176, 22	1.22 [0.66, 2.26]	0.52
CIDRδ1	176, 16	0.54 [0.17, 1.70]	0.29	176, 22	0.56 [0.30, 1.03]	0.06
DBLα1All	175, 15	1.03 [0.60, 1.76]	0.93	175, 22	0.81 [0.59, 1.11]	0.18
DBLα1.5/6/8	175, 16	0.62 [0.27, 1.46]	0.28	175, 22	0.78[0.47, 1.31]	0.36
DBLα2/1.1/2/4/7	176, 16	0.33 [0.09, 1.25]	0.10	176, 22	0.34 [0.15, 0.78]	0.01
DBLβ Motif 1	168, 13	1.36 [0.57, 3.22]	0.49	168, 21	0.77 [0.48, 1.23]	0.28
DBLβs3.1	177, 16	0.51 [0.19, 1.37]	0.18	177, 22	1.05 [0.78, 1.41]	0.77
DBLβs3.2	174, 16	1.03 [0.38, 2.77]	0.96	174, 22	0.88 [0.49, 1.56]	0.66
DBLβ5 S1	169, 14	1.18 [0.58, 2.42]	0.64	169, 22	1.38 [0.90, 2.12]	0.13
DBLε14	171, 15	4.24 [1.04, 17.25]	0.04	171, 21	0.71 [0.24, 2.07]	0.53
DBLζ2a	164, 15	1.31 [0.37, 4.66]	0.67	164, 20	1.17[0.55, 2.49]	0.68
DBLζ3	177, 16	0.75 [0.24, 2.32]	0.62	177, 22	0.59 [0.30, 1.13]	0.11
DBLζ4	175, 16	1.71 [0.64, 4.56]	0.29	175, 22	1.09 [0.60, 1.96]	0.78
DBLζ5	176, 16	0.56 [0.21, 1.51]	0.25	176, 21	0.72 [0.42, 1.25]	0.24
DBLζ6	177, 16	2.01 [0.61, 6.56]	0.25	177, 22	1.11 [0.54, 2.31]	0.77

Logistic regression model of log transformed *var* transcript levels and clinical outcomes.
[a]N obs, number of observations in regression model; N died, number of children that died; N pos, number of children with malaria retinopathy.

4.3.4 Var transcript levels of domains that interact with the endothelium are associated with levels of cytokines and sEPCR

CIDRα1 domains that bind EPCR and DBLβ domains that bind ICAM-1 are known to interact with the host endothelium during severe malaria. We wanted to see if the expression of these PfEMP1 domains were related to pro- or anti-inflammatory cytokines, markers of endothelial activation, or soluble forms of PfEMP1 binding partners. In children with severe malaria, increased expression of PfEMP1 group A EPCR-binding domains, particularly CIDRα1.6b (beta (95% CI), p-value; 0.18 [0.05, 0.32], p=0.01) and CIDRα1.7 (0.25 [0.07, 0.43], p=0.01), are associated with increased serum TNF-α levels (Figure 4.4). No significant associations were identified with IL-6, another pro-inflammatory cytokine involved in severe malaria pathogenesis. Increased transcript levels of every EPCR-binding domain tested, other than CIDRα1.5a, were associated with increased IL-10 levels, along with the ICAM-1 binder DBLβ S51 (Figure 4.4). We did not see consistent associations between these domains and markers of endothelial activation or soluble ICAM-1; however, transcript levels of all but three EPCR-binding domains and DBLβs3.2 were associated with decreased sEPCR levels in children with severe malaria (Table 4.8).

Figure 4.4. *var* transcript levels of EPCR and ICAM-1-binding domains are associated with serum levels of TNF-α and IL-10 in children with severe malaria.



Var transcript levels of EPCR-binding domains are ICAM-1-binding domains are selectively associated with increased serum TNF-α levels, and IL-10 levels but not IL-6 levels in children with any forms of severe malaria. Levels of *var* transcript and serum cytokines log10 transformed for linear regression analysis. *P<0.05 and below FDR threshold to correct for the number of domains.

Table 4.8. Associations between *var* transcript levels of EPCR-binding domains and ICAM-1-binding domains and levels of sEPCR and sICAM-1 in children with severe malaria.

	sEPCR			sICAM-1		
	n	**beta (95% CI)**	**P-value**	**n**	**beta (95% CI)**	**P-value**
CIDRα1 Domains	151	-0.83 [-1.18, -0.48]	**<0.0001**	161	0.17 [-0.49, 0.83]	0.61
Group A EPCR-binders	162	-0.84 [-1.14, -0.54]	**<0.0001**	172	0.16 [-0.44, 0.76]	0.61
CIDRα1.4	166	-0.47 [-0.76, -0.18]	**0.002**	176	0.38 [-0.17, 0.92]	0.17
CIDRα1.5a	165	-0.30 [-0.49, -0.10]	**0.004**	175	0.03 [-0.35, 0.41]	0.87
CIDRα1.5b	165	-0.09 [-0.24, 0.07]	0.28	175	0.02 [-0.28, 0.31]	0.92
CIDRα1.6b	165	-0.28 [-0.47, -0.09]	**0.004**	175	-0.06 [-0.42, 0.30]	0.75
CIDRα1.7	162	-0.66 [-0.90, -0.41]	**<0.0001**	172	-0.35 [-0.83, 0.14]	0.16
Group B EPCR-binders	154	-0.53 [-0.90, -0.15]	**0.01**	164	0.29 [-0.41, 0.98]	0.42
CIDRα1.1	166	-0.45 [-0.83, -0.06]	**0.02**	176	0.12 [-0.60, 0.84]	0.74
CIDRα1.8a	154	-0.28 [-0.56, -0.01]	0.05	164	0.24 [-0.28, 0.75]	0.36
CIDRα1.8b	165	-0.19 [-0.42, 0.03]	0.09	175	0.12 [-0.30, 0.55]	0.56
CIDRδ1	165	-0.35 [-0.58, -0.13]	**0.002**	175	0.15 [-0.29, 0.58]	0.50
DBLα1All	164	-0.62 [-1.06, -0.19]	**0.01**	174	0.76 [-0.06, 1.58]	0.07
DBLα1.5/6/8	164	-0.22 [-0.49, 0.05]	0.11	174	0.01 [-0.50, 0.51]	0.98
DBLα2/1.1/2/4/7	165	-0.41 [-0.58, -0.25]	**<0.0001**	175	-0.03 [-0.36, 0.30]	0.84
DBLβ Motif 1	159	-0.13 [-0.45, 0.19]	0.42	167	-0.11 [-0.68, 0.46]	0.70
DBLβs3.1	166	-0.40 [-0.88, 0.08]	0.10	176	-0.79 [-1.65, 0.07]	0.07
DBLβs3.2	163	-0.35 [-0.59, -0.12]	**0.003**	173	-0.43 [-0.88, 0.02]	0.06
DBLβ5 S1	158	-0.36 [-0.70, -0.01]	0.04	168	0.02 [-0.61, 0.65]	0.95

Levels of *var* transcript and serum cytokines log10 transformed for linear regression analysis. Analysis corrected for multiple comparisons using FDR threshold to correct for the number of domains, bolded P-values are below threshold.

4.4 Discussion

In this study, we measured transcript levels of PfEMP1 domains that bind to ECPR, ICAM-1 and additional group A and C terminal domains, to understand whether their expression differed in children with five forms of severe malaria. Of the domains measured in this study, only CIDRα1.1 was differentially expressed, with elevated transcript levels in children with malaria with repeated seizures (M/S) and prostration (Pro) compared to children with severe malarial anemia (SMA). We identified associations between these PfEMP1 domains and markers of severe disease, such as severe anemia and thrombocytopenia. We also demonstrated positive correlations between EPCR-binding domains and plasma levels of the proinflammatory cytokine TNF-α, anti-inflammatory cytokine IL-10, and a negative correlation between these domains and soluble plasma levels of EPCR. Overall, the results of this study show that the levels of transcript units of PfEMP1 domains do not different significantly between children with different forms of severe malaria, with the exception of CIDR1a, which was differentially expressed at higher levels in children with malaria with repeated seizures or prostration, and are associated, particularly the EPCR-binding domains, in complex ways with pro- and anti-inflammatory cytokines, resulting, somewhat counter-intuitively, in a decreased risk of severe anemia but an increased risk of thrombocytopenia.

Although transcript levels of EPCR-binding domains[113] and some ICAM-1 binding domains (chapter 3) were previously shown to be different in children with CM compared to children with SMA, this study showed similar levels of PfEMP1 domains across children with five forms of severe malaria. Transcript levels of CIDRα1.1 were elevated in children with M/S and Pro compared to SMA. CIDRα1.1 is the EPCR-binding domain of DC8 and has been consistently associated with severe malaria. Transcript levels of numerous EPCR-binding domains were elevated in children with CM compared to SMA, but not to significant levels. This is similar to results from our group's previous

study[113], in which CIDRα1.1 was significantly lower in children with SMA compared to CM. Thus, the lack of significant differences between severe malaria groups could be due to decreased number of children in each group and thus a decreased power to detect small differences in *var* transcripts. Children enrolled in this study are younger than children in the previous cohort and enrolled at a second study site with differing transmission rates and possible parasite genetics. These differences could result in alterations in *var* transcript patterns, although unlikely to alter the levels of specific transcripts across different groups of severe malaria. It is possible that levels of *var* transcripts are related to disease severity, not specific symptoms of severe malaria and thus elevated in more severe manifestations of severe malaria, such as CM, compared to other manifestations of severe malaria. These differences may not have been detected here because of symptom overlap within groups or the spectrum of manifestations of severe malaria included in the study.

However, the inclusion of additional groups of severe malaria beyond CM and SMA, to respiratory distress syndrome (RDS), M/S, and Pro, provides a more inclusive understanding of severe malaria. Studying transcript levels of PfEMP1 domains in this population allows for improved understanding of how the parasite is impacting these manifestations of severe disease; whether the parasite specifically targets different tissues or if widespread inflammation and endothelial activation can present with different symptoms. The similar transcript units across all five groups of severe malaria of the measured PfEMP1 domains suggest that in this population, PfEMP1 is playing a similar role across the different forms of severe malaria. Since PfEMP1 has been consistently linked to CM pathogenesis[117,128], it may be playing a similar role in less severe and less well characterized forms of severe malaria. We expected to see differences in expression of PfEMP1 domains with different severe malaria manifestations, particularly between manifestations of severe malaria with higher

mortality (CM and RDS) compared to less severe forms of severe malaria (SMA, M/S and Pro). However, the results are not surprising and suggest that the parasite is not targeting tissue-specific receptors in different forms of severe malaria. Instead, these finding suggested the parasite is benefiting from increased binding targets from general endothelial activation throughout the host.

We did not find associations between the transcript levels of PfEMP1 domains and mortality, parasite burden or malarial retinopathy, all outcomes previously associated with PfEMP1 domain expression[113,117,118]. Consistent with other studies[113,121], children in this study with any form of severe malaria who survived trended towards elevated PfEMP1 expression compared to children who died, although none of the differences were significant, likely due to limited sample size. Because binding of PfEMP1 to the endothelium contributes to sequestration during severe malaria, we expected *var* transcripts to be associated with parasite burden, measured by PfHRP2 levels; however, here only transcript levels of CIDRα1.1 and CIDRα1.4 were associated with increased parasite density, another measure of parasites in circulation that doesn't estimate sequestered parasites. Associations between the transcript levels of group A *var* transcripts trended towards a positive association with *Pf*HRP2, consistent with previous studies[117,118], demonstrating that associations could be identified with larger sample sizes.

However, in this population of children with severe malaria, elevated *var* transcripts of EPCR-binding domains were associated with an increased risk of thrombocytopenia, consistent with previous studies[117,118] and data in this dissertation (Chapter 3), along with a decreased risk of the presence of severe anemia. The relationship between the expression of PfEMP1 domains and increased risk thrombocytopenia or lower platelet counts is not well understood but because of the numerous roles of platelets in severe malaria pathogenesis, domains of PfEMP1 could

be involved in several processes. Briefly, activated platelets can aggregate and obstruct the microvasculature[34], bind to endothelial cells to provide receptors to infected erythrocytes[123], or inhibit parasite growth[35,36], as a protective mechanism to fight infection. More studies are needed to better understand how PfEMP1 domains are directly involved with platelets in children with severe malaria.

The association between EPCR-binding domains in children with severe malaria and select DBL domains and decreased risk of severe anemia, hemoglobin <5 g/dL, is surprising as anemia is a marker of severe malaria, and we would expect transcript levels of EPCR-binding domains to be related to disease severity. Previously, transcript levels of CIDRα1.4 were highest in children with CM and hemoglobin >8 g/dL[121], but not other measured domains. Elevated expression of PfEMP1 domains has not otherwise been related to increased hemoglobin levels, although decreased transcript levels of these domains have been seen in children with SMA compared to CM[113]. To understand the mechanism underlying the association between PfEMP1 domain expression and hemoglobin, the expression of these domains could relate to plasma levels of other markers within the hemolysis pathway, such as haptoglobin, lactate dehydrogenase or total bilirubin. Another mechanism to examine could be related to the availability of erythrocytes for PfEMP1 expression, with increased lysis of erythrocytes resulting in anemia leaving less erythrocytes for parasite infection and measurable *var* transcript expression.

Var transcript levels for group A and B EPCR-binding domains and DBLα domains were associated with increased concentrations of IL-10, while transcript levels of two group A EPCR-binding domains, CIDRα1.6a and CIDRα1.7 were associated with increased concentrations of TNF-α. IL-10 acts as an anti-inflammatory cytokine to decrease excessive inflammation during malaria infection and has been shown to be protective in mouse models of severe malaria[130] and CM[131]. High levels of IL-10 have

been seen in children with severe malaria[24]. Additionally, African children with severe anemia had lower levels of IL-10 than moderate anemia or CM, suggesting IL-10 may play a role in protection from severe anemia[132]. The ratio of TNF-α to IL-10 is also an important marker of the inflammatory status, as it can be a surrogate marker for the balance of pro- and anti-inflammatory signaling. The TNF-α/IL-10 ratio has been shown to be able to differentiate SMA from CM[132,133], with a higher ratio being associated with SMA. In this study, increased *var* transcripts were associated with a decreased TNF-α/IL-10 ratio (data not shown), increased IL-10 concentrations and decreased risk of anemia. This suggests that one reason why increased *var* transcripts are associated with a decreased risk of anemia is that they may influence the TNF- α/IL-10 ratio, possibly stimulating the release of IL-10 during endothelial activation, which is thought to stimulate hematopoiesis[134], thus possibly protecting from anemia. Conversely, these *var* transcripts could be bystanders in the interactions between TNF- α, IL-10 and the presence of anemia.

This study has numerous strengths, with an expansive dataset available for children in this cohort with respect to clinical and immunological markers to inform on the role of these domains in severe malaria. However, limited sample availability decreased the sample size, particularly with five groups of severe malaria, meaning we could have missed possible differences between children with different forms of severe malaria, as these differences are often small. However, 177 children with severe malaria were included in the final analysis, allowing us to detect meaningful associations between the expression of these PfEMP1 domains and markers of severe disease. Measuring the expression of PfEMP1 domains can be difficult with sequence diversity and complex domain structures, but the primers in this study are designed against sequences from many field isolates, accounting for variation in PfEMP1 domains and providing a more complete idea of PfEMP1 expression in this population.

In this study, we demonstrated that children with different manifestations of severe malaria, including manifestations not frequently included in these studies, have similar levels of *var* transcripts of EPCR-binding domains, ICAM-1-binding domains and additional DBL domains. We also linked the expression of these domains to some clinical outcomes such as severe anemia and thrombocytopenia, but not did not identify associations with mortality or parasite burden. The results of this study suggest that despite differences in severe malaria pathogenesis of various manifestations of severe malaria, PfEMP1 may be acting in a similar manner across these different manifestations, or the biggest differences between children with CM and non-cerebral severe malaria. Next steps for this research could include the development of a covariate model to understand how the various markers of disease severity, pro-inflammatory cytokines and soluble binding partners that were associated with *var* transcripts in this study could interacting to perpetuate severe malaria pathogenesis. The covariates selected would be ones that we hypothesize are directly or indirectly interacting with PfEMP1 molecules on the surface of the infected erythrocyte or the host endothelium. This type of analysis would allow for a better understanding of which of the covariates could be more significantly interacting with PfEMP1 than others. Results from this study could be also applied to future *in vitro* studies to understand possible interactions between these PfEMP1 domains and platelets and erythrocytes. Future studies that characterize possible binding of PfEMP1 to platelets or interactions with the hemolysis pathways, could inform about additional mechanisms resulting in thrombocytopenia or severe anemia in children with severe malaria.

Chapter 5: Antibodies to PfEMP1 and other *P. falciparum* antigens are maintained in children with asymptomatic parasitemia but not in children with severe malaria

5.1 Introduction

Malaria is among the leading causes of morbidity and mortality globally, causing an estimated 241 million cases across the globe in 2020[6]. Approximately 90% of these cases occurred in Africa, where *Plasmodium falciparum* is predominant over other *Plasmodium* species and is the leading cause of severe disease symptoms. Severe malaria caused more than estimated 600,000 deaths in Africa in 2020, primarily in children under 5 years old, who are at the greatest risk for severe malaria caused by *P. falciparum*[6].

A key virulence factor during severe malaria pathogenesis is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a protein expressed by the parasite on the surface of infected erythrocytes. Expression of specific domains of PfEMP1 mediates the binding of infected erythrocytes to endothelial cells, contributing to the sequestration of infected erythrocytes in blood vessels, perpetuating severe malaria pathogenesis. Group a *var* genes have been associated with severe malaria[86,126], particularly those express CIDRα1 domains that bind to endothelial protein C receptor (EPCR) on the host endothelium[75]. These EPCR-binding domains can be found in tandem with other domains, particularly Duffy binding-like (DBL) domains, to form domain cassettes (DC). DC8 and DC13 are examples of groups of domains that have been consistently associated with severe malaria[116,121].

PfEMP1 is a major source of antigenic variation during malaria infection, as it is encoded for by over 60 var genes within the parasite genome[56,57] and composed of diverse combinations of domains that can mediate different binding patterns with the

host endothelium[56,124]. As a contributor of antigenic variation during infection, PfEMP1 plays a role in immune evasion, altering expression of *var* genes in response to host immunity[135], adding to the success of the parasite. Despite its role in immune evasion, antibodies against PfEMP1 develop naturally with exposure to the parasite[98,99] and specific domains have been associated with protection from clinical malaria[99,101,102].

However, the role of antibodies to some blood-stage and pre-erythrocytic antigens in protection from clinical malaria is better characterized than PfEMP1 antigens. Antibodies to antigens on the surface of merozoites, such as merozoite surface protein (MSP) [48,136,137] and erythrocyte binding antigens (EBA)[138,139], have been associated with protection. For people living in malaria-endemic regions, these antibodies to malaria are naturally acquired after infection and years of repeated exposure. However, sterile immunity does not develop, resulting in continued risk of infection[140]. The lack of sterile immunity to *P. falciparum* infection, contributes to the presence of asymptomatic parasitemia (AP) in malaria endemic areas, often at submicroscopic levels. Asymptomatic infection has been associated with a reduced risk of infection in high transmission areas[141]. Additionally, asymptomatic parasitemia could lead to the persistence of antibodies against *P. falciparum* antigens and continued protection from severe malaria episodes. Yet, the development of antibodies to PfEMP1 antigens, compared to other blood-stage or pre-erythrocytic antigens, in children with severe malaria compared to asymptomatic parasitemia, and the persistence of antibodies over time in children with these very different states of *P. falciparum* infection and disease, has not be well characterized.

In previous studies, we have shown that asymptomatic community children (CC), including those with AP, are less likely to have subsequent severe or uncomplicated malaria than children with severe malaria. We hypothesized that children with AP develop antibodies to PfEMP1, other blood-stage and pre-erythrocytic antigens at

greater prevalence and levels than children with SM, and that these antibodies are associated with protection from clinical malaria. We tested this hypothesis in a cohort of Ugandan children with severe malaria, cerebral malaria or severe malarial anemia, comparing antibody prevalence and level in these children at time of disease and at 6- and 12-month follow-up to those in community children with AP or no parasitemia (NP) at enrollment. We then evaluated the risk of severe or uncomplicated malaria during 12-month follow-up period in all groups according to antibody presence or level.

5.2 Methods

5.2.1 Study population

This prospective study was performed at Mulago Hospital, Kampala, Uganda, from 2008 to 2015, as previously described[17]. Children enrolled in this study had severe malaria (SM) in the form of either cerebral malaria (CM) or severe malaria anemia (SMA) and were between 18 months and 12 years of age. CM was defined as (1) coma (Blantyre Coma Score ≤2); (2) *P. falciparum* on blood smear; and (3) no other known cause of coma (e.g., meningitis, a prolonged postictal state, or hypoglycemia-associated coma reversed by glucose infusion). Exclusion criteria for CM included prior history of coma, head trauma, hospitalization for malnutrition, or cerebral palsy. SMA was defined as a positive *P. falciparum* blood smear and serum hemoglobin ≤5 g/dL. Exclusion criteria for SMA included impaired consciousness, seizures prior to admission, or other clinical evidence of central nervous system involvement. All children underwent a medical history and physical examination at enrollment. Children with severe malaria were treated at Mulago Hospital according to Ugandan national treatment guidelines.

Asymptomatic children aged 18 months to 12 years were enrolled from the neighborhoods and extended households of those enrolled with severe malaria to act as community controls (CC). Children with active or recent illness, chronic illness requiring

medical care, or prior coma were excluded. Peripheral blood smears were assessed for *Plasmodium* species by microscopy with Giemsa staining using standard protocols.

All participants underwent scheduled follow-up visits at 6 and 12 months after discharge, with sample collection at each time point. During the 12-month follow-up, unscheduled interval ambulatory sick visits and/or hospitalizations were recorded. Children with clinical features of malaria were tested by blood smear or point-of-care rapid diagnostic testing (RDT) for malaria, and all uncomplicated malaria visits and readmissions for severe malaria were recorded.

5.2.2 Blood sample collection and detection of *P. falciparum* parasitemia

Blood samples were collected from all participants at enrollment and 6- and 12-month follow-up visits and stored at –80°C. *Plasmodium falciparum* parasitemia was confirmed with both thick and thin blood smears that were read independently by two experienced laboratory technicians, as previously described[17]. Parallel testing for *P. falciparum* histidine-rich protein 2 (HRP-2) was performed on all enrollment samples with available plasma as previously described[119]. CC were tested for submicroscopic *P. falciparum* infection at enrollment and scheduled 6- and 12-month follow-up visits using nested PCR of DNA extracted from whole blood, as previously described[120].

5.2.3 Measurement of antibodies to recombinant *P. falciparum* antigens

Immunoglobulin G (IgG) antibodies to 14 *P. falciparum* antigens previously associated with protection from clinical malaria and/or associated with severe disease were tested. Antigens tested included five PfEMP1 antigens associated with severe disease[142], including CIDRα1.1 and CIDRα1.4 that bind to EPCR, and DBLα2, DBLβ12 and DBLɣ6, commonly found in tandem with EPCR-binding domains; the erythrocyte binding antigens (EBA) EBA-140, EBA-175, and EBA-181[138,139]; merozoite surface protein (MSP) MSP2[136] and MSP3[48], including the MSP DLB1and MSP DBL2[137] antigens; serine repeat antigen 5 (SERA5)[143]; and the pre-erythrocytic stage antigen

circumsporozoite protein (CSP)[144]. Table 5.1 provides references and important features of the 14 *P. falciparum* antigens used to measure IgG antibodies.

Table 5.1. *P. falciparu*m antigens and PfEMP-1 variants.

***P. falciparum* antigen**	**Protein**	**Protein (μg) conjugated to 10^6 beads**	**MIA platform used**	**Reference**
CSP	CSP	0.5	MAGPIX™	Porter et. al.
EBA-140	EBA140	0.5	MAGPIX™	Thompson et. al.
EBA-175	EBA175	0.2	MAGPIX™	Reed et. al.
EBA-181	EBA181	0.2	MAGPIX™	Gilberber et. al.
MSP2	MSP2	0.2	MAGPIX™	Reddy et. al.
MSP3	MSP3	1	MAGPIX™	Tsai et al.
MSP DBL1	MSP DBL1	1	MAGPIX™	Lin et. al.
MSP DBL2	MSP DBL2	1	MAGPIX™	Lin et. al.
SERA5	SERA5	1	MAGPIX™	Sugiyama et. al.
PfEMP1	CIDRα1.1	1	BIO-PLEX®	Avril et. al.
PfEMP1	CIDRα1.4	1	BIO-PLEX®	Avril et. al.
PfEMP1	DBLα2	1	BIO-PLEX®	Avril et. al.
PfEMP1	DBLβ12	1	BIO-PLEX®	Avril et. al.
PfEMP1	DBLɣ6	1	BIO-PLEX®	Avril et. al.
	MBP-His_6 control	1	BIO-PLEX®	Avril et. al.

Proteins were conjugated to MAGPIX (Luminex) or non-magnetic microsphere bioplex beads (Bio-Rad) according to the manufacturer's instructions with slight modifications[145]. Briefly, varying concentrations of each protein (Table 5.1) were coupled to beads to obtain an optimal antibody signal determined by testing plasma pooled from 20 healthy adult residents of a malaria holoendemic region of western Kenya. Plasma was incubated with a master mix of antigen-conjugated beads at a 1:1 ratio for final dilutions of 1:100 and 1:1000. Antibody magnitude was quantified using the dilution within the linear range of the positive control pool. The secondary antibody was R-Phycoerythrin AffiniPure F(ab')2 fragment goat anti-human IgG F(ab')2 (Jackson ImmunoResearch Laboratories). Negative controls consisted of plasma from 12 North American malaria-naïve adults included on each plate. A positive antibody response to

an antigen was determined as mean fluorescent intensity (MFI) values greater than the mean MFI plus three standard deviations of malaria-naïve negative controls. MFI values below this value were set to 1, which is equivalent to malaria-naïve controls. For those with a positive response, the levels of antibodies were expressed as fold over negative control (FONC), calculated as the study participant MFI divided by the mean MFI of malaria naïve negative controls plus three standard deviations.

5.2.4 Statistical analysis

Participants were classified as having severe malaria (combined cerebral malaria and severe malarial anemia enrollment groups), asymptomatic parasitemia, or no parasitemia. PCR results were used to differentiate no-parasitemia community controls from asymptomatic parasitemia. Data were described using medians and interquartile ranges for continuous variables and counts and percentages for categorical variables. For continuous demographic variables, a Wilcoxon Rank Sum test was performed to compare differences between two groups, and a Kruskal–Wallis test was used to compare differences among all three groups. The categorical variable, sex, was analyzed using Chi-square tests.

Each antibody was analyzed separately. A logistic regression model was used for the prevalence of antibody response within study population, and a linear regression model was used for levels of antibodies among those with a positive response. Analysis was adjusted for age and corrected for multiple comparisons using the Benjamini-Hochberg false discover rate (FDR) approach with a significance level of 0.05[129]. Antibody levels were log-transformed for the continuous portion of the model due to non-Gaussian distribution. Comparison of antibody presence and levels to *P. falciparum* antigens and incidence of severe or uncomplicated malaria during follow-up was performed using negative binomial regression. The model was run with offset of natural log of years at risk of malaria while enrolled in the study. This analysis was adjusted for

age as a continuous variable and enrollment in a sub-study, in which transportation for clinical care and clinical care itself was covered by the study. StataSE version 14 (StataCorp) was used for all analyses. Graphics were generated using Prism GraphPad 9.

5.2.5 Ethics review

Written informed consent was obtained from parents or guardians of study participants. Ethical approval was granted by the institutional review boards for human studies at the Makerere University School of Medicine, the Uganda National Council for Science and Technology, and the University of Minnesota Medical School.

5.3 Results

5.3.1 Study population characteristics

We examined antimalarial antibodies in blood plasma from children at enrollment and at scheduled 6- and 12-month follow-up visits. Of these, 498 children presented with severe malaria (SM), which included children with cerebral malaria (CM, n=266) and severe malarial anemia (SMA, n=232). Mortality rates were higher in children with CM (12% 32/266) compared with SMA (0.4%, 1/232). Children with CM and SMA had comparable antimalarial antibody prevalence and levels and were thus combined into one SM group for analysis (Tables 5.2 and 5.3).

Table 5.2. Antibody prevalence of *P. falciparum* pre-erythrocytic, non-PfEMP1 blood-stage, and PfEMP1 variant antigens in children with CM or SMA over the course of follow-up.

P. falciparum antigen	Enrollment	6 months	12 months	
Cerebral Malaria	**n=262**	**n=222**	**n=220**	
CSP	185 (70.61)	102 (45.95)	88 (40.00)[$]	A
EBA-140	141 (53.82)	73 (32.88)	78 (35.45)	A
EBA-175	200 (76.34)	90 (40.54)[$]	90 (40.91)	A
EBA-181	149 (56.87)	80 (36.04)	82 (37.27)	A
MSP2	251 (95.80)	159 (71.62)	177 (80.45)[$]	A
MSP3	178 (67.94)[$]	129 (58.11)	110 (50.00)	B
MSP DBL1	249 (95.04)	187 (84.23)	187 (85.00)	A
MSP DBL2	238 (90.84)	145 (65.32)	140 (63.64)	A
SERA5	178 (67.94)[$]	121 (54.50)	110 (50.00)	A
CIDRα1.1	224 (85.82)	190 (85.59)	183 (83.18)	
CIDRα1.4	171 (65.52)	154 (69.37)	158 (71.82)	
DBLα2	228 (87.36)	173 (77.93)	179 (81.36)	C
DBLβ12	205 (78.54)	179 (80.63)	186 (84.55)	
DBLɣ6	96 (36.78)$	90 (40.54)	96 (43.64)	
Severe Malaria Anemia	**n=229**	**n=213**	**n=212**	
CSP	179 (78.17)	119 (55.87)	115 (54.25)	A
EBA-140	140 (61.14)	85 (39.91)	77 (36.32)	A
EBA-175	186 (81.22)	107 (50.23)	93 (43.87)	A
EBA-181	149 (65.07)	90 (42.25)	87 (41.04)	A
MSP2	226 (98.69)	172 (80.75)	190 (89.62)	D
MSP3	186 (81.22)	139 (65.26)	121 (57.08)	A
MSP DBL1	226 (98.69)	195 (91.55)	192 (90.57)	A
MSP DBL2	215 (93.89)	155 (72.77)	145 (68.40)	A
SERA5	185 (80.79)	120 (56.34)	101 (47.64)	A
CIDRα1.1	199 (86.90)	178 (83.57)	184 (86.79)	
CIDRα1.4	156 (68.12)	161 (75.59)	166 (78.30)	B
DBLα2	204 (89.08)	186 (87.32)	184 (86.79)	
DBLβ12	168 (73.36)	180 (84.51)	174 (82.08)	C
DBLɣ6	117 (51.09)	100 (47.17)	116 (54.72)	

Number of children with a positive response to *P. falciparum* antigens at enrollment, 6 and 12 months. Data presented as number positive (% prevalence). P-values from logistic regression model comparing two timepoints within the group or two groups at a specific time point. Significant results for p-values less than FDR threshold to correct for multiple comparisons. See key below for significant comparisons.

A: Enrollment is different than 6 and 12 months
B: Enrollment is different from 12 months
C: Enrollment is different than 6 months
D: All time points are different
[$]Cerebral Malaria is different than Severe Malarial Anemia

Table 5.3. Antibody levels of *P. falciparum* pre-erythrocytic, non-PfEMP1 blood-stage, and PfEMP1 variant antigens in children with CM or SMA that were had positive response per antigen.

	Enrollment, Median, (IQR) (n)	6 months, Median, (IQR) (n)	12 months, Median, (IQR) (n)	
Cerebral Malaria				
CSP	9.16 (5.82, 15.52) (185)[$]	7.34 (4.64, 14.29) (102)	8.08 (5.33, 15.01) (88)	
EBA-140	5.97 (4.05, 10.17) (141)	5.08 (3.79, 7.45) (73)	5.05 (3.39, 10.78) (78)	
EBA-175	22.51 (12.91, 40.77) (200)	23.55 (13.39, 37.37) (90)	19.65 (11.54, 34.31) (90)	
EBA-181	13.73 (9.85, 26.55) (149)	13.36 (9.23, 25.91) (80)	14.37 (8.60, 34.61) (82)	
MSP2	49.41 (22.08, 107.83) (251)	16.48 (8.26, 50.41) (159)	17.55 (6.98, 54.29) (177)	A
MSP3	9.29 (6.39, 19.67) (178)[$]	9.12 (6.12, 22.67) (129)	10.46 (6.11, 20.69) (110)	
MSP DBL1	24.59 (11.97, 77.12) (249)[$]	23.53 (9.42, 93.99) (187)	20.39 (8.02, 66.68) (187)	
MSP DBL2	10.38 (5.86, 19.94) (238) [$]	9.71 (5.10, 22.76) (145)	8.44 (4.56, 22.69) (140)[$]	
SERA5	13.48 (7.52, 26.41) (178)	8.60 (5.88, 19.61) (121)	11.13 (5.97, 25.91) (110)	
CIDRα1.1	57.93 (28.31, 244.41) (224)	74.15 (31.05, 216.36) (190)	35.78 (14.22, 93.26) (183)[$]	B
CIDRα1.4	287.00 (86.00, 929.25) (171) [$]	385.50 (88.57, 1445.50) (154)	272.75 (58.50, 1235.50) (158)	
DBLα2	23.66 (9.94, 48.17) (228)	17.56 (8.67, 37.49) (173)	16.41 (7.64, 33.47) (179)	C
DBLβ12	25.51 (11.66, 53.04) (205) [$]	37.17 (16.27, 87.85) (179)	26.31 (14.39, 60.39) (186)[$]	D
DBLɣ6	30.08 (13.76, 100.71) (96)	42.95 (16.54, 166.38) (90)	39.96 (11.88, 208.23) (96)	
Severe Malaria Anemia				
CSP	12.98 (6.42, 24.31) (179)	10.08 (7.13, 17.59) (119)	10.84 (6.03, 19.88) (115)	
EBA-140	6.72 (4.15, 12.59) (140)	5.49 (3.72, 10.49) (85)	5.85 (4.04, 11.79) (77)	
EBA-175	24.05 (13.21, 46.19) (186)	19.20 (11.92, 38.17) (107)	19.65 (13.51, 35.38) (93)	
EBA-181	13.73 (9.21, 26.67) (149)	12.77 (8.93, 23.26) (90)	14.71 (9.84, 30.12) (87)	
MSP2	54.51 (24.18, 116.86) (226)	28.01 (8.22, 67.07) (172)	23.85 (8.45, 61.09) (190)	A
MSP3	16.85 (8.41, 33.57) (186)	12.31 (6.34, 29.89) (139)	13.29 (6.45, 32.75) (121)	
MSP DBL1	56.18 (20.60, 130.69) (226)	36.18 (12.38, 128.18) (195)	28.19 (10.51, 100.61) (192)	C

MSP DBL2	15.08 (7.59, 29.88) (215)	12.74 (5.54, 29.84) (155)	14.51 (6.67, 34.13) (145)	
SERA5	15.85 (7.73, 41.07) (185)	8.92 (5.12, 16.57) (120)	11.25 (6.41, 20.09) (101)	D
CIDRα1.1	83.11 (32.44, 313.59) (199)	105.96 (43.66, 382.27) (178)	48.68 (18.82, 109.69) (184)	B
CIDRα1.4	407.38 (160.22, 2020.50) (156)	524.45 (106.50, 1737) (161)	359.57 (78.50, 1517.50) (166)	
DBLα2	26.52 (13.31, 51.94) (204)	19.44 (9.24, 44.92) (186)	15.11 (9.14, 43.38) (184)	A
DBLβ12	31.25 (15.84, 75.37) (168)	39.06 (16.32, 121.67) (180)	32.38 (15.30, 93.47) (174)	
DBLɣ6	42.10 (13.80, 178.50) (117)	47.66 (23.30, 145.65) (100)	49.80 (19.02, 219.36) (116)	

Presented as median and IQR for each antigen with, n= number of children that are positive for specific antigen at that time point. P-values from linear regression model of children with a positive response per antigen adjusted for age, comparing two timepoints within the group or two groups at a specific time point. Significant results for p-values less than FDR threshold to correct for multiple comparisons. See key below for significant comparisons.

A: Enrollment is different from 6 and 12 months
B: All three timepoints are different from each other
C: Enrollment is different from 12 months
D: Enrollment is different from 6 months
E: 6 months is different from enrollment and 12 months
F: 12 months is different from enrollment and 6 months
[$]CM is different than SMA

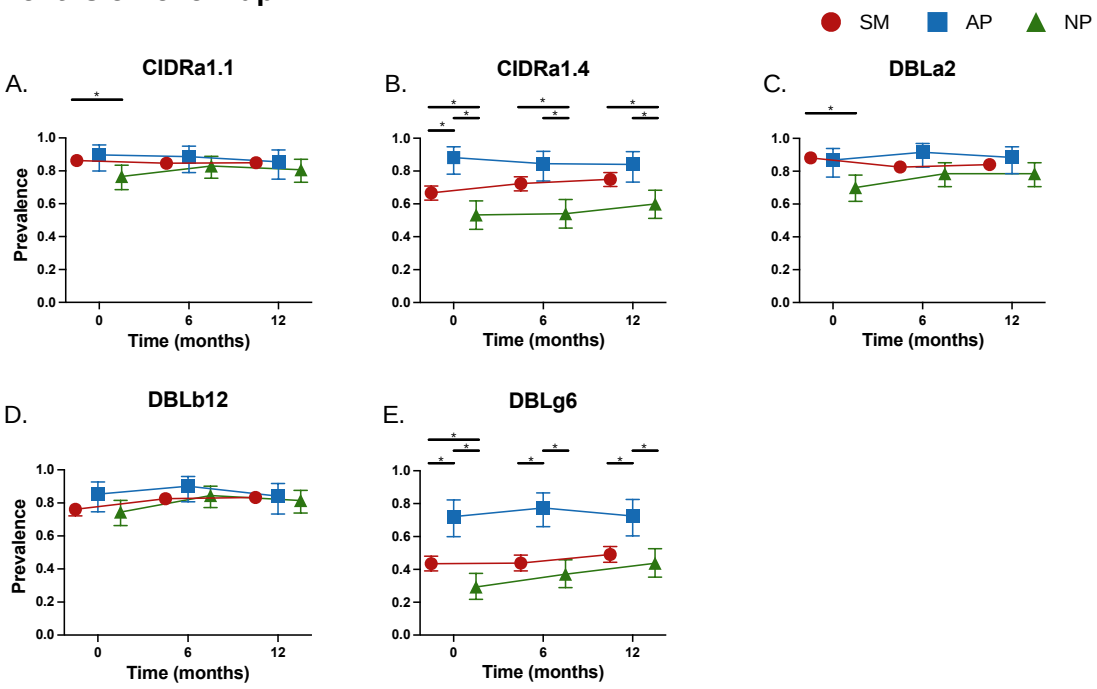
At enrollment, 72 of the 211 (34%) CC had asymptomatic parasitemia (AP) based on nested PCR testing for *P. falciparum* 18S DNA. Of this group, 29 of 72 were positive for P. falciparum on microscopy. The remaining 139 CC did not have detectable *P. falciparum* infection by PCR testing. All children with SM were positive at enrollment based on microscopy, using Giemsa staining of thick and thin blood smears. Children with SM and community children (CC) had similar rates of asymptomatic *P. falciparum* parasitemia by PCR testing at 6- and 12-month follow-up: 76/412 of children with SM (18%) and 35/163 (18%) of CC at 6 months, and 77/402 (19%) of children with SM and 40/197 (20%) of CC at 12 months. Enrollment parasitemia results in the CC group were used to define the AP and no parasitemia (NP) groups.

Enrollment demographics of the study population showed that children in the AP group were slightly older than children in either of the other groups, with a median age of 4.0 years, compared with 3.3 and 3.5 years, respectively, for children with SM or NP (p=0.003). There were more males in the SM group (59.8%), compared with the AP or NP groups (48.6% and 47.5%, respectively, p=0.013).

5.3.2 Prevalence of antibodies to PfEMP1 antigens over time

We next measured the presence and reactivity of antibodies specific against five PfEMP1 domains commonly expressed in severe malaria infection: two EPCR-binding domains (CIDRα1.1 and CIDRα1.4) and three found in tandem with EPCR-binding domains (DBLα2, DBLβ12 and DBLɣ6)[121,142]. Two distinct patterns emerged: a high proportion (70.1 to 91.6%) of children in all three groups had antibodies to CIDRα1.1, DBLα2, and DBLβ12 at all three timepoints (Figure 5.1, Table 5.4;), and proportions generally did not differ between groups. In contrast, a higher proportion of AP than NP or SM had antibodies to DBLɣ6 at all timepoints (Figure 5.1) and a higher proportion of AP than NP had antibodies to CIDRα1.4 at all time points (Figure 5.1).

Figure 5.1. Prevalence of antibodies to PfEMP1 family antigens in children with severe malaria, asymptomatic parasitemia, and no parasitemia, over 12 months of follow-up.



The prevalence of antibodies to PfEMP1 antigens (A) CIDRα1.1, (B) CIDRα1.4, (C) DBLα2, (D) DBLβ12, and (E) DBLɣ6 at enrollment, 6 and 12 months in children with severe malaria (SM), asymptomatic parasitemia at enrollment (AP) and no parasitemia at enrollment (NP). Plots depict frequency of the children in each group that were positive for the antigen and 95% confidence intervals.
*Denotes p-value<0.05 based on logistic regression comparing two groups at each timepoint; adjusted for age and FDR to correct for multiple comparisons.

Table 5.4. Antibody prevalence of *P. falciparum* pre-erythrocytic, non-PfEMP1 blood-stage, and PfEMP1 variant antigens in children with SM, AP, and NP.

P. falciparum antigen	Enrollment	6 months	12 months	
Severe Malaria	n= 491	n=435	n=432	
CSP	364 (74.13)$^{\$}$	221 (50.80)$^{\&\&}$	203 (46.99)$^{\&\&}$	A
EBA-140	281 (57.23) $^{\$}$	158 (36.32)$^{\$\$}$	155 (35.88)$^{\$}$	A
EBA-175	386 (78.62) $^{\$}$	197 (45.29)$^{\%}$	183 (42.36)$^{\%}$	A
EBA-181	298 (60.69) $^{\$}$	170 (39.08)$^{\%\%}$	169 (39.12)$^{\%\%}$	A
MSP2	477 (97.15) $^{\$\$}$	331 (76.09)$^{\&}$	367 (84.95)$^{\$}$	B
MSP3	364 (74.13) $^{\$}$	268 (61.61)$^{\%\%}$	231 (53.47)$^{\%}$	B
MSP DBL1	475 (96.74) $^{\$}$	382 (87.82)$^{\$}$	379 (87.73)$^{\$}$	A
MSP DBL2	453 (92.26) $^{\$\$}$	300 (68.97)$^{\$\$}$	285 (65.97)$^{\%}$	A
SERA5	363 (73.93) $^{\$}$	241 (55.40)$^{\$}$	211 (48.84)$^{\%\%}$	A
CIDRα1.1	423 (86.33)$^{\&}$	368 (84.60)	367 (84.95)	
CIDRα1.4	327 (66.73) $^{\$\$}$	315 (72.41)$^{\$}$	324 (75.00)$^{\$}$	C
DBLα2	432 (88.16)$^{\&}$	359 (82.53)	363 (84.03)	
DBLβ12	373 (76.12)	359 (82.53)	360 (83.33)	A
DBLɣ6	213 (43.47) $^{\$\$}$	190 (43.78)$^{\%}$	212 (49.07)$^{\%}$	
Asymptomatic Parasitemia	n=68	n=71	n=69	
CSP	49 (72.06)	48 (67.61)	45 (65.22)	
EBA-140	34 (50.00)	41 (57.75)	34 (49.28)	
EBA-175	52 (76.47)	47 (66.20)	44 (63.77)	
EBA-181	38 (55.88)	36 (50.70)	37 (53.62)	
MSP2	60 (88.24)	50 (70.42)	61 (88.41)	D
MSP3	48 (70.59)	52 (73.24)	52 (75.36)	
MSP DBL1	62 (91.18)	66 (92.96)	64 (92.75)	
MSP DBL2	56 (82.35)	62 (87.32)	59 (85.51)	
SERA5	45 (66.18)	42 (59.15)	42 (60.87)	
CIDRα1.1	61 (89.71)	63 (88.73)	59 (85.51)	
CIDRα1.4	60 (88.24)	60 (84.51)	58 (84.06)	
DBLα2	59 (86.76)	65 (91.55)	61 (88.41)	
DBLβ12	58 (85.29)	64 (90.14)	58 (84.06)	
DBLɣ6	49 (72.06)	55 (77.46)	50 (72.46)	
No Parasitemia	n=137	n=135	n=135	
CSP	62 (45.26)	66 (48.89)	66 (48.89)	
EBA-140	29 (21.17)	34 (25.19)	33 (24.44)	
EBA-175	45 (32.85)	54 (40.00)	51 (37.78)	
EBA-181	37 (27.01)	40 (29.63)	38 (28.15)	
MSP2	84 (61.31)	77 (57.04)	93 (68.89)	
MSP3	61 (44.53)	69 (51.11)	65 (48.15)	
MSP DBL1	97 (70.80)	101 (74.81)	96 (71.11)	
MSP DBL2	65 (47.45)	73 (54.07)	77 (57.04)	
SERA5	50 (36.50)	52 (38.52)	52 (38.52)	
CIDRα1.1	105 (76.64)	112 (82.96)	109 (80.74)	
CIDRα1.4	73 (53.28)	73 (54.07)	81 (60.00)	
DBLα2	96 (70.07)	106 (78.52)	106 (78.52)	
DBLβ12	102 (74.45)	114 (84.44)	110 (81.48)	
DBLɣ6	40 (29.20)	50 (37.04)	59 (43.70)	C

Number of children with a positive response to *P. falciparum* antigens at enrollment, 6, and 12 months. Data presented as number positive (% prevalence). p-values from logistic regression model comparing two timepoints within the group or two groups at a specific time point. Significant results for p-values less than FDR threshold to correct for multiple comparisons. See key below for significant comparisons.

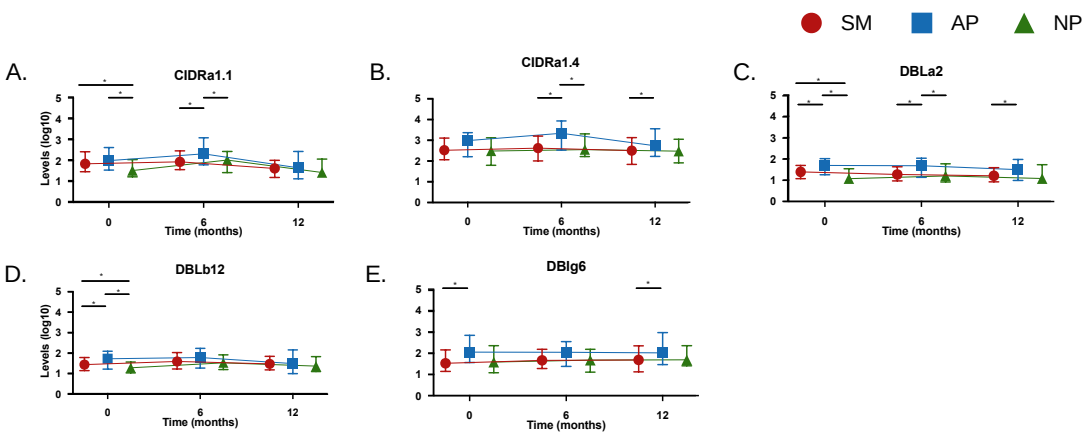
A: Enrollment is different from 6 and 12 months
B: All timepoints are different
C: 0 different from 12 months
D: 6 months is different from enrollment and 12 months

[$]NP is different from SM and AP
[$$]All groups are different
[&]SM is different than NP
[&&]SM is different than AP
[%]AP is different from SM and NP
[%%]AP is different from NP

5.3.3 Levels of antibodies to PfEMP1 antigens over time among children who were antibody positive

Among children who had antibodies to PfEMP1 antigens, a different pattern emerged with levels of antibodies. For 3 of the 5 antigens (CIDRα1.1, DBLα2, and DBLβ12), children with AP or SM had higher levels of antibodies than children with NP, and children with AP had higher levels of antibodies to DBLɣ6 than children with SM at enrollment (Figure 5.2, Table 5.5). However, by 12 months only differences between AP and SM were seen, with higher levels in AP than SM for CIDRα1.4, DLBα2 and DLBɣ6 (Figure 5.2, Table 5.5). Together the findings showed maintenance of antibodies and higher antibody levels to PfEMP1 antigens in children with AP as compared to SM.

Figure 5.2 Levels of antibodies to PfEMP1 family antigens.



Data presented as log 10 transformed levels of antibody levels for children with severe malaria (SM), asymptomatic parasitemia at enrollment (AP) and no parasitemia at enrollment (NP) that had a positive antibody response for each PfEMP1 antigen, (A) CIDRα1.1, (B) CIDRα1.4, (C) DBLα2, (D) DBLβ12, and (E) DBLɣ6. The overlayed box plots depict median and IQR levels of antibodies. *Denotes p-value<0.05 based on linear regression comparing two groups at each timepoint; adjusted for age and FDR to correct for multiple comparisons.

Table 5.5. Antibody levels of *P. falciparum* pre-erythrocytic, non-PfEMP1 blood-stage, and PfEMP1 variant antigens in children with SM, AP, and NP over a 1-year period for those that are positive for antibodies.

	Enrollment, Median, (IQR) (n)	6 months, Median, (IQR) (n)	12 months, Median, (IQR) (n)	
Severe Malaria (SM)				
CSP	10.67 (6.20, 18.80) (364)	8.77 (5.57, 15.04) (221)[&&]	9.11 (5.65, 17.64) (203)	A
EBA-140	6.47 (4.14, 11.06) (281)	5.26 (3.72, 9.06) (158)[&&]	5.21 (3.86, 11.71) (155)	
EBA-175	23.08 (13.04, 43.12) (386)	21.57 (12.39, 37.37) (197)[&&]	19.65 (12.48, 34.93) (183)	
EBA-181	13.73 (9.45, 26.67) (298)	13.08 (9.03, 24.84) (170)	14.71 (9.33, 30.12) (169)	
MSP2	51.17 (23.63, 110.18) (477)[$]	20.27 (8.23, 58.95) (331)[&&]	22.07 (7.67, 60.15) (367)[$$]	B
MSP3	11.52 (6.76, 26.58) (364)[$$]	10.48 (6.25, 26.81) (268)[$$]	11.49 (6.28, 29.35) (231)[&&]	
MSP DBL1	37.26 (15.42, 103.66) (475)[&]	31.07 (11.29, 107.76) (382)[$$]	23.90 (9.09, 81.13) (379)[$$]	C
MSP DBL2	11.84 (6.51, 24.86) (453)[$$]	10.57 (5.22, 26) (300)[$$]	10.87 (5.28, 29.04) (285)[$$]	
SERA5	14.13 (7.52, 28.73) (363)[$]	8.64 (5.73, 17) (241)[&&]	11.22 (6.17, 23.29) (211)	A
CIDRα1.1	70.74 (29.32, 266.85) (423)[$]	87.81 (37.28, 293.88) (368)[$$]	42.88 (15.79, 104.13) (367)	D
CIDRα1.4	323.50 (113.17, 1231) (327)	407.50 (99.35, 1544) (315)[$$]	307.26 (67.58, 1311.50) (324)[&&]	
DBLα2	24.62 (11.83, 49.37) (432)[&]	18.63 (9.17, 42.74) (359)[$$]	16.11 (8.48, 38.45) (363)[&&]	B
DBLβ12	26.78 (13.64, 58.92) (373)[&]	37.54 (16.27, 102.46) (359)	28.95 (14.70, 67.64) (360)	A
DBLɣ6	33.28 (13.76, 139.95) (213)[&&]	45.79 (19.11, 147.54) (190)	47.82 (13.13, 219.20) (212)[&&]	
Asymptomatic parasitemia (AP)				
CSP	12.48 (9.26, 26.14) (49)	12.40 (7.13, 36.66) (48)	13.38 (8.29, 30.08) (45)	
EBA-140	9.71 (4.82, 14.52) (34)	8.03 (4.72, 15.89) (41)	6.96 (4.18, 14.78) (34)	
EBA-175	21.25 (13.09, 66.17) (52)	52.60 (14.86, 164.94) (47)	21.45 (11.18, 81.70) (44)	
EBA-181	13.48 (8.62, 33.38) (38)	19.70 (10.65, 45.40) (36)	15.67 (11.81, 34.97) (37)	
MSP2	58.72 (19.44, 184.02) (60)	31.33 (10.96, 104.58) (50)	41.35 (15.79, 147.93) (61)	
MSP3	38.60 (9.90, 64.32) (48)	33.80 (15.24, 76.48) (52)	23.34 (8.59, 66.72) (52)	
MSP DBL1	83.95 (30.35, 243.73) (62)	118.05 (26.54, 359.45) (66)	80.17 (22.94, 224.60) (64)	

MSP DBL2	31.04 (12.20, 58.53) (56)	36.31 (10.79, 68.23) (62)	28.73 (10.06, 51.32) (59)	
SERA5	18.12 (8.32, 32.81) (45)	16.58 (8.80, 40.46) (42)	12.79 (7.33, 32.28) (42)	
CIDRα1.1	99.96 (36.35, 404.91) (61)	213.08 (63.44, 1241.05) (63)	45.60 (13.68, 274.33) (59)	E
CIDRα1.4	924.16 (158.14, 2266.75) (60)	2107.75 (333.51, 7968.79) (60)	535.00 (171.61, 3388.39) (58)	
DBLα2	49.34 (18.03, 103.16) (59)	47.86 (13.91, 107.03) (65)	31.05 (10.00, 92.52) (61)	
DBLβ12	52.17 (16.08, 113.42) (58)	59.49 (18.23, 158.33) (64)	29.95 (9.68, 136.89) (58)	
DBLɣ6	110.78 (36.45, 646) (49)	109.86 (23.54, 344.83) (55)	102.31 (33.89, 912.13) (50)	
No Parasitemia (NP)				
CSP	9.16 (6.38, 18.61) (62)	9.93 (6.29, 22.75) (66)	9.55 (5.91, 24.10) (66)	
EBA-140	5.89 (4.06, 14.62) (29)	6.19 (3.99, 10.16) (34)	5.35 (3.81, 11.76) (33)	
EBA-175	16.06 (12.33, 33.68) (45)	25.49 (15.06, 54.34) (54)	22.01 (11.18, 45.16) (51)	
EBA-181	11.73 (8.26, 25.92) (37)	13.29 (7.93, 26.54) (40)	16.34 (9.83, 28.87) (38)	
MSP2	16.67 (6.72, 71.52) (84)	21.16 (7.06, 50.83) (77)	13.35 (5.34, 47.37) (93)	
MSP3	12.78 (6.66, 25.66) (61)	9.41 (6.52, 23.56) (69)	11.88 (6.89, 29.77) (65)	
MSP DBL1	19.95 (5.98, 60.75) (97)	18.35 (5.72, 93.03) (101)	18.43 (7.24, 70.88) (96)	
MSP DBL2	12.22 (5.17, 24.31) (65)	10.85 (4.88, 23.06) (73)	9.23 (4.90, 25.61) (77)	
SERA5	6.32 (4.54, 19.23) (50)	10.89 (5.28, 20.61) (52)	10.80 (6.38, 23.07) (52)	
CIDRα1.1	33.59 (16.92, 112.38) (105)	107.79 (26.87, 273.94) (112)	27.26 (16.41, 116.71) (109)	F
CIDRα1.4	297.50 (60.50, 1157) (73)	345.50 (166.50, 1724.45) (73)	287.50 (79.00, 1060.50) (81)	
DBLα2	11.85 (7.00, 33.41) (96)	15.84 (8.33, 58.61) (106)	12.00 (8.19, 53.41) (106)	
DBLβ12	18.68 (10.64, 35.30) (102)	32.57 (15.13, 79.88) (114)	22.57 (12.27, 63.77) (110)	B
DBLɣ6	38.01 (12.12, 208.35) (40)	46.63 (12.88, 147.80) (50)	48.57 (25.18, 223.53) (59)	

Presented as median and IQR for each antigen with, n= number of children that are positive for specific antigen at that time point. p-values from linear regression model of children with a positive response per antigen adjusted for age, comparing two timepoints within the group or two groups at a specific time point. Significant results for p-values less than FDR threshold to correct for multiple comparisons. See key below for significant comparisons.

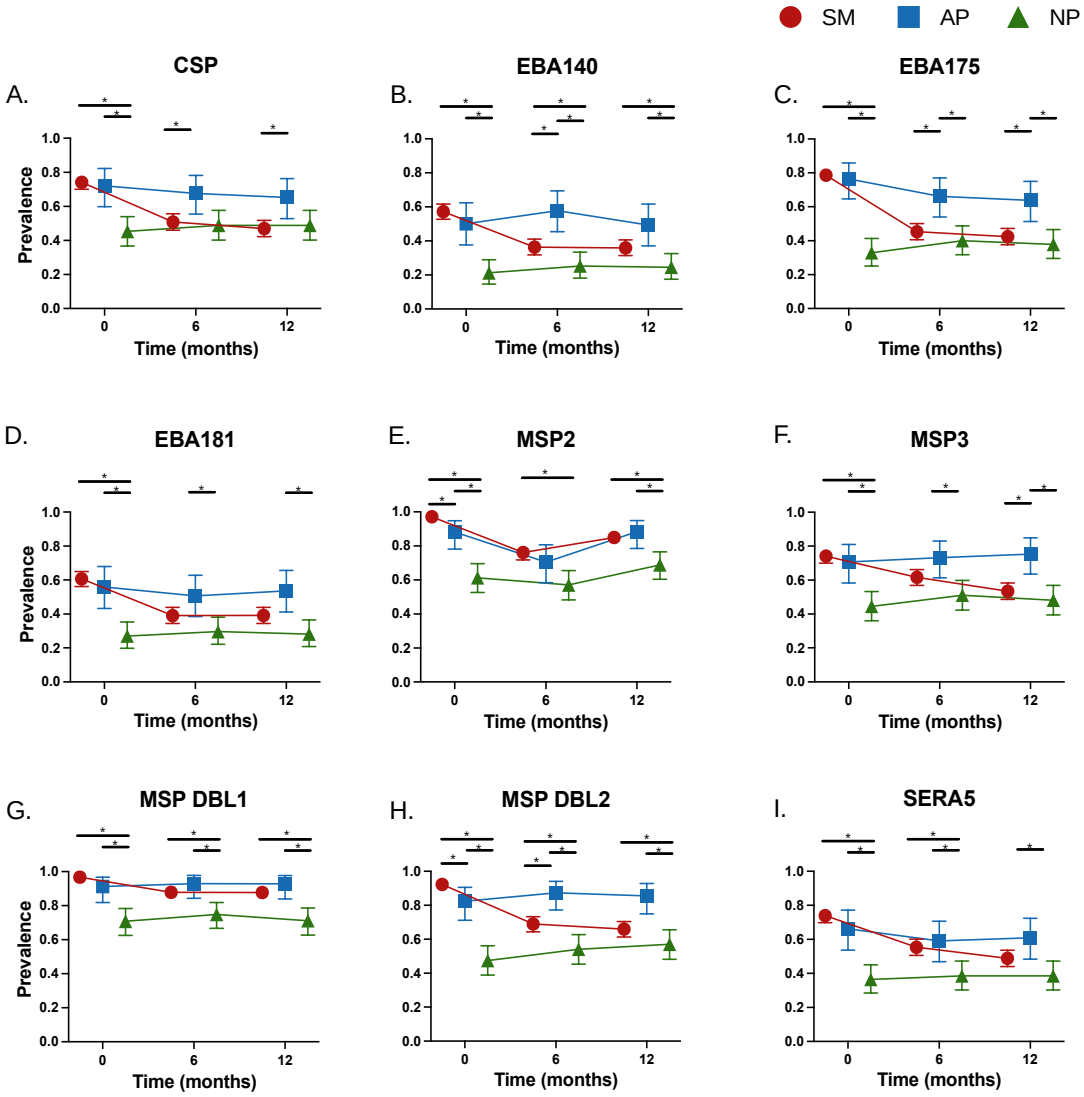
A: Enrollment is different from 6 months
B: Enrollment is different from 6 and 12 months
C: Enrollment is different from 12 months
D: 12 months is different from enrollment and 6 months
E: 6 months is different than 12 months
F: 6 months is different from enrollment and 12 months

$NP is different from SM and AP
$$AP is different from SM and NP
&All groups are different
&&SM is different than AP

5.3.4 Prevalence of antibodies to CSP and non-PfEMP1 blood-stage antigens over time

In contrast to antibodies to PfEMP1 antigens, antibodies to CSP and all the 8 non-PfEMP1 blood-stage antigens tested were higher in prevalence in children with SM or AP than NP at enrollment, and were similar in SM and AP at enrollment, except for antibodies to MSP2 and MSP DBL2, which were higher in SM than AP (Figure 5.3). Antibody prevalence remained similar to enrollment for all antigens in children with AP, and at similar lower prevalence rates in children with NP, but by 12 months had decreased significantly in children with SM compared to AP for CSP, EBA-175, and MSP-3 (Figure 5.3, Table 5.4).

Figure 5.3. Prevalence of antibodies against CSP and non-PfEMP1 *P. falciparum* blood-stage antigens in Ugandan children with SM, AP, and NP at enrollment and over 12-month follow-up.

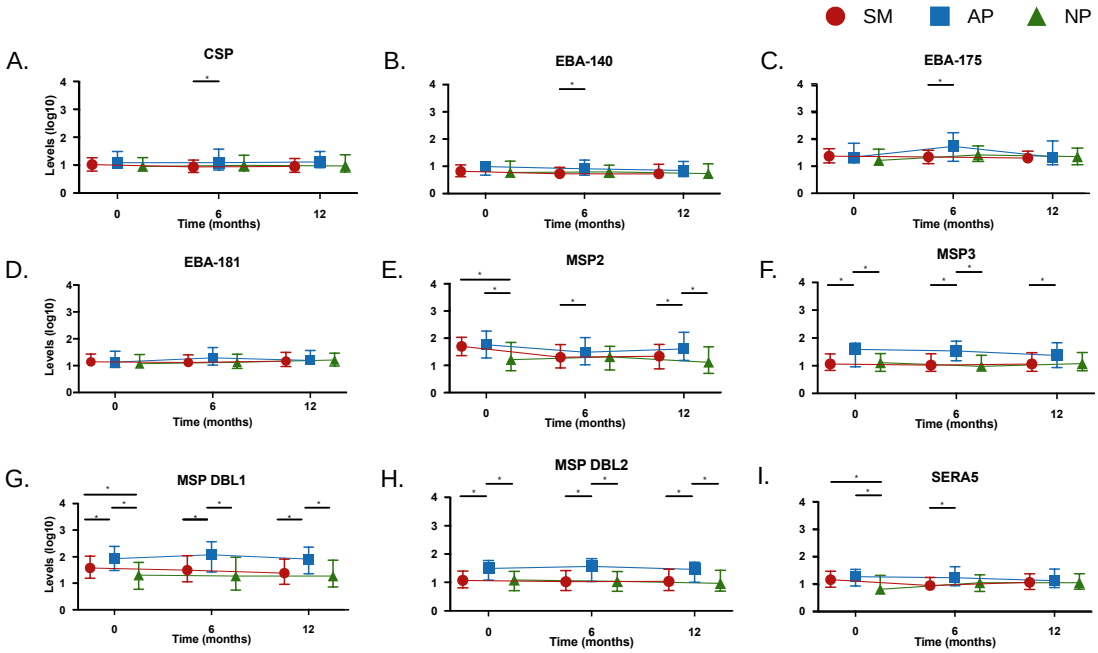


The prevalence of antibodies to *P. falciparum* antigens (A) CSP, (B) EBA140, (C) EBA 175, (D) EBA 181, (E) MSP2, (F) MSP 3, (G) MSP DBL1, (H) MSP DBL 2, and (I) SERA5 at enrollment, 6, and 12 months in children with severe malaria (SM), asymptomatic parasitemia at enrollment (AP), and no parasitemia at enrollment (NP). Plots depict the frequency of the children in each group that were positive for the antigen and 95% confidence intervals.
*Denotes p-value<0.05 based on logistic regression comparing two groups at each timepoint; adjusted for age and FDR to correct for multiple comparisons.

5.3.5 Levels of antibodies to CSP and non-PfEMP1 blood-stage antigens over time among children who were antibody positive

Levels of antibodies to CSP and *P. falciparum* blood-stage antigens, other than PfEMP1, over time among children with antibodies to these antigens followed four patterns: 1) no difference between SM, AP and NP in antibody levels at any time point (EBA-181); 2) higher levels in AP than SM only at the 6-month time point (CSP, EBA-140 and EBA-175); 3) similar levels in AP and SM at baseline, both higher than NP, with subsequent higher levels in AP than SM at 6 months (SERA5) or 6 and 12 months (MSP2); or 4) higher levels in AP than SM at all time points (MSP3, MSP DBL1, MSP DBL2) (Figure 5.4). Together, the findings showed that, as for PfEMP1 antigens, prevalence and levels of antibodies to CSP and multiple other blood-stage antigens were maintained better in children with AP than in children with SM.

Figure 5.4. Levels of antibodies against CSP and other blood-stage *P. falciparum* antigens.



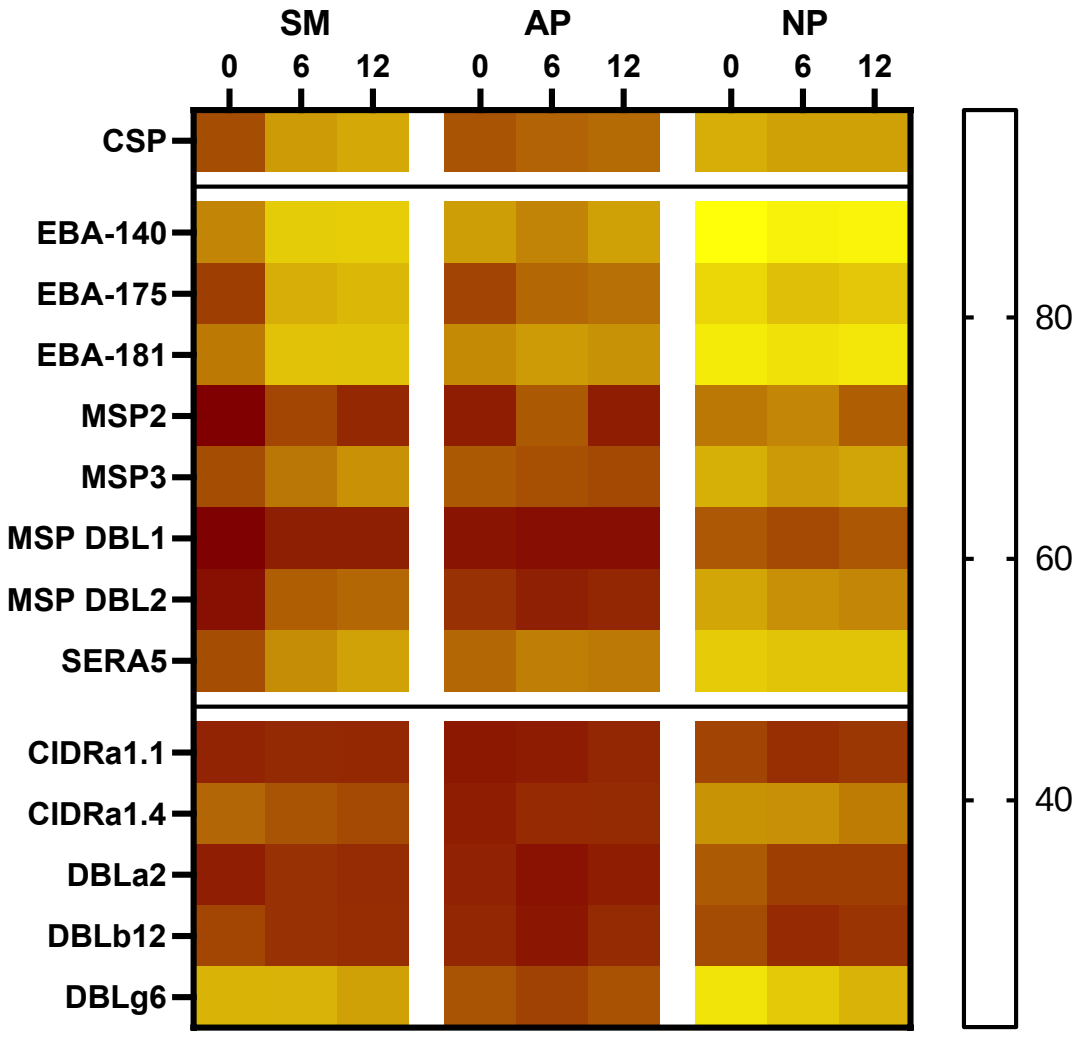
Data are presented as log10 transformed levels of antibody levels for children with severe malaria (SM), asymptomatic parasitemia at enrollment (AP), and no parasitemia at enrollment (NP) that had a positive antibody response for each specific antigen; (A) CSP, (B) EBA140, (C) EBA 175, (D) EBA 181, (E) MSP2, (F) MSP 3, (G) MSP DBL1, (H) MSP DBL 2, and (I) SERA5. The overlayed box plots depict median and IQR levels of antibodies.
*Denotes p-value<0.05 based on logistic regression comparing two groups at each timepoint; adjusted for age and FDR to correct for multiple comparisons.

5.3.6 Summary of the prevalence of antibodies against 14 tested *P. falciparum* antigens

Figure 5.5 shows a heat map summary of the changes in prevalence among the three groups over time for all 14 antigens. Overall, the prevalence of antibodies in children with SM decreased for CSP and all 8 blood-stage antigens, other than PfEMP1, but none of the PfEMP1 antigens, while for AP prevalence decreased modestly only for CSP and EBA-175. Children with NP had lower prevalence of antibodies to CSP, the

other blood-stage antigens and 2 of 5 PfEMP1 antigens at all time points than children with AP but did not have a decrease in prevalence of antibodies over time for any antigen.

Figure 5.5. Heat map of prevalence of antibodies to 14 tested *P. falciparum* antigens in Ugandan children with SM, AP, and no parasitemia at time of enrollment, 6 months, and 12 months of follow-up.



Heat map of the prevalence of the 14 tested *P. falciparum* antigens (left) for children with severe malaria (SM), asymptomatic parasitemia (AP), and no parasitemia (NP). Overall, the prevalence of antibodies to many of these antigens decreased in children with severe malaria but were maintained at higher levels in children with asymptomatic parasitemia compared to no parasitemia at enrollment. Legend on the right shows the gradation of percent of children in the group that have a positive antibody response, from 100% (dark red) to 0% (light yellow).

5.3.7 Presence or level of antibodies to *P. falciparum* antigens and risk of severe malaria

12% (56/461) of children with SM were readmitted with SM within 12 months, while 2.8% (2/72) children with AP and 2.2% (3/139) of children with NP developed SM in the same time period.

Antibodies to most of the *P. falciparum* antigens tested were present in high proportions of the children with SM at enrollment. In children with SM, the presence of antibodies to *P. falciparum* was not associated with risk of post-discharge SM for any of the 14 antigens tested (Table 5.6).

Table 5.6. Risk of severe malaria within 12 months of discharge or at enrollment, according to presence of antibodies to *P. falciparum* antigens.

	Antibody +	Antibody –	Risk of SM		
	N obs, N events		IRR	95% CI	P value
CSP	341, 56	113, 16	1.26	(0.66, 2.42)	0.48
EBA-140	262,44	192, 28	0.98	(0.56, 1.72)	0.96
EBA-175	358, 53	96, 19	0.79	(0.42, 1.49)	0.47
EBA-181	280, 45	174, 27	1.08	(0.61, 1.90)	0.79
MSP2	444, 70	10, 2	0.58	(0.11, 3.13)	0.52
MSP3	336, 61	118, 11	1.91	(0.94, 3.91)	0.08
MSP DBL1	443, 68	11, 4	0.42	(0.11, 1.64)	0.21
MSP DBL2	424, 68	30, 4	1.13	(0.35, 3.62)	0.83
SERA5	339, 47	115, 25	0.68	(0.38, 1.22)	0.20
CIDRα1.1	392, 63	61, 9	0.96	(0.43, 2.18)	0.93
CIDRα1.4	307, 51	146, 21	1.38	(0.76, 2.52)	0.30
DBLα2	400, 61	53, 11	0.89	(0.41, 1.95)	0.78
DBLβ12	344, 60	109, 12	1.62	(0.80, 3.27)	0.18
DBLɣ6	199, 38	254, 34	1.72	(0.98, 3.00)	0.06

Incidence rate ratios adjusted for age and sub-study enrollment. Calculated using negative binomial regression models of presence or absence of *P. falciparum* antibodies in children with a positive antibody response per antigen. N obs: Number of observations included in the model; N events: number of severe malaria episodes.

When antibody levels were compared to risk of post-discharge SM, children with SM had an increased risk of post-discharge SM with increased levels of MSP3 (incidence rate ratio (IRR) [95% confidence interval (CI)], 1.90 [1.20, 3.01], p-value, <0.01, Table 5.7). No other antibody levels were associated with risk of post-discharge SM. Children with AP and NP had too few follow-up episodes of SM to evaluate SM as an outcome.

Table 5.7. Risk of severe malaria within 12 months of discharge among children with severe malaria at enrollment, according to levels of antibodies to *P. falciparum* antigens.

	N obs, N events	Risk of SM IRR (95% CI)	P value
CSP	454, 72	1.39 (0.86, 2.25)	0.18
EBA-140	454, 72	0.87 (0.49, 1.55)	0.64
EBA-175	454, 72	0.76 (0.51, 1.14)	0.19
EBA-181	454, 72	0.93 (0.60, 1.44)	0.74
MSP2	454, 72	1.54 (0.90, 2.62)	0.12
MSP3	454, 72	1.90 (1.20, 3.01)	**0.01**
MSP DBL1	454, 72	1.88 (1.16, 3.04)	0.01
MSP DBL2	454, 72	2.00 (1.08, 3.72)	0.03
SERA5	454, 72	0.73 (0.47, 1.12)	0.15
CIDRα1.1	453, 72	0.93 (0.70, 1.24)	0.62
CIDRα1.4	453, 72	1.20 (0.98, 1.47)	0.07
DBLα2	453, 72	0.91 (0.58, 1.44)	0.70
DBLβ12	453, 72	1.38 (0.96, 2.00)	0.09
DBLɣ6	453, 72	1.45 (1.12, 1.88)	0.01

Incidence rate ratios adjusted for age and sub-study enrollment. Calculated using negative binomial regression models of log 10 transformed levels of *P. falciparum* antibodies in children with a positive antibody response per antigen. Bolded p-values are significant based on FDR threshold to correct for multiple comparisons. N obs: Number of observations included in the model; N events: number of severe malaria episodes.

5.3.8 Presence or level of antibodies to *P. falciparum* antigens and risk of uncomplicated malaria

Antibody prevalence was not significantly associated with risk of uncomplicated malaria in children with SM, AP or NP (Table 5.8). The levels of CIDRα1.4 in children with NP were associated with an increased risk of post-discharge UM (1.63 [1.21, 2.20], 0.001, Table 5.9). No other levels of antibodies were associated with risk of post-discharge uncomplicated malaria.

Table 5.8. Risk of uncomplicated malaria within 12 months of discharge or at enrollment, according to presence of antibodies to *P. falciparum* antigens.

Children with Severe Malaria					
	Antibody +	**Antibody –**	**Risk of UM**		
	N obs, N events		**IRR**	**95% CI**	**P value**
CSP	341, 126	113, 43	1.02	(0.72, 1.46)	0.91
EBA-140	262, 92	192, 77	0.82	(0.60, 1.12)	0.21
EBA-175	358, 126	96, 43	0.83	(0.58, 1.18)	0.29
EBA-181	280, 90	174, 79	0.73	(0.53, 0.99)	0.05
MSP2	444, 165	10, 4	0.84	(0.30, 2.33)	0.74
MSP3	336, 119	118, 50	0.86	(0.61, 1.21)	0.39
MSP DBL1	443, 165	11, 4	1.08	(0.39, 2.99)	0.88
MSP DBL2	424, 158	30, 11	0.99	(0.53, 1.86)	0.98
SERA5	339, 119	115, 50	0.83	(0.59, 1.16)	0.28
CIDRα1.1	392, 145	61, 24	0.96	(0.62, 1.49)	0.86
CIDRα1.4	307, 113	146, 56	1.05	(0.75, 1.46)	0.78
DBLα2	400, 150	53, 19	1.23	(0.75, 2.01)	0.41
DBLβ12	344, 118	109, 51	0.76	(0.54, 1.06)	0.11
DBLɣ6	199, 62	254, 107	0.79	(0.57, 1.09)	0.15
Children with AP at enrollment					
	Antibody +	**Antibody –**	**Risk of UM**		
	N obs, N events		**IRR**	**95% CI**	**P value**
CSP	49, 8	19, 7	0.41	(0.13, 1.27)	0.12
EBA-140	34, 6	34, 9	0.57	(0.17, 1.89)	0.36
EBA-175	52, 10	16, 5	0.38	(0.10, 1.41)	0.15
EBA-181	38, 6	30, 9	0.49	(0.16, 1.52)	0.22
MSP2	60, 12	8, 3	0.38	(0.09, 1.61)	0.19
MSP3	48, 9	20, 6	0.59	(0.19, 1.85)	0.37
MSP DBL1	62, 15	6, 0	-	-	-
MSP DBL2	56, 11	12, 4	0.48	(0.13, 1.82)	0.28

SERA5	45, 11	23, 4	1.26	(0.37, 4.34)	0.71
CIDRα1.1	61, 14	7, 1	1.67	(0.19, 14.75)	0.65
CIDRα1.4	60, 13	8, 2	0.76	(0.14, 3.97)	0.74
DBLα2	59, 13	9, 2	1.00	(0.20, 5.10)	1.00
DBLβ12	58, 13	10, 2	1.11	(0.22, 5.59)	0.90
DBLɣ6	49, 12	19, 3	1.40	(0.36, 5.40)	0.63
Children with NP at enrollment					
	Antibody +	**Antibody –**	**Risk of UM**		
	N obs, N events		**IRR**	**95% CI**	**P value**
CSP	62, 15	74, 14	1.52	(0.63, 3.66)	0.35
EBA-140	28, 7	108, 22	1.35	(0.49, 3.73)	0.57
EBA-175	45, 11	91, 18	1.59	(0.62, 4.06)	0.33
EBA-181	37, 10	99, 19	1.6	(0.63, 4.03)	0.32
MSP2	84, 21	52, 8	1.82	(0.72, 4.64)	0.21
MSP3	61, 16	75, 13	1.59	(0.68, 3.71)	0.29
MSP DBL1	96, 25	40, 4	2.82	(0.90, 8.90)	0.08
MSP DBL2	65, 11	71, 18	0.86	(0.33, 2.25)	0.76
SERA5	50, 16	86, 13	2.25	(0.96, 5.26)	0.06
CIDRα1.1	105, 25	31, 4	1.77	(0.55, 5.69)	0.34
CIDRα1.4	73, 21	63, 8	2.74	(1.10, 6.83)	0.03
DBLα2	96, 19	40, 10	0.93	(0.37, 2.37)	0.88
DBLβ12	102, 23	34, 6	1.34	(0.48, 3.75)	0.58
DBLɣ6	40, 7	96, 22	0.95	(0.34, 2.64)	0.92

Incidence rate ratios adjusted for age and sub-study enrollment. Calculated using negative binomial regression models of presence or absence of *P. falciparum* antibodies in children with a positive antibody response per antigen. N obs: Number of observations included in the model; N events: number of uncomplicated malaria episodes.

Table 5.9. Risk of post-discharge uncomplicated malaria within 12 months of discharge or at enrollment, according to level antibodies to *P. falciparum* antigens.

Children with severe malaria			
	N obs, N events	**Risk of UM IRR (95% CI)**	**P value**
CSP	454, 169	1.13 (0.86, 1.48)	0.38
EBA-140	454, 169	0.84 (0.60, 1.16)	0.28
EBA-175	454, 169	0.82 (0.65, 1.03)	0.09
EBA-181	454, 169	0.79 (0.62, 1.01)	0.06
MSP2	454, 169	0.87 (0.65, 1.14)	0.31
MSP3	454, 169	0.82 (0.64, 1.06)	0.14
MSP DBL1	454, 169	0.88 (0.67, 1.16)	0.36
MSP DBL2	454, 169	0.88 (0.62, 1.24)	0.46
SERA5	454, 169	0.80 (0.63, 1.02)	0.07
CIDRα1.1	454, 169	0.89 (0.76, 1.04)	0.15

CIDRα1.4	454, 169	1.07 (0.95, 1.20)	0.26
DBLα2	454, 169	1.12 (0.85, 1.46)	0.42
DBLβ12	454, 169	0.84 (0.69, 1.02)	0.08
DBLɣ6	454, 169	0.93 (0.78, 1.10)	0.37
Children with AP at enrollment			
	N obs, N events	**Risk of UM IRR (95% CI)**	**P value**
CSP	68, 15	0.71 (0.29, 1.69)	0.44
EBA-140	68, 15	0.65 (0.21, 2.01)	0.46
EBA-175	68, 15	0.59 (0.26, 1.35)	0.21
EBA-181	68, 15	0.58 (0.24, 1.43)	0.24
MSP2	68, 15	0.78 (0.39, 1.55)	0.48
MSP3	68, 15	1.01 (0.49, 2.09)	0.98
MSP DBL1	68, 15	0.87 (0.39, 1.92)	0.73
MSP DBL2	68, 15	0.74 (0.31, 1.74)	0.49
SERA5	68, 15	1.06 (0.45, 2.49)	0.90
CIDRα1.1	68, 15	1.24 (0.65, 2.37)	0.51
CIDRα1.4	68, 15	1.05 (0.65, 1.70)	0.83
DBLα2	68, 15	0.89 (0.40, 1.99)	0.78
DBLβ12	68, 15	1.25 (0.60, 2.63)	0.55
DBLɣ6	68, 15	1.36 (0.84, 2.21)	0.21
Children with NP at enrollment			
	N obs, N events	**Risk of UM IRR (95% CI)**	**P value**
CSP	136, 29	1.47 (0.70, 3.08)	0.30
EBA-140	136, 29	1.43 (0.48, 4.24)	0.52
EBA-175	136, 29	1.43 (0.75, 2.74)	0.28
EBA-181	136, 29	1.66 (0.77, 3.55)	0.20
MSP2	136, 29	1.75 (1.06, 2.90)	0.03
MSP3	136, 29	1.47 (0.78, 2.79)	0.23
MSP DBL1	136, 29	1.94 (1.06, 3.57)	0.03
MSP DBL2	136, 29	1.09 (0.50, 2.39)	0.83
SERA5	136, 29	2.06 (0.96, 4.41)	0.06
CIDRα1.1	136, 29	1.32 (0.86, 2.02)	0.20
CIDRα1.4	136, 29	1.63 (1.21, 2.20)	**0.001**
DBLα2	136, 29	1.42 (0.73, 2.75)	0.31
DBLβ12	136, 29	1.08 (0.55, 2.10)	0.83
DBLɣ6	136, 29	1.15 (0.71, 1.86)	0.56

Incidence rate ratios adjusted for age and iron treatment sub-study enrollment. Calculated using negative binomial regression models of log 10 transformed levels of *P. falciparum* antibodies. Bolded p-values are significant based on FDR threshold to correct for multiple comparisons. N obs: Number of observations included in the model; N events: number of uncomplicated malaria episodes.

5.4 Discussion

Expression of PfEMP1 antigens is associated with severe malaria (SM), and in numerous prior studies, antibodies to PfEMP1 antigens and to other blood-stage and pre-erythrocytic antigens have been associated with protection from uncomplicated malaria (UM). Protection from severe malaria is less well studied, because it is a less frequent event, but some studies also show association of antibodies to SERA5[143] and PfEMP1 variants[100] with protection from SM. Most of these studies have evaluated antibodies in children who are asymptomatic and well-appearing at the time of antibody testing. Prevalence and level of antibodies during severe malaria is less well-studied, and there are few studies evaluating the duration of antibody presence and level in children with SM. In this study we show for the first time that children with SM are less likely to maintain antibodies to other blood-stage antigens and CSP than children with asymptomatic parasitemia (AP), but do maintain antibodies to PfEMP1 antigens, though the prevalence of antibodies to DBLɣ6 was always lower in children with SM than children with AP. In addition, we show that children with no parasitemia (NP) generally have a lower prevalence of antibodies to *P. falciparum* antigens than children with SM or AP, but maintain those antibodies at rates similar to children with AP and higher than children with SM. The finding that many children with SM are unable to maintain antibodies to key blood-stage *P. falciparum* antigens, and are less able to produce antibodies to the PfEMP1 antigen DBLɣ6, suggests multiple areas for future study, including evaluation of antibody subclass and antibody-mediated mechanisms of immune protection in children with SM compared to AP or NP, evaluation of whether loss of antibodies is associated with increased susceptibility to SM or UM, and assessment of the factors that contribute to decreased antibodies to the PfEMP1 antigen DBLɣ6.

Our finding that there were no antibodies or antibody levels in children with SM that were associated with protection from subsequent SM or UM further support more detailed evaluation of antibody subclass and mechanism in the different groups. We were not able to evaluate risk of SM in children with AP or NP, because so few developed SM. This suggests that children with AP or NP, who were from the same household or neighborhoods as children with SM, either had lower exposure to *P. falciparum*, a finding not supported by the data showing similar levels of parasitemia in children in the three groups in follow-up or had mechanisms of immunity not reflected in measurement IgG antibodies to *P. falciparum* antigens by Luminex. These findings and the finding of a lack of difference in risk of SM or UM among children with AP vs. NP argue for further evaluation of antibody type and mechanism in these two groups, since IgG presence and levels to almost all antigens studied were higher in children with AP vs. NP, yet the risk of SM or UM did not differ in the two groups.

The possible persistence of antibodies over time in children with AP, but not those with SM or with no parasitemia, suggests that this group have developed some levels of clinical immunity to *P. falciparum* infection not seen in children recovering from severe malaria. However, despite the persistence of antibodies in this group, not all children with asymptomatic parasitemia at enrollment maintained asymptomatic parasitemia over the 12 months. At enrollment, 72 children were positive for asymptomatic *P. falciparum*; 31 of those children were also positive at 6 months, and only 15 were positive at 12 months. Children with parasitemia at multiple time points could either have new infections or be maintaining low levels of the parasite over the year of the study. However, at both 6 and 12 months, the percentage of children in each of the three groups with asymptomatic parasitemia via PCR was similar. This suggests that children had similar levels of exposure to the parasite and thus similar risks of developing asymptomatic parasitemia during the 12-month follow-up. It has been

demonstrated previously that antibodies to *P. falciparum* antigens declined at similar rates in children with and without chronic asymptomatic infection during the dry season in Mali[146]. These results contrast with the findings in our study, where antibodies persisted in children with asymptomatic parasitemia compared to children with no parasitemia. Parasite genetic studies would be required to look at the rate of new infections in this population and the number of parasite clones per infection. Being exposed to multiple parasite strains over time could impact the antibody response mounted by children living in malaria-endemic regions.

The PfEMP1 family antigens tended to have higher prevalence and levels over time across all groups and presented the most differences in these measures over time compared with the other antigens tested. This could be because antibodies against PfEMP1 antigens develop before other blood-stage antigens[98], particularly to domains of the protein related to severe malaria[98]. Antibodies to PfEMP1 antigen DBLɣ6, though maintained in children with SM over time, were consistently lower in children with SM than AP. Among PfEMP1 antigens, only antibodies to this antigen were lower at all time points in children with SM than in children with AP. This antigen is found in tandem with EPCR-binding domains as a part of DC8[121], which are associated with severe malaria pathogenesis in children[112,121]. The inability to develop antibodies to this antigen could predispose children with SM to develop SM again. However, antibodies to DBLɣ6 were not associated with protection, again raising the question as to whether the antibody subclass, affinity or ability to mediate phagocytosis or complement fixation may also differ in children with SM who develop these antibodies as compared to those with AP or NP.

In this study, antibodies to MSP3 were associated with increased risk of post-discharge SM in children with SM, and no antibodies were associated with an altered risk of post-discharge/post-enrollment risk of UM in children with SM, AP or NP. The increased risk associated with MSP3 is unlikely to relate to exposure or true increased risk of disease, since prevalence of antibodies to this antigen was similar in children with AP, yet they did not develop SM. We also found no association between antibodies or antibody levels to any antigen and risk of UM in children with AP or NP. This is in contrast to studies by our group and others showing protection from UM in children with antibodies or elevated antibody levels to GLURP (glutamate-rich protein), MSP, and AMA-1(apical membrane antigen)[48], and EBA 175[139]. In the current study, antibodies were generally associated with an IRR greater than 1, though confidence intervals were wide, and no association was significant. The associations suggest the antibodies in this study may be acting as markers of exposure. We previously showed, in a cohort in an area of higher malaria endemicity, that evaluating risk of malaria in comparison to antibody level once parasitemia is detectable, reduced some of the confounding introduced by exposure-related antibody levels[147]. In this study, we did not find associations with protection from clinical malaria in children with AP, but we were not doing monthly surveillance for parasitemia, and so could not use the same definition for outcome (clinical malaria only in children who developed parasitemia). Small numbers of children with UM in the AP and NP groups limited our ability to detect all but large associations with protection in these groups, but it is still unclear why most associations showed an increase in risk and no antibodies were associated with protection, unless in this study antibody prevalence and level was almost exclusively a marker for exposure.

A number of other measures of antibody-related protection from clinical malaria have been described, including IgG subclass[79], measurement of IgM[148], and evaluation of antibody-mediated monocyte phagocytosis[52,149] or antibody-mediated complement

fixation[53,150]. Future studies should investigate whether these measures of antibody function protection differ in these three groups, and whether these assessments provide better correlates of immune protection from severe or uncomplicated malaria in children in malaria endemic areas.

In conclusion, we established the persistence of higher antibody levels to 14 different *Plasmodium falciparum* antigens in children with AP compared to children with SM or without asymptomatic *P*. *falciparum* infection at enrollment. At 6 months, children that recovered from SM had antibody responses closer to the children with NP, while those with asymptomatic parasitemia maintained higher levels at 6 and 12 months after enrollment. The results of this study show that the presence of antibodies to *P. falciparum* antigens, including PfEMP1, persisted in children with AP at enrollment. These results support the hypothesis that AP may stimulate an antibody response to PfEMP1 and other antigens in asymptomatic children. The presence of parasites in the blood, even at low levels, may improve the development of an efficient immune response to malaria infection.

Chapter 6: The presence of IgG antibodies to PfEMP1 CIDR domains is highest in children with severe malarial anemia compared to other forms of severe malaria

6.1 Introduction

In 2020, malaria caused more than an estimated 600,000 deaths[6], 75% of which were children, who are at the greatest risk of severe malaria caused by *Plasmodium falciparum*. Severe malaria symptoms include severe anemia, seizures, respiratory distress, and coma. Cytoadherence of infected erythrocytes to the host endothelium is a key mediator of severe malaria and can occur throughout the host. Certain interactions between infected erythrocytes and the host endothelium are related to different disease states, such as the binding of endothelial protein C receptor (EPCR) in the brain during cerebral malaria[151]. These interactions of *P. falciparum* with host proteins are mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is expressed on the surface of infected erythrocytes and is a primary immunity target of natural immunity[62]. PfEMP1 is a significant source of antigenic variation for the parasite, as there are approximately 60 *var* genes that encode the protein and generate a wide diversity of protein domain structures[56,66].

PfEMP1 molecules are composed of combinations of two to nine Duffy binding-like (DBL) and cysteine-rich interdomain region (CIDR) domains that are anchored on the surface of the infected erythrocyte at the C terminal end of the protein as a part of the knob structure[72]. DBL and CIDR domains can be subdivided into seven DBL (α, β, γ, δ, ε, ξ, x) and three CIDR (α, β, γ) main sequence classes based on sequence similarity, with further subdivisions[73]. CIDRα domains are found towards the N-terminal end of the protein, in tandem with DBLα domains, and are responsible for binding to host EPCR[74,75] CIDRα1 domains or CD36 CIDRα2-6 domains[78,79]. CIDRδ1 domains can alternatively be found in tandem with DBLα domains, which are thought to be involved in rosetting—the

clumping of infected and uninfected red blood cells in the vasculature—but have unknown host binding partners[76,77]. The expression of EPCR-binding domains in PfEMP1 has been associated with severe malaria[114,116], while the expression of domains that bind to CD36 is related to uncomplicated malaria[85,152].

The relationships between PfEMP1 expression and malaria disease manifestation suggest that PfEMP1 is playing a role in pathogenesis of disease, but PfEMP1 also plays a role in evading the immune system via its antigenic variation contributed by the *var* genes. PfEMP1 is a natural immune target, with antibodies forming over time with exposure, alongside antibodies to other *P. falciparum* antigens[98,99]. Antibodies against EPCR-binding domains develop prior to those against CD36-binding domains and other *P. falciparum* antigens[98,99]. Immunity to non-cerebral severe malaria is suggested to develop after one or two infections[38]. Combined with the rapid development of antibodies to EPCR-binding domains, this is thought to contribute to why severe malaria is primarily seen only in children, especially in high transmission areas. Antibodies against PfEMP1 have been associated with protection from severe and clinical malaria[101,153]. The associations between PfEMP1 antibodies and protection range from noting that a broad repertoire of antibodies to PfEMP1 are required for preventing malaria episodes[154] to highlighting individual domains or groups of domains and their relationship to malarial protection[99]. Recently, immunity to severe malaria was shown to be related to a subset of conserved PfEMP1 EPCR-binding domains, but clinical immunity to malaria required a broad repertoire of antibodies to PfEMP1 domains[100].

In this study, we aimed to improve our understanding of severe malaria immunity, particularly with respect to antibodies to different regions of the PfEMP1 protein. Using plasma samples from Ugandan children with severe malaria, we measured the IgG antibody response to 18 PfEMP1 domains, including nine EPCR-binding domains, six

CD36-binding domains, and three CIDRδ1 domains; using a broad repertoire of CIDR antigens to capture the diversity of PfEMP1 antigens. The study included children with five manifestations of severe malaria—cerebral malaria, respiratory distress, malaria with repeated seizures, severe malarial anemia, and prostration—allowing for an inclusive examination of the antibody response to severe malaria. Additionally, we evaluated associations between the presence of these antibodies and protection from additional episodes of severe and uncomplicated malaria. We expected the antibody response to ECPR-binding domains to be elevated in children with severe malaria and related to severity of disease in this population and protection from additional severe malaria. Understanding the specific antigenic immune targets associated with severe malaria could inform about possible vaccine targets for severe malaria or help to reduce the rate of hospital readmissions for severe malaria in children. In this study we investigate the presence of antibodies to PfEMP1 at enrollment in children with severe malaria and the maintenance of these antibodies at 12 months follow-up.

6.2 Methods

6.2.1 Study design

Between 2014 and 2017, 600 children with severe malaria and 120 community children were enrolled in a prospective cohort study at two sites in Uganda, Mulago National Referral and Teaching Hospital in Kampala, Uganda, and Jinja Regional Referral Hospital in Jinja, Uganda. This study is a part of a larger study designed to evaluate the impact of the five most common forms of severe malaria on neurocognition at 12-months follow-up in children <5 years of age. Children were enrolled with one of five forms of severe malaria: cerebral malaria (CM), respiratory distress (RDS), malaria with repeated seizures (M/S), severe malarial anemia (SMA), and prostration (Pro). A hierarchy based on disease severity was used to place children with multiple symptoms

into groups, with CM being the most severe. Inclusion criteria included children aged six months to four years of age with malaria (positive rapid diagnostic test for *Plasmodium falciparum* histidine-rich protein-2 (HRP-2) or direct visualization of parasites by Giemsa microscopy) who required hospitalization for coma, respiratory distress, two or more seizures in 24 hours, severe anemia defined as hemoglobin <5g/dL or prostration. The definition of malaria using either rapid diagnostic test or microscopy aligns with the Uganda Clinical Guidelines on malaria diagnosis. Exclusion criteria included known chronic illness requiring medical care, known developmental delay, or history of coma, head trauma, and prior hospitalization for malnutrition. Comatose children with lumbar puncture findings suggestive of another cause of coma were excluded. For asymptomatic controls, 120 community children (CC) from the nuclear family, extended family, or household area of the children with severe malaria were enrolled. Additional exclusion criteria for the controls included an illness requiring medical care within the previous four weeks, a major medical or neurologic abnormality at screening physical examination, or active illness. On enrollment, children had a complete history and physical exam, and venous blood draw. Children were managed according to Ugandan National Treatment guidelines for severe malaria, including intravenous artesunate followed by oral artemisinin-combination therapy.

6.2.2 Blood sample collection and detection of *P. falciparum* parasitemia

Blood samples were collected from all participants at enrollment and in limited participants at 12-month follow-up visit and stored at –80°C. *P. falciparum* parasitemia was confirmed with both thick and thin blood smears that were read independently by two experienced laboratory technicians, as previously described[17]. Parallel ELISA testing for *P. falciparum* histidine-rich protein 2 (HRP-2) was performed on all enrollment samples with available plasma (Malaria Ag Cellabs, Brookvale, Australia), as previously described[119]. DNA extracted from whole blood samples from all participating children

was tested for submicroscopic *P. falciparum* infection at enrollment using nested PCR, as previously described[120].

6.2.3 Measurement of antibodies to PfEMP1 domains

A total of 18 His-tagged CIDR domains of PfEMP1 and three blood stage antigens (Table 6.1) were expressed and purified as previously described[155,156]. Total IgG levels were measured using a Luminex assay[156]. Briefly, serum was diluted 1:40 in assay buffer E (0.1% bovine serum albumin, 0.05% Tween-20 in phosphate-buffered saline, pH 7.4). One each plate, a standard curve was generated from serially diluted pooled plasma of hyperimmune individuals. Mean fluorescent intensities were measured using Luminex system to determine antibody levels of each PfEMP1 domain.

Table 6.1. List of antigens.

Antigen	Genotype	Family	Binding
EPCR Binders		PfEMP1 Group A	EPCR
CIDRα1.1 (1)	IT4var06	PfEMP1 Group A	EPCR
CIDRα1.1 (2)	raj116_var8	PfEMP1 Group A	EPCR
CIDRα1.4 (1)	1994-2	PfEMP1 Group A	EPCR
CIDRα1.4 (2)	HB3var03	PfEMP1 Group A	EPCR
CIDRα1.4 (3)	IT4var22	PfEMP1 Group A	EPCR
CIDRα1.5	1965-2	PfEMP1 Group A	EPCR
CIDRα1.6b	>ERS010031_NODE_1881_4_length_3948_cov_7.379179_rframe3_ORF	PfEMP1 Group A	EPCR
CIDRα1.7	2083-1	PfEMP1 Group A	EPCR
CIDRα1.8a	>ERS010178_NODE_17177_length_7471_cov_8.698702_rframe2_ORF	PfEMP1 Group A	EPCR
CD36 Binders		PfEMP1 Group B/C	CD36
CIDRα2.1	IT4var30	PfEMP1 Group B/C	CD36
CIDRα2.2	IT4var24	PfEMP1 Group B/C	CD36
CIDRα2.4	IT4var33	PfEMP1 Group B/C	CD36
CIDRα2.7	IT4var61	PfEMP1 Group B/C	CD36
CIDRα2.9	IT4var45	PfEMP1 Group B/C	CD36
CIDRα3.3	IT4var26	PfEMP1 Group B/C	CD36

CIDRδ1 Group		PfEMP1 Group A	Unknown
CIDRδ1 (1)	HB3var35	PfEMP1 Group A	Unknown
CIDRδ1 (2)	HB3var05	PfEMP1 Group A	Unknown
CIDRδ1 (3)	IT4var02	PfEMP1 Group A	Unknown
CSP		Pre-erythrocytic	
GLURP		Erythrocytic	
SERA 5		Erythrocytic	

6.2.4 Statistical analysis

Using the pooled plasma from hyperimmune individuals, a standard curve was generated with an arbitrary value of 1000 relative units assigned to the highest concentration. IgG concentrations were interpolated from the standard curves and used to calculate a positive response per antigen per individual. An individual was recorded as having a positive response if their antibody response was at least two standard deviations above the mean arbitrary units of 40 Danish negative controls. Data are presented as the percent prevalence of antibodies to PfEMP1 domains. For individuals with a positive response, levels of antibodies are expressed as fold over negative control (FONC), calculated as the study participant arbitrary units divided by the mean response of malaria naïve negative controls plus three standard deviations. Group antigen variables, EPCR binders and CD36 binders, were given a positive result if a child had a positive response for at least one of the antigens included in the group.

A Chi-squared test was used to compare the prevalence of antibodies at enrollment. McNemar's test was used for matched analyses to compare the prevalence of antibodies at baseline and 12 months. For continuous variables, a Kruskal-Wallis test was used to compare all five severe malaria groups, with the Dunn test as post hoc analysis. For dichotomous variables, when comparing all five severe malaria and community control groups, multiple comparisons were adjusted for using the Bonferroni correction, with $p<0.003$ as the threshold for significance. In other analyses, multiple

comparisons were adjusted for using the Benjamini-Hochberg false discover rate (FDR) approach with a significance level of 0.05[129]. Comparison of antibody presence to *P. falciparum* antigens and incidence of severe or uncomplicated malaria at 12 months follow-up was performed using negative binomial regression. The model was run with offset of natural log of years at risk of malaria while enrolled in the study. This analysis was adjusted for age, sex, study site, and severe malaria group when applicable. StataSE version 14 (StataCorp) was used for all analyses. Graphics were generated using Graph Pad Prism 9.

6.2.5 Ethics review

Written informed consent was obtained from the parents or legal guardians of study participants after providing emergency clinical care to stabilize children. Ethical approval was granted by Institutional Review Boards at Makerere University School of Medicine, the University of Minnesota, and Indiana University School of Medicine. The Uganda National Council for Science and Technology approved the study.

6.3 Results

6.3.1 Demographic characteristics of study participants

Demographic and baseline clinical characteristics for children enrolled in all six groups are shown in Table 6.2. Demographic data, including age, sex and study site were similar across all groups (Table 6.2). In this population, the rate of asymptomatic parasitemia (AP) was 27.5%, with 33 of 120 asymptomatic community children testing positive for *P. falciparum* infection by nested PCR.

Table 6.2. Clinical and demographic data for children with severe malaria and community children included in antibody testing.

	SMA (n=155)	CM (n=85)	RDS (n=120)	M/S (n=160)	Pro (n=76)	CC (n=120)	P-value[a]
Age, years	1.92 (1.43, 2.83)	2.16 (1.57, 3.09)	1.86 (1.12, 2.51)	2.06 (1.43, 2.99)	2.22 (1.56, 2.95)	2.16 (1.39, 3.08)	0.53
Sex, n female (%)	69 (44.52)	38 (44.71)	47 (39.17)	71 (44.38)	35 (46.05)	56 (46.67)	0.54
Study Site, n Jinja (%)	58 (37.42)	37 (43.53)	67 (55.83)	64 (40.00)	41 (53.95)	53 (44.17)	0.90
P. *falciparum* RDT or blood smear positive, n (%)[b]	155 (100)	85 (100)	120 (100)	160 (100)	76 (100)	N/A	
Blood smear *P. falciparum* positive (%)	93 (60.00)	69 (81.18)	86 (71.67)	127 (79.38)	63 (82.89)	13 (11.02)	**<0.0001[c]**
PCR *P. falciparum* positive (%)	146 (94.19)	82 (96.47)	114 (95.00)	155 (96.88)	73 (96.05)	33 (27.50)	**<0.0001[d]**

[a]P-value from on Wilcoxon Rank Test for age and remaining Chi-squared for dichotomous variables, comparing all severe malaria groups and community control children.
[b]Children in severe malaria groups were positive based on RDT, if RDT were not performed, children were determined to be positive for *P. falciparum* infection using microscopy. CC's were not tested using RDT
[c]Blood smear positivity is significantly different for all severe malaria groups compared to CC in pairwise comparisons. Additional significant comparisons include: CM vs SMA, M/S vs SMA and SMA vs Pro.
[d]PCR positivity is significantly different for all severe malaria groups compared to CC in pairwise comparisons.

6.3.2 Prevalence of antibodies to PfEMP1 in children with different forms of severe malaria

We measured the presence of IgG antibodies at study enrollment to nine CIDRα1 domains that bind ECPR, six CIDRα2-6 domains that bind to CD36, and three CIDRδ1 domains of PfEMP1. Additionally, three non-PfEMP1 *P. falciparum* antigens were included to examine immunity at different stages of the parasite life cycle: CSP (pre-erythrocytic stage), and GLURP and SERA5 (erythrocytic stage) (Supplemental Table A5.1). Children with any of the five forms of severe malaria had higher prevalence rates

of antibodies to 12 of 18 PfEMP1 antigens and to GLURP and SERA5 but not CSP, compared to CC (Table 6.3). When comparing among the different forms of severe malaria, the antibody response to all measured domains of PfEMP1 was similar among these forms (Table 6.4), except for severe malarial anemia (SMA), in which prevalence rates were higher for 16 of 18 PfEMP1 antigens, CSP and SERA5, when compared to all other forms of severe malaria (Table 6.5). 90.2% of children with SMA had antibodies to at least one of the nine EPCR-binding antigens, as compared to 79.3% of individuals with any other form of severe malaria ($p=0.001$, Table 6.5). Compared to children with other forms of severe malaria, children with SMA had greater prevalence of antibodies to all nine EPCR-binding domains, CIDRδ1 domains, CSP and SERA5 but only to four of the seven CD36-binding domains (Table 6.5). The levels of antibodies in children with a positive response were similar across the six study groups (Table 6.6).

Table 6.3. Prevalence of antibodies to PfEMP1 in children with any form of severe malaria compared to asymptomatic community children (CC).

	Severe Malaria (n=596)	CC (n=120)	P-value[a]
EPCR Binders	491 (82.38)	83 (69.17)	**<0.0001**
CIDRα1.1 (1)	108 (18.12)	17 (14.17)	0.30
CIDRα1.1 (2)	313 (52.52)	49 (40.83)	**0.02**
CIDRα1.4 (1)	323 (54.19)	52 (43.33)	**0.03**
CIDRα1.4 (2)	389 (65.27)	62 (51.67)	**0.005**
CIDRα1.4 (3)	398 (66.78)	56 (46.67)	**<0.0001**
CIDRα1.5	213 (35.74)	37 (30.83)	0.30
CIDRα1.6b	441 (73.99)	56 (46.67)	**<0.0001**
CIDRα1.7	340 (57.05)	45 (37.50)	**<0.0001**
CIDRα1.8a	324 (54.36)	48 (40.00)	**0.004**
CD36 Binders	482 (80.87)	69 (57.50)	**<0.0001**
CIDRα2.1	347 (58.22)	47 (39.17)	**0.0001**
CIDRα2.2	112 (18.79)	17 (14.17)	0.23
CIDRα2.4	279 (46.81)	34 (28.33)	**0.0002**
CIDRα2.7	29 (4.87)	3 (2.50)	0.25
CIDRα2.9	340 (57.05)	44 (36.67)	**<0.0001**
CIDRα3.3	273 (45.81)	30 (25.00)	**<0.0001**
CIDRδ1 Group	329 (55.20)	49 (40.83)	**0.004**
CIDRδ1 (1)	170 (28.52)	30 (25.00)	0.43
CIDRδ1 (2)	154 (25.84)	24 (20.00)	0.18
CIDRδ1 (3)	280 (46.98)	41 (34.17)	**0.01**
CSP	117 (19.63)	18 (15.00)	0.24
GLURP	413 (69.30)	48 (40.00)	**<0.0001**
SERA 5	398 (66.78)	51 (42.50)	**<0.0001**

Number of children and percent prevalence (in parentheses) of positive antibody response to the measured PfEMP1 and *P. falciparum* antigens in children with five forms of severe malaria and asymptomatic community controls.

[a]Chi-squared test comparing all severe malaria groups (n=596) with community children.

Table 6.4. Prevalence of antibodies to PfEMP1 antigens at enrollment in children with five forms of severe malaria.

	SMA (n=155)	CM (n=85)	RDS (n=120)	M/S (n=160)	Pro (n=76)	P-value
EPCR Binders	141 (90.97)	73 (85.88)	95 (79.17)	118 (73.75)	64 (84.21)	**0.001**
CIDRα1.1 (1)	37 (23.87)	16 (18.82)	15 (12.50)	27 (16.88)	13 (17.11)	0.18
CIDRα1.1 (2)	100 (64.52)	47 (55.29)	55 (45.83)	74 (46.25)	37 (48.68)	**0.01**
CIDRα1.4 (1)	98 (63.23)	46 (54.12)	65 (54.17)	76 (47.50)	38 (50.00)	0.07
CIDRα1.4 (2)	116 (74.84)	57 (67.06)	75 (62.50)	88 (55.00)	53 (69.74)	**0.005**
CIDRα1.4 (3)	124 (80.00)	59 (69.41)	76 (63.33)	91 (56.88)	48 (63.16)	**0.0004**
CIDRα1.5	68 (43.87)	26 (30.59)	43 (35.83)	51 (31.87)	25 (32.89)	0.15
CIDRα1.6b	129 (83.23)	70 (82.35)	82 (68.33)	106 (66.25)	54 (71.05)	**0.002**
CIDRα1.7	109 (70.32)	50 (58.82)	67 (55.83)	72 (45.00)	42 (55.26)	**0.0003**
CIDRα1.8a	103 (66.45)	47 (55.29)	60 (50.00)	75 (46.88)	39 (51.32)	**0.01**
CD36 Binders	133 (85.81)	70 (82.35)	97 (80.83)	121 (75.62)	61 (80.26)	0.25
CIDRα2.1	106 (68.39)	48 (56.47)	66 (55.00)	83 (51.88)	44 (57.89)	0.04
CIDRα2.2	34 (21.94)	12 (14.12)	16 (13.33)	35 (21.88)	15 (19.74)	0.23
CIDRα2.4	90 (58.06)	40 (47.06)	51 (42.50)	59 (36.88)	39 (51.32)	**0.003**
CIDRα2.7	8 (5.16)	4 (4.71)	4 (3.33)	7 (4.38)	6 (7.89)	0.69
CIDRα2.9	102 (65.81)	53 (62.35)	59 (49.17)	81 (50.62)	45 (59.21)	**0.02**
CIDRα3.3	85 (54.84)	44 (51.76)	51 (42.50)	61 (38.12)	32 (42.11)	**0.03**
CIDRδ1 Group	105 (67.74)	51 (60.00)	60 (50.00)	71 (44.38)	42 (55.26)	**0.001**
CIDRδ1 (1)	55 (35.48)	20 (23.53)	32 (26.67)	38 (23.75)	25 (32.89)	0.11
CIDRδ1 (2)	54 (34.84)	24 (28.24)	24 (20.00)	33 (20.62)	19 (25.00)	**0.02**
CIDRδ1 (3)	91 (58.71)	45 (52.94)	52 (43.33)	56 (35.00)	36 (47.37)	**0.001**
CSP	41 (26.45)	13 (15.29)	24 (20.00)	30 (18.75)	9 (11.84)	0.07
GLURP	117 (75.48)	56 (65.88)	77 (64.17)	113 (70.62)	50 (65.79)	0.26

SERA 5	130 (83.87)	57 (67.06)	86 (71.67)	82 (51.25)	43 (56.58)	**<0.0001**

Number of children and percent prevalence (in parentheses) of positive antibody response to the measured PfEMP1 and *P. falciparum* antigens in children with five forms of severe malaria and asymptomatic community controls.

Table 6.5. The prevalence of antibodies to PfEMP1 domains in children with SMA compared to all other forms of severe malaria.

	SMA (n=155)	All other forms of severe malaria (n=441)	P-value
EPCR Binders	141 (90.97)	350 (79.37)	**0.001**
CIDRα1.1 (1)	37 (23.87)	71 (16.10)	**0.03**
CIDRα1.1 (2)	100 (64.52)	213 (48.30)	**0.001**
CIDRα1.4 (1)	98 (63.23)	225 (51.02)	**0.01**
CIDRα1.4 (2)	116 (74.84)	273 (61.90)	**0.004**
CIDRα1.4 (3)	124 (80.00)	274 (62.13)	**0.0001**
CIDRα1.5	68 (43.87)	145 (32.88)	**0.01**
CIDRα1.6b	129 (83.23)	312 (70.75)	**0.002**
CIDRα1.7	109 (70.32)	231 (52.38)	**0.0001**
CIDRα1.8a	103 (66.45)	221 (50.11)	**0.0004**
CD36 Binders	133 (85.81)	349 (79.14)	0.07
CIDRα2.1	106 (68.39)	241 (54.65)	**0.003**
CIDRα2.2	34 (21.94)	78 (17.69)	0.24
CIDRα2.4	90 (58.06)	189 (42.86)	**0.001**
CIDRα2.7	8 (5.16)	21 (4.76)	0.84
CIDRα2.9	102 (65.81)	238 (53.97)	**0.01**
CIDRα3.3	85 (54.84)	188 (42.63)	**0.01**
CIDRδ1 Group	105 (67.74)	224 (50.79)	**0.0003**
CIDRδ1 (1)	55 (35.48)	115 (26.08)	**0.03**
CIDRδ1 (2)	54 (34.84)	100 (22.68)	**0.003**
CIDRδ1 (3)	91 (58.71)	189 (42.86)	**0.001**
CSP	41 (26.45)	76 (17.23)	**0.01**
GLURP	117 (75.48)	296 (67.12)	0.05
SERA 5	130 (83.87)	268 (60.77)	**<0.0001**

Number of children and percent prevalence (in parentheses) of positive antibody response to measured PfEMP1 and *P. falciparum* antigens in children with five forms of severe malaria and asymptomatic community controls.
[a]Chi-squared test comparing the prevalence of antibodies in SMA vs. all other forms of severe malaria.

Table 6.6. Levels of antibodies to PfEMP1 antigens in children that had a positive response with five forms of severe malaria and asymptomatic community children at enrollment.

	CM	RDS	M/S	SMA	Pro	CC	P-value[a]
EPCR Binders	2.54 (1.56-4.76) (73)	2.94 (1.63-6.68) (95)	2.24 (1.54-4.56) (118)	3.59 (1.84-7.34) (141)	2.41 (1.69-4.28) (64)	2.66 (1.56-6.47) (83)	0.03
CIDRα1.1 (1)	1.49 (1.12-2.28) (16)	1.66 (1.14-4.77) (15)	1.73 (1.32-2.36) (27)	1.75 (1.26-2.90) (37)	1.35 (1.17-2.00) (13)	1.58 (1.19-2.24) (17)	0.69
CIDRα1.1 (2)	2.53 (1.52-5.45) (47)	2.19 (1.72-3.45) (55)	2.36 (1.46-4.72) (74)	2.68 (1.57-5.23) (100)	2.51 (1.42-4.18) (37)	2.51 (1.68-7.60) (49)	0.66
CIDRα1.4 (1)	3.71 (1.82-13.93) (46)	3.56 (1.53-22.29) (65)	3.30 (1.62-7.89) (76)	6.12 (2.91-41.95) (98)	3.07 (1.74-10.26) (38)	4.98 (1.57-16.60) (52)	0.008
CIDRα1.4 (2)	3.99 (1.57-11.68) (57)	4.03 (2.05-15.04) (75)	3.92 (2.01-9.21) (88)	4.71 (2.32-24.12) (116)	3.39 (1.71-9.24) (53)	4.95 (1.55-15.31) (62)	0.21
CIDRα1.4 (3)	3.44 (1.64-13.58) (59)	4.67 (2.39-24.30) (76)	3.90 (1.85-10.02) (91)	5.72 (2.44-28.73) (124)	3.55 (1.74-7.81) (48)	4.97 (2.22-23.24) (56)	0.03
CIDRα1.5	2.13 (1.48-4.17) (26)	3.03 (1.77-8.38) (43)	1.86 (1.24-4.00) (51)	3.25 (1.53-8.31) (68)	1.79 (1.38-4.13) (25)	3.01 (1.43-10.97) (37)	0.13
CIDRα1.6b	3.65 (1.62-8.63) (70)	3.65 (2.19-6.94) (82)	2.83 (1.64-6.37) (106)	4.70 (2.35-10.91) (129)	3.12 (1.67-6.20) (54)	4.36 (1.81-10.88) (56)	0.03
CIDRα1.7	3.50 (1.77-7.37) (50)	2.73 (1.64-4.48) (67)	2.63 (1.71-5.60) (72)	3.90 (1.78-7.11) (109)	2.94 (2.05-4.37) (42)	3.94 (2.43-6.27) (45)	0.12
CIDRα1.8a	2.61 (1.43-6.62) (47)	2.75 (1.60-4.79) (60)	2.50 (1.43-4.90) (75)	3.10 (1.82-7.35) (103)	3.39 (1.72-5.29) (39)	2.93 (1.72-11.20) (48)	0.46
CD36 Binders	1.97 (1.37-2.65) (70)	1.69 (1.29-2.66) (97)	1.84 (1.45-2.73) (121)	2.24 (1.69-3.71) (133)	1.97 (1.34-2.96) (61)	1.84 (1.31-3.32) (69)	0.005
CIDRα2.1	2.71 (1.38-5.23) (48)	2.01 (1.24-3.57) (66)	1.85 (1.34-3.77) (83)	3.21 (1.69-5.30) (106)	1.97 (1.25-4.79) (44)	2.14 (1.34-4.46) (47)	0.003
CIDRα2.2	1.45 (1.16-2.67) (12)	1.49 (1.15-2.47) (16)	1.61 (1.38-3.95) (35)	1.94 (1.28-3.00) (34)	1.59 (1.32-2.33) (15)	1.91 (1.24-2.61) (17)	0.76
CIDRα2.4	2.04 (1.54-2.80) (40)	1.58 (1.18-2.60) (51)	2.16 (1.32-3.73) (59)	1.81 (1.30-3.81) (90)	1.99 (1.41-3.59) (39)	1.67 (1.19-2.97) (34)	0.33

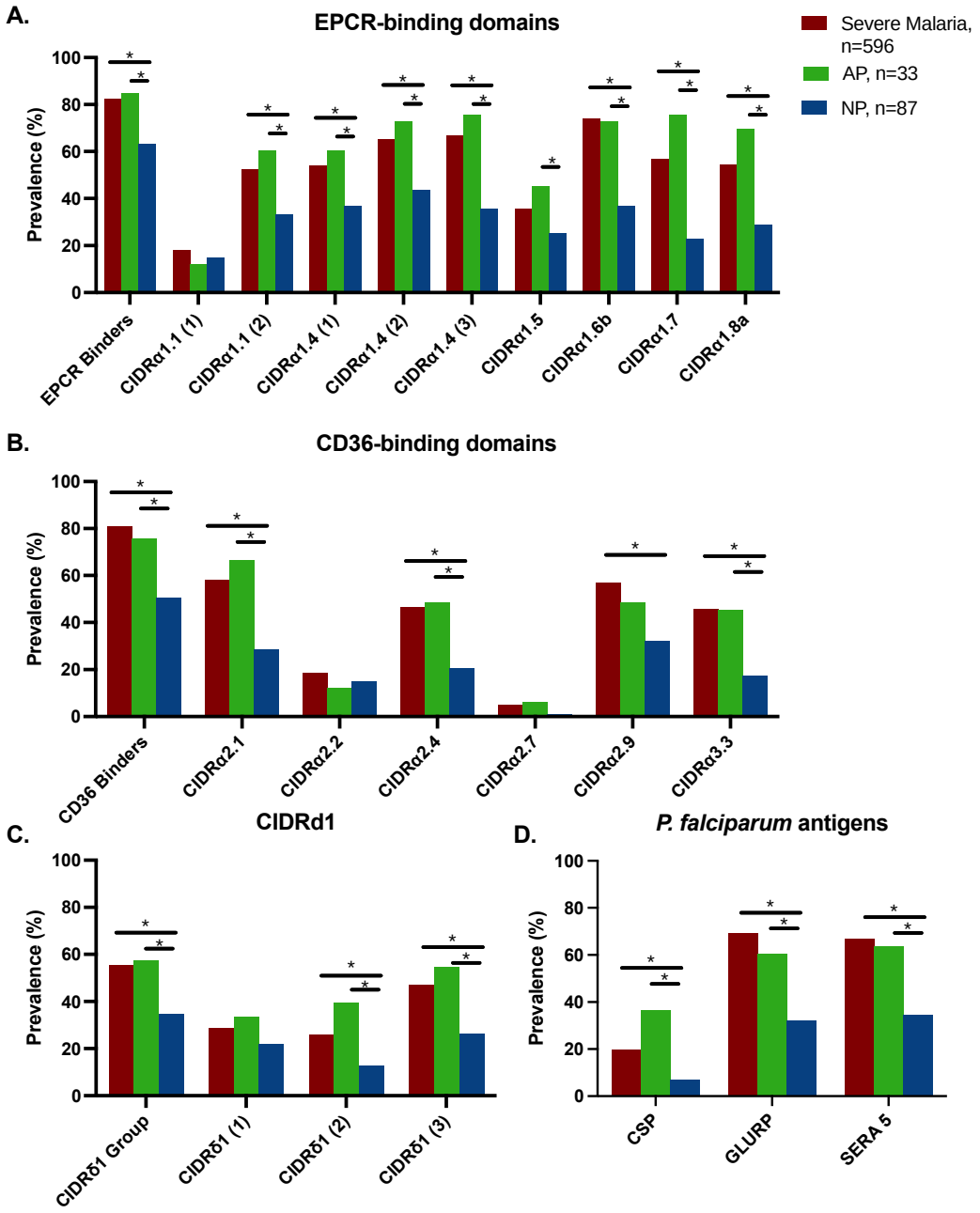
CIDRα2.7	1.67 (1.37-1.67) (4)	1.67 (1.41-1.67) (4)	1.67 (1.67-1.67) (7)	1.67 (1.48-1.67) (8)	1.67 (1.67-1.67) (6)	1.67 (1.00-1.67) (3)	0.99
CIDRα2.9	2.15 (1.35-3.31) (53)	2.12 (1.37-4.38) (59)	2.24 (1.59-4.09) (81)	2.45 (1.55-4.34) (102)	2.46 (1.50-4.06) (45)	2.14 (1.51-4.57) (44)	0.68
CIDRα3.3	2.26 (1.35-3.97) (44)	1.73 (1.34-2.85) (51)	1.80 (1.38-4.94) (61)	2.79 (1.86-4.54) (85)	1.87 (1.33-3.17) (32)	2.14 (1.34-4.13) (30)	0.01
CIDRδ1 Group	2.20 (1.27-4.35) (51)	2.08 (1.44-3.97) (60)	2.17 (1.37-3.94) (71)	2.89 (1.75-4.71) (105)	1.99 (1.37-3.51) (42)	2.89 (1.47-8.86) (49)	0.19
CIDRδ1 (1)	3.34 (2.09-21.97) (20)	3.44 (1.61-15.78) (32)	2.52 (1.22-8.05) (38)	3.05 (1.48-10.16) (55)	1.70 (1.20-3.77) (25)	10.83 (2.01-22.17) (30)	0.05
CIDRδ1 (2)	1.85 (1.25-4.15) (24)	1.67 (1.32-3.88) (24)	1.85 (1.35-3.16) (33)	2.43 (1.41-4.71) (54)	2.06 (1.34-3.46) (19)	1.75 (1.28-4.40) (24)	0.48
CIDRδ1 (3)	2.05 (1.27-7.00) (45)	2.78 (1.46-5.25) (52)	2.15 (1.44-6.94) (56)	3.56 (1.46-7.81) (91)	2.02 (1.35-4.58) (36)	4.55 (1.63-9.09) (41)	0.11
CSP	1.75 (1.30-3.11) (13)	2.22 (1.18-3.84) (24)	1.97 (1.47-3.94) (30)	1.99 (1.37-3.57) (41)	1.70 (1.24-1.92) (9)	2.49 (1.96-3.28) (18)	0.65
GLURP	5.09 (1.87-16.69) (56)	4.91 (1.93-14.92) (77)	6.47 (1.84-21.29) (113)	5.77 (2.17-18.41) (117)	6.49 (2.01-19.44) (50)	7.79 (3.04-21.66) (48)	0.88
SERA 5	4.82 (2.40-15.77) (57)	5.16 (2.42-14.98) (86)	4.29 (1.98-12.29) (82)	6.86 (2.78-16.00) (130)	5.17 (2.41-15.95) (43)	4.09 (2.34-11.17) (51)	0.26

Levels of antibodies to PfEMP1 domains and other blood-stage *P. falciparum* antigens for children with a positive antibody response per antigen, presented in arbitrary units as fold change over negative control threshold. Data presented as median [IQR] (n).
[a]Kruskal-Wallis test to compare levels across all six groups. No significant differences after adjusting for multiple comparisons for the number of domains using FDR.

6.3.3 Prevalence of antibodies to PfEMP1 in children with asymptomatic parasitemia

Because the IgG antibody response to PfEMP1 was similar among children with different forms of severe malaria, aside from children with SMA, we looked at children with asymptomatic parasitemia (AP) at enrollment to see if children with asymptomatic *P. falciparum* infection were mounting a different response from those with no measurable infection. Of the 120 asymptomatic community control children enrolled in the study, 33 had asymptomatic *P. falciparum* infection based on nested PCR results. These children had prevalence rates of antibodies to PfEMP1 domains that were similar to those of children with severe malaria and significantly higher than those with no parasitemia (NP), for 14 of 18 PfEMP1 antigens, as well as CSP, GLURP and SERA5 (Figure 6.1).

Figure 6.1. Prevalence of antibodies to PfEMP1 antigens in children with severe malaria, asymptomatic parasitemia, and no parasitemia.



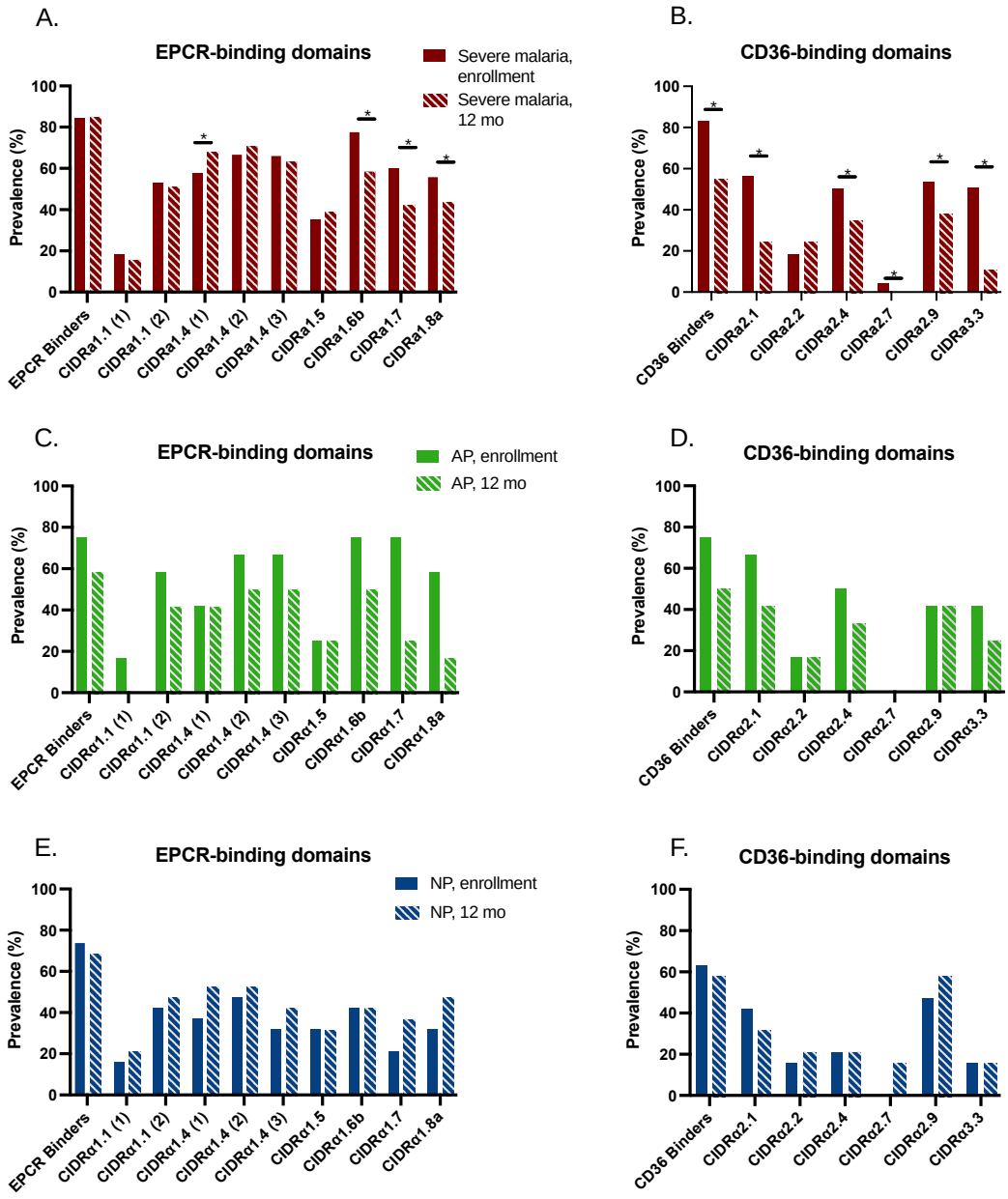
The prevalence of antibodies to EPCR-binding domains of PfEMP1 (A), CD36-binding domains (B), CIDRδ1 domains (C), and additional *P. falciparum* antigens (D) in children with any form of severe malaria (SM, red), asymptomatic parasitemia (AP, green) at enrollment, or no parasitemia (NP, blue) at enrollment. *P<0.05 and below FDR threshold to correct for multiple comparisons.

6.3.4 Prevalence of antibodies to PfEMP1 domains at 12 months post-infection

Next, we wanted to determine if IgG antibodies to PfEMP1 persist up to 12 months after discharge (for children with severe malaria) or study enrollment (for asymptomatic community children). Samples were collected in 178 of the 716 individuals at the 12-month visit, so children with any form of severe malaria were grouped together (n=147) and compared to children with AP (n=12) or NP (n=19) at enrollment. At enrollment, 84.4% of children with SM had antibodies to at least one of the nine EPCR-binding antigens tested, and at 12 months post-infection the prevalence of antibodies to EPCR-binding antigens was maintained at 85.0% (Figure 6.2A). At enrollment, 83.0% of children with severe malaria had antibodies to one or more of the six CD36-binding antigens but the prevalence of antibodies to CD36 antigens decreased significantly at the 12-month visit (55.1%, $p<0.001$ compared to enrollment) (Figure 6.2B).

Children with AP had decreases in antibody prevalence to 11 of 15 PfEMP1 domains at 12 months compared to enrollment, but none of the decreases was statistically significant, likely because of the small sample size. In contrast, children with NP had similar or increased prevalence of antibodies to PfEMP1 antigens at 12 months compared to enrollment, and a small decrease in prevalence of antibodies to only one of six CD36 antigens at the 12-month compared to enrollment visit. In both cases, sample sizes were too small for detection of any but very large differences in prevalence. Overall, we showed that children with severe malaria had persistent antibodies to PfEMP1 EPCR-binding antigens but decreased prevalence of antibodies to CD36-binding antigens over time.

Figure 6.2. Prevalence of antibodies to PfEMP1 antigens in children with severe malaria, AP and NP at enrollment compared to 12-month follow-up.



The prevalence of antibodies to EPCR-binding (A, C, E) and CD36-binding (B, D, F) domains of PfEMP1 in children with severe malaria (n=147; A, B), asymptomatic parasitemia at enrollment (n=12, C, D) and no parasitemia at enrollment (n=19, E, F) at enrollment and 12-month follow-up (n=31). *P<0.05 and below FDR threshold to correct for multiple comparisons.

6.3.5 Protection from additional malaria episodes in children with severe malaria

Participants were followed for 12 months post-enrollment. Any return to the hospital, resulting either in admission for severe malaria or clinic visit for uncomplicated malaria, was documented. Of the 551 children that recovered from severe malaria, 49% (270/551) returned with severe malaria within 12 months. There were no differences among the five groups of severe malaria with respect to the rate of readmission with severe malaria. However, the rate of admission for severe malaria in community children was significantly lower, at 25% (30/120, $p<0.005$). We wanted to see if the presence or levels of antibodies to PfEMP1 was related to protection from severe malaria in children who recovered from a previous episode of severe malaria. Additionally, because the prevalence of antibodies to PfEMP1 was significantly higher in children with severe malarial anemia compared to other forms of severe malaria, we looked at risk of readmission in children who recovered from any form of severe malaria and those that recovered from severe malarial anemia. Among children with any form of severe malaria, the presence or levels antibodies to PfEMP1 antigens or CSP, GLURP or SERA5 were not associated with protection from recurrent severe malaria (Tables 6.7 and 6.8). Among children with severe malarial anemia, who had the highest presence of antibodies to PfEMP1 antigens, antibodies to CIDRα2.1 were associated with protection from recurrent severe malaria (IRR [95% CI], p value, 0.49 [0.29, 0.81], 0.006), and trends toward protection were seen with antibodies to CIDRα1.8a and CIDRδ1 (Table 6.7). The levels of CIDRα2.1 in children with SMA were associated with protection, although not after correcting for multiple comparisons (0.49 [0.26, 0.95], $p=0.03$; Table 6.8). The frequency of uncomplicated malaria over 12 months of follow-up did not differ in children with severe malaria (30.8%, 170/551) compared with community children (30.8%, 37/120). No significant associations with protection from uncomplicated malaria

were found in children with any form of severe malaria or children with severe malarial anemia (Tables 6.9 and 6.10).

Table 6.7. Risk of readmission with severe malaria among children based on the presence of antibodies to PfEMP1 and other *P. falciparum* antigens.

A. Children with any form of severe malaria

	Antibody + N obs, N events	Antibody – N obs, N events	Risk of Severe Malaria IRR (95% CI)	P-value
EPCR Binders	459, 426	98, 85	1.07 (0.74, 1.53)	0.73
CIDRα1.1 (1)	101, 96	456, 415	1.05 (0.74, 1.48)	0.80
CIDRα1.1 (2)	291, 241	266, 270	0.81 (0.62, 1.06)	0.12
CIDRα1.4 (1)	304, 267	253, 244	0.89 (0.68, 1.18)	0.43
CIDRα1.4 (2)	363, 330	194, 181	0.95 (0.71, 1.26)	0.71
CIDRα1.4 (3)	369, 342	188, 169	1.03 (0.77, 1.38)	0.83
CIDRα1.5	199, 152	358, 359	0.75 (0.56, 1.00)	0.05
CIDRα1.6b	414, 387	143, 124	1.07 (0.78, 1.48)	0.66
CIDRα1.7	317, 275	240, 236	0.89 (0.68, 1.17)	0.40
CIDRα1.8a	299, 266	258, 245	0.92 (0.70, 1.20)	0.52
CD36 Binders	449, 415	108, 96	1.05 (0.74, 1.49)	0.78
CIDRα2.1	319, 267	238, 244	0.84 (0.64, 1.10)	0.20
CIDRα2.2	104, 109	453, 402	1.16 (0.83, 1.62)	0.40
CIDRα2.4	259, 229	298, 282	0.94 (0.72, 1.23)	0.65
CIDRα2.7	29, 31	528, 480	1.18 (0.65, 2.12)	0.58
CIDRα2.9	318, 284	239, 227	0.93 (0.71, 1.22)	0.60
CIDRα3.3	253, 214	304, 297	0.90 (0.68, 1.18)	0.44
CIDRδ1 Group	306, 280	251, 231	0.99 (0.75, 1.30)	0.94
CIDRδ1 (1)	160, 129	397, 382	0.83 (0.61, 1.12)	0.22
CIDRδ1 (2)	142, 106	415, 405	0.75 (0.54, 1.03)	0.08
CIDRδ1 (3)	260, 242	297, 269	1.03 (0.79, 1.36)	0.81
CSP	110, 89	447, 422	0.87 (0.61, 1.24)	0.44
GLURP	386, 333	171, 178	0.81 (0.61, 1.08)	0.15
SERA 5	372, 349	185, 162	1.09 (0.82, 1.46)	0.55

B. Children with severe malarial anemia

	Antibody + N obs, N events	Antibody – N obs, N events	Risk of Severe Malaria IRR (95% CI)	P-value
EPCR Binders	133, 123	10, 7	1.23 (0.42, 3.58)	0.71
CIDRα1.1 (1)	33, 31	110, 99	0.95 (0.52, 1.73)	0.86
CIDRα1.1 (2)	95, 76	48, 54	0.62 (0.36, 1.04)	0.07

CIDRα1.4 (1)	92, 87	51, 43	0.98 (0.57, 1.69)	0.95
CIDRα1.4 (2)	108, 93	35, 37	0.70 (0.39, 1.25)	0.23
CIDRα1.4 (3)	116, 105	27, 25	0.76 (0.39, 1.50)	0.43
CIDRα1.5	66, 50	77, 80	0.61 (0.36, 1.03)	0.06
CIDRα1.6b	122, 106	21, 24	0.65 (0.32, 1.33)	0.24
CIDRα1.7	104, 85	39, 45	0.61 (0.35, 1.06)	0.08
CIDRα1.8a	96, 75	47, 55	0.58 (0.35, 0.99)	0.05
CD36 Binders	123, 108	20, 22	0.71 (0.35, 1.45)	0.35
CIDRα2.1	98, 70	45, 60	0.49 (0.29, 0.81)	**0.006**
CIDRα2.2	31, 35	112, 95	1.20 (0.64, 2.23)	0.57
CIDRα2.4	84, 72	59, 58	0.76 (0.45, 1.29)	0.31
CIDRα2.7	8, 3	135, 127	0.32 (0.08, 1.29)	0.11
CIDRα2.9	95, 79	48, 51	0.69 (0.40, 1.20)	0.19
CIDRα3.3	79, 62	64, 68	0.68 (0.40, 1.16)	0.15
CIDRδ1 Group	98, 93	45, 37	0.93 (0.52, 1.68)	0.82
CIDRδ1 (1)	52, 44	91, 86	0.82 (0.48, 1.40)	0.47
CIDRδ1 (2)	53, 34	90, 96	0.51 (0.29, 0.88)	0.02
CIDRδ1 (3)	84, 83	59, 47	1.08 (0.62, 1.86)	0.79
CSP	40, 24	103, 106	0.53 (0.28, 1.00)	0.05
GLURP	107, 95	36, 35	0.81 (0.45, 1.47)	0.50
SERA 5	121, 115	22, 15	1.18 (0.55, 2.51)	0.67

Adjusted for age, sex, study site, and disease group for children any form of severe malaria (A). (N obs) Number of observations included in the model; (N events) number of malaria episodes.

Table 6.8. Risk of readmission with severe malaria among children based on the levels of antibodies to PfEMP1 and other *P. falciparum* antigens.

A. Children with any form of severe malaria

	N obs, N events	Risk of SM IRR (95% CI)	P-value
CIDRα1.1 (1)	557, 511	1.14 (0.47, 2.75)	0.77
CIDRα1.1 (2)	557, 511	0.90 (0.61, 1.33)	0.59
CIDRα1.4 (1)	557, 511	0.79 (0.61, 1.02)	0.07
CIDRα1.4 (2)	557, 511	0.94 (0.73, 1.20)	0.60
CIDRα1.4 (3)	557, 511	0.87 (0.69, 1.10)	0.25
CIDRα1.5	557, 511	0.81 (0.56, 1.17)	0.27
CIDRα1.6b	557, 511	0.94 (0.69, 1.27)	0.68
CIDRα1.7	557, 511	0.89 (0.63, 1.26)	0.50
CIDRα1.8a	557, 511	0.91 (0.65, 1.28)	0.61
CIDRα2.1	557, 511	0.79 (0.52, 1.18)	0.25
CIDRα2.2	557, 511	1.02 (0.49, 2.14)	0.96
CIDRα2.4	557, 511	1.27 (0.81, 2.00)	0.30
CIDRα2.7	557, 511	2.77 (0.15, 50.01)	0.49
CIDRα2.9	557, 511	0.95 (0.66, 1.36)	0.77
CIDRα3.3	557, 511	1.13 (0.74, 1.72)	0.57
CIDRδ1 (1)	557, 511	0.73 (0.51, 1.05)	0.09
CIDRδ1 (2)	557, 511	0.54 (0.26, 1.13)	0.10
CIDRδ1 (3)	557, 511	0.86 (0.60, 1.22)	0.39
CSP	557, 511	0.86 (0.44, 1.71)	0.68
GLURP	557, 511	0.90 (0.72, 1.12)	0.33
SERA 5	557, 511	1.03 (0.81, 1.30)	0.81

B. Children with severe malarial anemia

	N obs, N events	Risk of SM IRR (95% CI)	P-value
CIDRα1.1 (1)	143, 130	0.74 (0.18, 3.01)	0.68
CIDRα1.1 (2)	143, 130	0.92 (0.49, 1.74)	0.81
CIDRα1.4 (1)	143, 130	1.01 (0.68, 1.50)	0.96
CIDRα1.4 (2)	143, 130	0.97 (0.63, 1.48)	0.88
CIDRα1.4 (3)	143, 130	0.96 (0.63, 1.45)	0.84
CIDRα1.5	143, 130	0.95 (0.54, 1.67)	0.87
CIDRα1.6b	143, 130	0.67 (0.39, 1.14)	0.14
CIDRα1.7	143, 130	0.65 (0.36, 1.19)	0.16

ClDRα1.8a	143, 130	0.74 (0.41, 1.33)	0.32
ClDRα2.1	143, 130	0.49 (0.26, 0.95)	0.03
ClDRα2.2	143, 130	1.10 (0.35, 3.51)	0.87
ClDRα2.4	143, 130	1.07 (0.46, 2.47)	0.87
ClDRα2.7	143, 130	0.01 (0.00, 8.42)	0.18
ClDRα2.9	143, 130	0.98 (0.55, 1.75)	0.95
ClDRα3.3	143, 130	0.85 (0.43, 1.67)	0.63
ClDRδ1 (1)	143, 130	0.98 (0.56, 1.69)	0.93
ClDRδ1 (2)	143, 130	0.41 (0.13, 1.25)	0.12
ClDRδ1 (3)	143, 130	0.77 (0.42, 1.40)	0.39
CSP	143, 130	0.25 (0.07, 0.98)	0.05
GLURP	143, 130	0.65 (0.42, 1.02)	0.06
SERA 5	143, 130	1.23 (0.80, 1.90)	0.35

Adjusted for age, sex, study site, and disease group for children any form of severe malaria (A). (N obs) Number of observations included in the model; (N events) number of malaria episodes.

Table 6.9. Risk of return sick visit with uncomplicated malaria among children based on the presence of antibodies to PfEMP1 and other *P. falciparum* antigens.

A. Children with any form of severe malaria

	Antibody + N obs, N events	Antibody – N obs, N events	Risk of Uncomplicated Malaria IRR (95% CI)	P-value
EPCR Binders	459, 218	98, 40	1.27 (0.82, 1.98)	0.29
CIDRα1.1 (1)	101, 62	456, 196	1.47 (0.99, 2.17)	0.05
CIDRα1.1 (2)	291, 133	266, 125	1.06 (0.76, 1.47)	0.72
CIDRα1.4 (1)	304, 131	253, 127	0.93 (0.67, 1.30)	0.68
CIDRα1.4 (2)	363, 160	194, 98	0.97 (0.68, 1.37)	0.86
CIDRα1.4 (3)	369, 169	188, 89	1.04 (0.73, 1.47)	0.83
CIDRα1.5	199, 99	358, 159	1.17 (0.83, 1.64)	0.37
CIDRα1.6b	414, 195	143, 63	1.19 (0.81, 1.73)	0.38
CIDRα1.7	317, 143	240, 115	0.98 (0.71, 1.37)	0.91
CIDRα1.8a	299, 134	258, 124	1.02 (0.73, 1.42)	0.91
CD36 Binders	449, 210	108, 48	1.10 (0.73, 1.67)	0.65
CIDRα2.1	319, 139	238, 119	0.88 (0.63, 1.21)	0.43
CIDRα2.2	104, 57	453, 201	1.31 (0.88, 1.96)	0.18
CIDRα2.4	259, 125	298, 133	1.11 (0.80, 1.54)	0.55
CIDRα2.7	29, 16	528, 242	1.19 (0.59, 2.37)	0.63
CIDRα2.9	318, 147	239, 111	1.02 (0.73, 1.41)	0.92
CIDRα3.3	253, 138	304, 120	1.31 (0.95, 1.81)	0.10
CIDRδ1 Group	306, 165	251, 93	1.53 (1.10, 2.13)	0.01
CIDRδ1 (1)	160, 81	397, 117	1.22 (0.86, 1.74)	0.27
CIDRδ1 (2)	142, 75	415, 183	1.28 (0.89, 1.83)	0.19
CIDRδ1 (3)	260, 150	297, 108	1.64 (1.19, 2.27)	0.003
CSP	110, 71	447, 187	1.59 (1.09, 2.33)	0.02
GLURP	386, 194	171, 64	1.36 (0.95, 1.95)	0.09
SERA 5	372, 171	185, 87	0.98 (0.69, 1.39)	0.92

B. Children with severe malarial anemia

	Antibody + N obs, N events	Antibody – N obs, N events	Risk of Uncomplicated Malaria IRR (95% CI)	P-value
EPCR Binders	133, 59	10, 3	1.55 (0.40, 5.95)	0.53
CIDRα1.1 (1)	33, 19	110, 43	1.39 (0.70, 2.75)	0.34
CIDRα1.1 (2)	95, 42	48, 20	1.06 (0.54, 2.06)	0.87
CIDRα1.4 (1)	92, 40	51, 22	0.95 (0.50, 1.82)	0.88
CIDRα1.4 (2)	108, 49	35, 13	1.24 (0.58, 2.64)	0.57
CIDRα1.4 (3)	116, 50	27, 12	0.86 (0.38, 1.91)	0.70
CIDRα1.5	66, 31	77, 31	1.04 (0.55, 1.96)	0.91
CIDRα1.6b	122, 54	21, 8	1.31 (0.53, 3.22)	0.56
CIDRα1.7	104, 46	39, 16	1.02 (0.50, 2.08)	0.95

CIDRα1.8a	96, 45	47, 17	1.27 (0.64, 2.51)	0.49
CD36 Binders	123, 55	20, 7	1.40 (0.55, 3.55)	0.48
CIDRα2.1	98, 39	45, 23	0.75 (0.40, 1.40)	0.36
CIDRα2.2	31, 15	112, 47	1.23 (0.60, 2.55)	0.57
CIDRα2.4	84, 44	59, 18	1.56 (0.81, 2.98)	0.18
CIDRα2.7	8, 2	135, 60	0.50 (0.10, 2.46)	0.39
CIDRα2.9	95, 44	48, 18	1.34 (0.68, 2.63)	0.40
CIDRα3.3	79, 38	64, 24	1.12 (0.60, 2.09)	0.73
CIDRδ1 Group	98, 47	45, 15	1.42 (0.69, 2.90)	0.34
CIDRδ1 (1)	52, 24	91, 38	1.09 (0.58, 2.05)	0.78
CIDRδ1 (2)	53, 22	90, 40	0.87 (0.46, 1.65)	0.67
CIDRδ1 (3)	84, 42	59, 20	1.51 (0.78, 2.92)	0.22
CSP	40, 18	103, 44	1.05 (0.49, 2.24)	0.90
GLURP	107, 49	36, 13	1.23 (0.59, 2.57)	0.59
SERA 5	121, 52	22, 10	0.88 (0.38, 2.05)	0.77

Adjusted for age, sex, study site, and disease group for children any form of severe malaria (A). (N obs) Number of observations included in the model, (N events) number of malaria episodes.

Table 6.10. Risk of sick visit with uncomplicated malaria among children based on the levels of antibodies to PfEMP1 and other *P. falciparum* antigens.

A. Children with any form of severe malaria

	N obs, N events	Risk of UM IRR (95% CI)	P-value
CIDRα1.1 (1)	557, 258	3.01 (1.24, 7.31)	0.02
CIDRα1.1 (2)	557, 258	1.31 (0.85, 2.04)	0.22
CIDRα1.4 (1)	557, 258	1.05 (0.78, 1.42)	0.74
CIDRα1.4 (2)	557, 258	1.18 (0.88, 1.60)	0.27
CIDRα1.4 (3)	557, 258	1.13 (0.85, 1.50)	0.40
CIDRα1.5	557, 258	1.46 (0.97, 2.18)	0.07
CIDRα1.6b	557, 258	1.23 (0.86, 1.77)	0.26
CIDRα1.7	557, 258	1.06 (0.70, 1.62)	0.78
CIDRα1.8a	557, 258	1.07 (0.72, 1.60)	0.73
CIDRα2.1	557, 258	1.05 (0.65, 1.68)	0.85
CIDRα2.2	557, 258	1.53 (0.66, 3.54)	0.32
CIDRα2.4	557, 258	1.30 (0.74, 2.29)	0.36
CIDRα2.7	557, 258	2.65 (0.09, 78.54)	0.57
CIDRα2.9	557, 258	1.26 (0.85, 1.85)	0.25
CIDRα3.3	557, 258	1.74 (1.10, 2.76)	0.02
CIDRδ1 (1)	557, 258	1.04 (0.68, 1.59)	0.86
CIDRδ1 (2)	557, 258	1.27 (0.55, 2.96)	0.57
CIDRδ1 (3)	557, 258	1.34 (0.88, 2.04)	0.18
CSP	557, 258	1.33 (0.60, 2.94)	0.48
GLURP	557, 258	1.06 (0.81, 1.38)	0.68
SERA 5	557, 258	1.21 (0.92, 1.59)	0.17

B. Children with severe malarial anemia

	N obs, N events	Risk of UM IRR (95% CI)	P-value
CIDRα1.1 (1)	143, 62	2.22 (0.53, 9.27)	0.27
CIDRα1.1 (2)	143, 62	1.23 (0.59, 2.57)	0.58
CIDRα1.4 (1)	143, 62	1.14 (0.70, 1.86)	0.59
CIDRα1.4 (2)	143, 62	1.20 (0.70, 2.03)	0.51
CIDRα1.4 (3)	143, 62	1.23 (0.75, 2.02)	0.42
CIDRα1.5	143, 62	1.86 (1.01, 3.43)	0.05
CIDRα1.6b	143, 62	1.30 (0.69, 2.43)	0.42

ClDRα1.7	143, 62	1.32 (0.66, 2.64)	0.43
ClDRα1.8a	143, 62	1.12 (0.57, 2.20)	0.75
ClDRα2.1	143, 62	0.86 (0.40, 1.85)	0.70
ClDRα2.2	143, 62	1.52 (0.42, 5.47)	0.52
ClDRα2.4	143, 62	1.58 (0.62, 4.05)	0.34
ClDRα2.7	143, 62	0.08 (0.00, 151.46)	0.51
ClDRα2.9	143, 62	1.67 (0.93, 2.99)	0.08
ClDRα3.3	143, 62	1.37 (0.67, 2.84)	0.39
ClDRδ1 (1)	143, 62	0.88 (0.43, 1.81)	0.73
ClDRδ1 (2)	143, 62	0.83 (0.21, 3.25)	0.79
ClDRδ1 (3)	143, 62	0.73 (0.33, 1.59)	0.43
CSP	143, 62	0.80 (0.17, 3.64)	0.77
GLURP	143, 62	1.16 (0.71, 1.90)	0.55
SERA 5	143, 62	1.42 (0.87, 2.32)	0.16

Adjusted for age, sex, study site, and disease group for children any form of severe malaria (A). (N obs) Number of observations included in the model; (N events) number of malaria episodes.

6.4 Discussion

In this study, we examined the host IgG response to PfEMP1, a key antigen during *P. falciparum* infection, and how it relates to different forms of severe malaria. Using serum samples from a cohort of Ugandan children, we measured antibodies to 18 CIDR domains of PfEMP1, including EPCR-binding domains, which are more prevalent in severe malaria and CD36-binding domains, that are associated with uncomplicated malaria. Of the five manifestations of severe malaria we tested, children with severe malarial anemia had the greatest prevalence of antibodies to 16 of 18 measured PfEMP1 domains compared to children with any other form of severe malaria. We also demonstrated the persistence of antibodies to EPCR-binding domains, but not CD36-binding domains, in children with severe malaria up to 12 months post-infection. In children with severe malarial anemia, but not other forms of severe malaria, antibodies to one CD36-binding domain, CIDRα2.2, were associated with protection from recurrent severe malaria, and trends toward protection were seen for antibodies to EPCR-binding domains CIDRα1.8a. In contrast, antibodies to PfEMP1 domains did not correlate with protection against uncomplicated malaria in children with prior severe malarial anemia or other forms of severe malaria.

Although previous studies have highlighted the differences in PfEMP1 immunity between uncomplicated malaria and severe malaria[153,154], no other studies to date have identified differences in antibody responses to PfEMP1 between different forms of severe malaria. In our study, children with five different types of malaria were enrolled to ensure we had the most inclusive definition of severe malaria and to understand differences in immunity and pathogenesis between the various forms of severe malaria. Antibodies to all but two CD36-binding domains were more prevalent in SMA compared with all other types of severe malaria in this study, suggesting that children with SMA are

mounting a greater response to these domains than the other forms of SM. This is surprising, because children with cerebral malaria have greater transcript levels of EPCR-binding domains compared to children with SMA[113], therefore they would be expected to have more exposure to the antigens that children with SMA. The elevated antibody response in children with SMA may not be related to pathogenesis of the disease. However, African children with severe anemia, the most common cause being malaria, have been found to have higher rates of readmission and death than children without anemia[157-159]. It is possible that children with SMA have more episodes of malaria during their lifetime than children with other manifestations of severe malaria, resulting in elevated antibody responses, especially to domains associated with severe disease, such as the EPCR-binding domains of PfEMP1. Alternatively, this elevation in antibodies to PfEMP1 in children with SMA could be related to antibody kinetics and the onset of disease in children with SMA, which is more gradual than the acute onset of CM. Thus, antibodies in children with SMA could have more time to reach peak levels compared to in other forms of severe malaria, where children were admitted to the hospital closer to initial infection. This could be tested looked at repeated samples over time, although collecting numerous samples during hospitalization can be difficult on patients and study personal.

The results of this study are different than many other studies that have looked at antibodies to *P. falciparum* antigens in children with severe malaria, where the primary comparisons are between children with severe malaria or CM and mild disease. Previously, we showed similar antibody responses between children with CM and SMA and they were pooled together to compare with asymptomatic children (Chapter 5). One study showed that children with SMA had significantly lower IgG antibody levels to the blood-stage antigens AMA-1, MSP-1, and MSP-2 compared to children with uncomplicated malaria and CM[160]. This is different from the findings here for antibodies

to PfEMP1 antigens and other blood-stage antigens, when comparing children with SMA to children with CM or AP. In that study, children with SMA were younger than children with CM, suggesting that age or exposure may also be playing a role in antibody development. Although the average age of children in this study was similar to that of children with SMA in the previous study[160], we saw no significant differences in age in this study between children with different forms of severe malaria. However, the variants used in the previous study have not been tested in this population of children with severe malaria suggesting that the elevated antibody response to PfEMP1 in children with SMA observed here could be variant-specific, highlighting a possible difference in severe malaria immunity.

In this study, children with AP had prevalence rates of antibodies to PfEMP1 similar to seropositivity rates in children with severe malaria. This was true for both EPCR-binding and CD36-binding domains. Although expression of EPCR-binding domains is relatively low in children with AP[113], there could be enough PfEMP1 expression on infected erythrocytes to generate antibodies. It could be that having a parasite infection, despite severity of disease, means that you develop antibodies to PfEMP1, but these antibodies may be more effective in children with AP. Although we identified no associations with protection in this cohort. Antibodies to PfEMP1, do develop with exposure naturally, not only with disease[98], so perhaps these children with AP have naturally encountered these antigens and developed a clinical immunity not yet present in children that developed severe malaria.

We identified an association between the presence and levels of antibodies to CIDRα2.2, a CD36-binding domain, in children with SMA and risk of additional severe malaria episode. We did not identify other associations with risk of severe or uncomplicated malaria in children with other forms of severe malaria or other PfEMP1 domains, including EPCR-binding domains, or blood stage antigens. Although we would

anticipate associations between risk of severe malaria and EPCR-binding domains, which are associated with severe malaria pathogenesis[114,116], the relationship between risk of severe malaria and one CD36-binding domain suggests that PfEMP1 domains could be related to severe malaria immunity beyond EPCR-binding domains. Previous studies have identified a relationship to protection from clinical malaria and the presence of antibodies to CIDRα1 domain[101] and CIDRɣ3 [99]. Additionally, using a parasite strain lacking PfEMP1 expression, levels of antibodies to infected erythrocytes surface antigens, particularly PfEMP1, have been related to reduced risk of clinical malaria in children[161]. The lack of similar associations in this study could be due to the young age of the study participants and lack of exposure and time to develop effective immunity, although antibodies to PfEMP1 domains, in particular EPCR-binding domains develop in young children living in malaria endemic areas[98]. In this study, we see higher prevalence of antibodies in children with severe malaria, but it could be that the antibodies are not functional or the risk class of IgG to contribute effectively to protection.

The study design could also contribute to the lack of association with protection from malaria; it is possible that measuring antibodies at the time of a severe malaria episode is not related to antibody response required for protection. Most studies that identify relationships between PfEMP1 domains and protection are longitudinal studies, that follow an asymptomatic population over time[99,101], unlike the current study that enrolled children at the time of their malaria episode. Although we did not identify specific indications of protection in this population, it is possible that malaria protection requires a breadth of antibodies to PfEMP1, not a singular domain. We propose to conduct additional analysis to investigate how the breadth of antibody response to these antigens related to the risk of severe or uncomplicated malaria. The antigenic variation and diversity of PfEMP1 is one of the difficulties of studying the protein. In this study, we attempted to have an inclusive study of PfEMP1 antibodies by measuring 18 CIDR

domains that are involved in binding to the host endothelium or rosetting, yet there are many other domains that could be related to protection or immunity to severe malaria.

In this study, we showed that children with SMA have an elevated antibody response to PfEMP1 domains compared to children with other manifestations of severe malaria, including CM and in children with SMA, antibodies to CIDRα2.2 are associated with protection from severe malaria. Future studies to understand why this group of children is mounting a different response could include measuring additional *P. falciparum* antigens to see if this response is maintained with other antigens or if functional antibodies differ in this population that could be impacting relationships with protection.

Chapter 7: Summary & Discussion

PfEMP1 is the primary source of antigenic variation during *P. falciparum* infection, contributing to pathogenesis through cytoadherence of the parasite to the host endothelium and aiding in immune evasion of the parasite. Group A *var* genes that encode PfEMP1 molecules, particularly PfEMP1 proteins with EPCR-binding domains, are associated with severe malaria[86,126]. Antibodies to these domains are naturally acquired with exposure to the parasite, and develop prior to other PfEMP1 domains[98]. However, EPCR-binding domains represent only a subset of PfEMP1 domains and does not address how other PfEMP1 domains could be playing a role in pathogenesis or immunity of severe malaria. In this dissertation, blood samples from Ugandan children with severe malaria enrolled in two separate prospective observation cohorts were used to elucidate the role of PfEMP1 EPCR-binding domains and additional PfEMP1 domains in severe malaria pathogenesis and immunity. The large sample size and detailed study design, including thorough follow-up of both cohorts, allowed for the investigation of differences in PfEMP1 expression and antibody response. Information about PfEMP1 domain expression and antibody responses could be used to differentiate forms of severe malaria, and understand associations with clinical outcomes and protection.

To understand the role of PfEMP1 domains in children with severe malaria, we measured the transcript levels of various domains of PfEMP1 in children with CM or SMA. Previously, in children with CM or SMA in study 1 (CM-SMA study), transcript levels of group A *var* genes and EPCR-binding domains were elevated in children with CM compared to SMA, particularly CIDRα1.1[113]. Additionally, elevated expression of these group A *var* genes and EPCR-binding domains were elevated in children with CM with malarial retinopathy compared to no retinopathy and in children with severe malaria that survived compared to those who died[113]. However, this earlier study did not include

ICAM-1-binding domains of PfEMP1, the expression of which have been related to severe malaria, particularly CM[93]. We hypothesized that ICAM-1-binding domains of PfEMP1 would be elevated in children with CM compared to children with SMA and that these domains would be associated with markers of endothelial activation, as a sign of PfEMP1–ICAM-1-binding. We observed elevated expression of one ICAM-1-binding domain, DBLβS3.1, in children with CM compared to SMA. Of the additional DBLε/DBLζ domains, we studied that are located towards the C terminal end of PfEMP1, DBLε14, DBLζ3, DBLζ4, and DBLζ6 were elevated in children with CM compared to SMA. When looking at markers of endothelial activation, only sVCAM-1 was associated with increased transcript levels of DBLβ motif 1 in children with severe malaria. However, elevated transcript levels of DBLβ motif 1, DBLβS3.1 and DBLζ3 were associated with an increased risk of thrombocytopenia in children with severe malaria. These results demonstrate differences in PfEMP1 domain expression, beyond EPCR-binding domains, in children with CM compared to children with SMA and highlight the association between PfEMP1 domains and thrombocytopenia, consistent with previous studies with EPCR-binding domains[117,118].

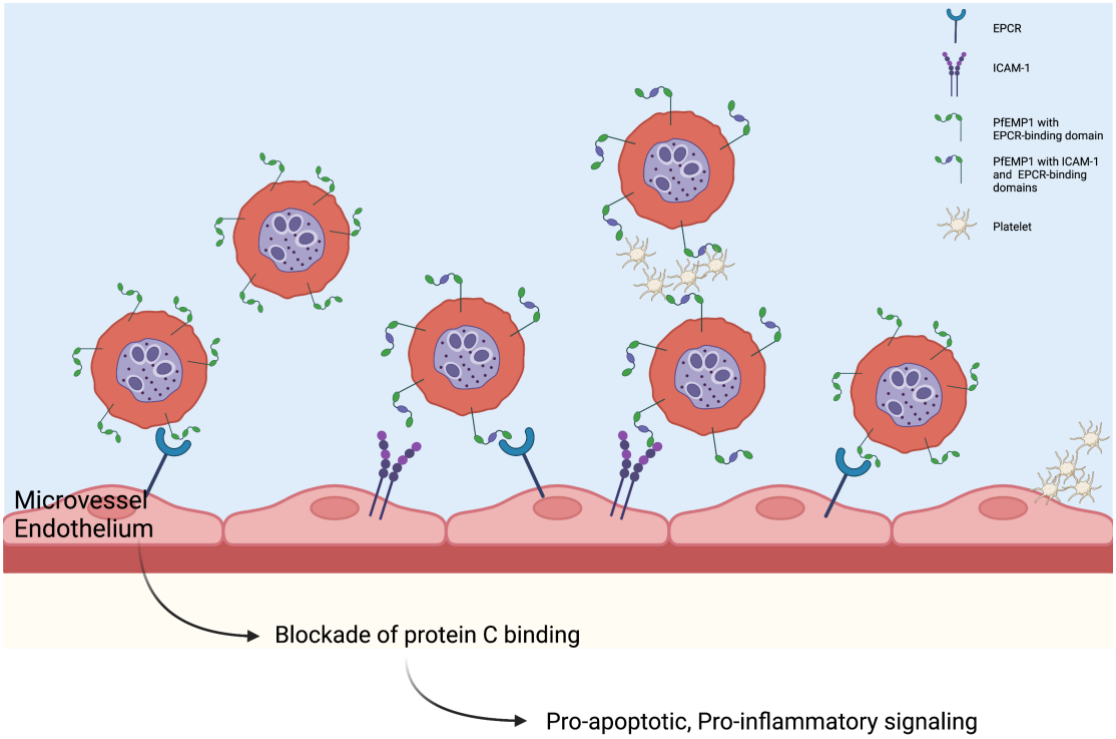
CM and SMA represent only two manifestations of severe malaria, limiting our understanding of the role of PfEMP1 in other forms of disease. Study population 2 (5 SM cohort) enrolled children with five forms of severe malaria: CM, RDS, malaria with repeated seizures, SMA and prostration. Using samples from this cohort, we measured the expression of the group A *var* genes and EPCR-binding domains tested previously[113], along with the ICAM-1-binding, and additional C terminal domains measured in, Chapter 3. Although we hypothesized differences in transcript levels of these domains, particularly EPCR-binding domains, in children with different forms of severe malaria, we saw similar expression of these domains across the five forms of severe malaria. Only CIDRα1.1 was significantly decreased in children with SMA

compared to M/S and Pro. Detecting differences in PfEMP1 transcript levels across different groups of severe malaria in this population is difficult, as the sample size was limited, differences between transcript levels can be small, and comparing multiple groups required more stringent statistics. However, these results do demonstrate that PfMEP1 is playing a role in pathogenesis in these additional, less understood manifestations of severe malaria. The results of this study also highlight associations between these domains and increased risk of thrombocytopenia and decreased risk of severe anemia, particularly EPCR-binding domains, DBLβ5 S1, and DBLζ4, highlighting possible roles of PfEMP1 in impacting clinical outcomes. Although we did not see associations between EPCR and ICAM-1-binding domains and markers of endothelial activation, increased transcript levels of group A EPCR-binding domains, CIDRα1.6b and CIDRα1.7, were associated with increased levels of serum TNF-α and nearly all measured EPCR-binding domains, but not ICAM-1-binding domains, were associated with increased serum IL-10 levels. Overall, these data suggest that PfEMP1 is acting similarly across different manifestations of severe malaria and is binding host receptors at an increased rate in severe malaria due to endothelial activation throughout the host, as opposed to tissue-specific responses. The results of this study differ from the previous study measuring EPCR-binding transcripts in children with CM and SMA in cohort 1. The lack of significant differences between severe malaria groups could be due to decreased number of children in each group and thus a decreased power to detect small differences in *var* transcripts. It is possible that levels of *var* transcripts are related to disease severity, not specific symptoms of severe malaria and thus elevated in more severe manifestations of severe malaria, such as CM, compared to other manifestations of severe malaria. These differences may not have been detected here because of symptom overlap within groups or how the groups were defined.

PfEMP1 binding to EPCR via CIDRα1 domains is thought to perpetuate severe malaria pathogenesis by blocking protein C binding to its receptor EPCR. The binding of PfEMP1 instead of protein C decreases the anti-inflammatory and anti-apoptotic signaling, perpetuating endothelial activation and barrier damage[87-89]. The binding of infected erythrocytes to EPCR can occur in brain endothelial cells[142], contributing to cerebral malaria. However, this binding can also occur in other tissues throughout the body, including in bone marrow and lung cells[127]. The presence of CD36-binding domains of PfEMP1, conversely, are associated with uncomplicated malaria, highlighting a difference in pathogenesis from severe malaria contributed by PfEMP1. Additionally, CD36 has lower expression on brain endothelial cells compared to EPCR. The binding of PfEMP1 DBLβ domains to ICAM-1 could also be an important aspect of disease progression in severe malaria, with the binding of infected erythrocytes contributing to endothelial activation. Unlike EPCR and CD36 binding domains, ICAM-1 binding occurs at a different portion of the PfEMP1 protein and can be found on the same PfEMP1 molecules as CD36 or EPCR-binding domains. In this study we did not find as many associations between ICAM-1 binding domains and the selected covariates. This could be because ICAM-1 binding domains of PfEMP1 are a relatively new discovery and thus less well characterized. It is possible that the primers to these domains are not as well designed as the EPCR-binding domains, meaning some associations could have been missed. Additionally, these ICAM-1 binding domains are not as strongly associated with severe malaria as EPCR-binding domains. It is possible that the ICAM-1 binding domains are not related to severity and thus the markers of severe disease in the same manner as EPCR-binding domains. Overall, the results from this study add to our understanding of PfEMP1 binding to the host endothelium during severe malaria by demonstrating that the transcripts of EPCR-binding domains are similarly elevated

across different forms of severe malaria and could be inhibiting protein C binding throughout the host to worsen the severe malaria.

Figure 7.1. Model of infected erythrocytes binding to EPCR and ICAM-1 during severe malaria



Infected erythrocytes expressing EPCR-binding and ICAM-1 binding domains of PfEMP1 can bind to EPCR and ICAM-1 on the surface of microvasculature endothelium throughout the body. The binding of infected erythrocytes to EPCR blocks protein C binding, resulting in pro-apoptotic and pro-inflammatory signaling to exacerbate endothelial activation and barrier damage. Platelet levels have been associated with var transcripts of EPCR and ICAM-1 binding domains. Platelets could be binding directly to PfEMP1 on infected erythrocytes or contributing to clumping and endothelial activation, generating microvasculature obstructions.
Created with BioRender.com.

Results from these studies of the transcript levels of PfEMP1 domains in children with severe malaria could be used in future *in vitro* studies to characterize infected erythrocyte binding to platelets, hemoglobin and other host factors. These *in vitro* studies could elucidate mechanisms underlying the associations shown here between PfEMP1 transcript levels and thrombocytopenia or severe anemia. For example, specific parasite

lab strains can be used that expressed EPCR-binding or EPCR-ICAM-1 dual binding domains of PfEMP1. These could be cultured with platelets or within a cell model of severe or cerebral malaria, allowing us to better understand if platelets and infected erythrocytes are directly interacting via PfEMP1 and how their interaction could be impacting endothelial cells. The only direct link between PfEMP1 and platelets shows that platelets can bind to endothelial cells and provide receptors for infected erythrocytes, likely through CD36-binding domains of PfEMP1[123]; however, this binding was not further characterized. Since the previous study, other binding partners of PfEMP1 have been identified, suggesting that EPCR-binding domains and additional PfEMP1 domains found to be associated with thrombocytopenia, could be binding platelets and impacting thrombocytopenia.

Further analysis could be done using the data on *var* transcript levels from both cohorts to address additional questions presented in this data. For example, a model could be developed to include multiple covariates assessed in this project that were associated with *var* transcripts to understand how different covariates could be impacting each other or *var* transcript expression whether a significant relationship of one covariate with var expression is independent of potential confounding covariates. Although transcript units were similar across the different forms of severe malaria, it is possible that a more in depth or complex analysis could reveal differences in groups of *var* transcripts or highlight specific manifestations of severe malaria being associated with certain *var* profiles.

Aside from the expression of PfEMP1 domains, investigating the antibody response to PfEMP1 domains during severe malaria is important in understanding severe malaria immunity. Antibodies to PfEMP1 are acquired in an ordered manner[98,99], naturally with exposure to the parasite. Antibodies to PfEMP1 domains have also been associated with clinical protection[100,161]. However, little is known about how these

antibodies compare in children with different forms of severe malaria and how they relate to severe malaria protection. In this dissertation, we aimed to measure the presence and levels of IgG antibodies to PfEMP1 domains in children with CM or SMA. Five PfEMP1 antigens were selected because they are found in DC8 and DC13, tandem sets of domains that include EPCR-binding domains, which have been associated with severe malaria[112,121]. Nine *P. falciparum* antigens from the pre-erythrocytic and erythrocytic stages of the parasite life cycles were also selected. Antibody prevalence to the PfEMP1 antigens CIDRα1.4 and DBLɣ6 was higher in children with AP than SM at enrollment and remained higher in follow-up for DBLɣ6. Antibody prevalence to non-PfEMP1 blood-stage antigens and to CSP was similar or higher in children with SM compared to AP at baseline. Over time, at 6- and 12-month follow-up visits, children with AP maintained antibodies to these antigens, while children who recovered from severe malaria demonstrated a significant decline in antibodies over time, to responses similar to children with NP. However, neither antibody prevalence nor level to any antigen was associated with protection from severe or uncomplicated malaria over 12 months in children with SM, AP or no parasitemia. Although we did not find evidence of these antibodies being associated with protection from additional episodes of malaria, this study demonstrated that children with AP are boosting their immune response to *P. falciparum* to maintain asymptomatic infections. Additionally, we highlighted that PfEMP1 domains may elicit a different antibody response than other *P. falciparum* antigens.

Because PfEMP1 is highly complex and demonstrates significant sequence and domain diversity, studying more than the five antigens studied in chapter 5, provides better information about the antibody response to PfEMP1. Therefore, to further study the PfEMP1 antibody response, we used serum samples from children with five forms of severe malaria enrolled in study 2 (5 SM study) to measure the IgG response to 18 CIDR PfEMP1 antigens and three additional *P. falciparum* antigens, including two used

in the previous cohort. The PfEMP1 antigens selected in this study include nine EPCR-binding domains, six CD36-binding domains, and three CIDRδ1 domains. In this cohort, we demonstrated that children with SMA had higher prevalence of antibodies to the measured PfEMP1 domains than any other form of severe malaria and that at enrollment, children with AP had similar prevalence rates as children with severe malaria. Data also showed that in children who recover from an episode of severe malaria, the prevalence of antibodies to ECPR-binding domains are maintained at 12-month follow-up, while the prevalence of antibodies to CD36-binding domains decreased. In children with SMA, but not other forms of severe malaria, antibodies to one CD36-binding domain, CIDRα2.2, were associated with protection from recurrent severe malaria, and trends toward protection were seen for antibodies to EPCR-binding domain CIDRα1.8a. Results from this study highlight children with SMA mounting an elevated antibody response to PfEMP1. Children with SMA who could be mounting elevated antibody responses is likely related to the fact that children are more likely to have multiple episodes of SMA than other manifestations of malaria and, therefore, be boosting their antibody response to domains associated with severe disease, such as the EPCR-binding domains of PfEMP1. Alternatively, children with SMA may have slower onset of disease compared to children with other manifestations of malaria. Therefore, children with SMA have more time for antibody levels to reach their peak before seeking treatment and thus appear to have higher concentrations of antibodies to PfEMP1 compared to other more acute manifestations of severe malaria. Future studies to elucidate the differences in the antibody response in children with SMA compared to other forms of malaria could provide understanding about the importance of this difference in response. Studies could look into additional *P. falciparum* antigens or whether children with SMA are more likely to develop functional antibodies than other children with SM.

Neither study of antibodies against PfEMP1 or other *P. falciparum* antigens showed significant associations between presence or levels of antibodies and reduced risk of additional malaria episodes during 12-month post-discharge follow-up. Associations with protection were anticipated because antibodies to a number of erythrocytic stage antigens included in this study[48,137,139] and other PfEMP1 domains[99,101,102] have been previously associated with protection. The lack of associations in this dissertation could be due to sample size, with a limited number of children returning to the clinic with malaria. However, in study 2 (5 SM study), a large proportion of the study population returned with severe or uncomplicated malaria. In many studies that identify associations with protection, samples are collected as part of a longitudinal study, collecting samples in asymptomatic individuals over time. Measuring antibodies during an episode of severe malaria may not be indicative of the lasting immunity required for protection, with antibody responses rapidly rising before and during the severe malaria episode, peaking after initial presentation, then decreasing during convalescence. Therefore, measuring antibodies at the time of illness may not be indicative of the steady state antibody response. Additionally, protection may be related to the breadth of antibodies, not a single domain or antigen, particularly for PfEMP1. Additional statistical modelling is required in these populations to understand how antibodies to multiple antigens could relate to clinical malaria protection.

The two cohort studies used in this dissertation were designed to study the differences between the multiple types of severe malaria at the time of presentation as well their differential clinical outcomes. The inclusion of two cohorts conducted over non-overlapping time periods increases the generalizability our findings. Thus, the results of this project may be broadly applicable to children with severe malaria in similar regions of high-malaria transmission in sub-Saharan Africa. Conversely, in low or unstable transmission settings, the epidemiology and pathogenesis of severe malaria differs such

that even adults can succumb to its severe manifestations. In these settings, PfEMP1 may be playing a slightly different role in disease, although there is also evidence of EPCR-binding domain expression in adults with CM[118]. The results from this study on the *var* gene expression and antibodies to PfEMP1 can be used to improve our understanding of SM pathogenesis beyond this cohort to children with severe malaria and be used to design studies involving other cohorts of children with severe malaria. Designing these types of cohort studies can be difficult, and it is important to consider what questions you are asking and how they can be answered by the study design and the samples that are collected. For the purposes of this study, the cohorts and samples collected could not be changed as the studies occurred a number of years ago, but the inclusion of blood samples are readmission visits or sample collection at follow-up for all children in the second study could answer some of the questions related to the protection and maintenance of antibodies to PfEMP1 in children with severe malaria.

Overall, the work presented in this dissertation identifies PfEMP1 as a key player in severe malaria pathogenesis and immunity. Despite some differences in children with CM or SMA, the transcript levels of PfEMP1 domains were relatively similar in children with different forms of severe malaria. This implies that despite distinct differences in the pathogenesis of different manifestations of severe malaria, PfEMP1 is not acting differently in the manifestations tested here. However, despite similarities in PfEMP1 expression, children with SMA could be mounting an elevated antibody response to PfEMP1, demonstrating a difference in PfEMP1 related immunity with respect to different manifestations of severe malaria. PfEMP1 may not be a differentiation factor between manifestations of severe malaria, but it is important in severe malaria pathogenesis and immunity. Therefore, developing a vaccine that blocks PfEMP1 binding to EPCR or boosts antibodies to these domains is possible in preventing any form of severe malaria, although not without its difficulties, including a lack of an animal

model of PfEMP1 and significant antigenic variation of PfEMP1. Severe malaria vaccines targeting PfEMP1 could greatly improve childhood morbidity and mortality in malaria endemic regions. However, the antigenic variation within PfEMP1 would be difficult to overcome and require the development of cross-reactive antibodies that could recognize diversity within CIDRα1 domains.

Results of this dissertation highlight the importance of PfEMP1, but it is crucial to recognize the role of other parasite and host factors that could be impacting pathogenesis and outcomes of severe malaria. For example, there are other variant surface antigens, such as RIFINs and STEVORs that could be contributing to cytoadherence of infected erythrocytes and immune evasion. Additionally, host immune factors play a critical role in severe malaria pathogenesis, including cytokines and chemokines. Interleukin-33 (IL-33) is one such cytokines whose role in severe malaria and murine experimental cerebral malaria (ECM) models has been studied. In ECM models, IL-33 and its receptor ST2 have been demonstrated to act is opposite manners, both alleviating and exacerbating CM pathogenesis. Appendix 1 demonstrates the role of ST2 and IL-33 in severe malaria and the relationship between ST2 and cognitive deficits in children with CM.

Appendix 1: Elevated plasma ST2 levels are associated with neuronal injury and neurocognitive impairment in children with cerebral malaria

A1.1 Introduction

Cerebral malaria (CM) and severe malarial anemia (SMA) are among the most devastating manifestations of *Plasmodium falciparum* infection. Globally, approximately two thirds of malaria deaths are estimated to occur in children <5 years old, resulting in almost 600,000 deaths in Africa in 2020[6]. CM and SMA have also been associated with long-term neurocognitive impairment (NCI) in children that survive CM[13-16] or SMA[17]. However, the mechanisms underlying disease severity and NCI in CM and SMA are not fully understood.

Microvascular obstruction, endothelial activation, and excessive systemic inflammation that occur, in part, from parasite burden and sequestration of infected erythrocytes are thought to underlie the pathogenesis of both CM and SMA[105-108,162-164]. The excessive production of pro-inflammatory cytokines is an important component of systemic inflammation during severe disease, particularly CM[164]. Interleukin-33 (IL-33), an alarmin molecule released early in response to tissue or endothelial barrier damage in several inflammatory diseases[165], is a member of the IL-1 cytokine family and binds to its receptor, ST2[166]. ST2 has two splice variants: a membrane-bound form, which initiates IL-33 intracellular signaling; and the soluble form, soluble ST2 (sST2), which binds IL-33 in circulation, inhibiting the intracellular signaling of IL-33[167]. IL-33 and ST2 are widely expressed throughout the human body, and low plasma or serum levels of IL-33 or high levels of sST2 are seen in numerous diseases, including cardiovascular diseases, particularly heart failure[168,169] and stroke[170,171]; and infectious diseases, including sepsis[172,173] and recently COVID-19[174,175]. However, elevated IL-33 has also

been associated in human studies with increased endothelial activation and neuroinflammation[176,177], suggesting that it may have a pleiotropic effect.

Studies of experimental cerebral malaria (ECM) in mice using *Plasmodium berghei* ANKA have identified IL-33 and ST2 as key molecules involved in ECM pathogenesis. Studies support a role for endogenous IL-33 in increasing inflammation and contributing to development of ECM[178] and associated cognitive impairments[179]. However, exogenous IL-33 has been shown to reduce inflammation and mediate protection from ECM[180,181]. Taken together, the murine ECM studies and human studies of the role of the IL-33/ST2 pathway in infectious processes highlight the complex interplay of this pathway in the host response to infection.

In human studies, sST2, which acts as a decoy receptor to bind IL-33 and reduce the intracellular effects of IL-33, is often measured in place of IL-33, because it is present in higher levels in plasma or serum and is a better predictor of disease severity and mortality than IL-33[167,182]. However, plasma and central nervous system (CNS) levels of sST2 have not been assessed to date in children with severe malaria. We hypothesized that children with severe malaria would have elevated plasma and CNS levels of sST2, which would correlate with markers of endothelial activation and neuronal damage and predict NCI. To test these hypotheses, we measured plasma and cerebrospinal fluid (CSF) sST2 levels in children with CM and plasma IL-33 and sST2 levels in children with CM or SMA, comparing them with biomarkers of endothelial activation and neuronal damage, and with neurocognitive scores, over 2-year follow-up.

A1.2 Methods

A1.2.1 Study population

This prospective study was performed at Mulago Hospital, Kampala, Uganda, from 2008 to 2015. Children with cerebral malaria (CM) or severe malarial anemia

(SMA) were enrolled if they were between 18 months and 12 years of age. CM was defined as (1) coma (Blantyre Coma Score ≤2); (2) *P. falciparum* on blood smear; and (3) no other known cause of coma (e.g., meningitis, a prolonged postictal state, or hypoglycemia-associated coma reversed by glucose infusion). Exclusion criteria for CM included prior history of coma, head trauma, hospitalization for malnutrition, or cerebral palsy. SMA was defined as a positive *P. falciparum* blood smear and serum hemoglobin ≤5 g/dL. Exclusion criteria for SMA included impaired consciousness, seizures prior to admission, or other clinical evidence of CNS involvement. Asymptomatic children aged 18 months to 12 years were enrolled from the neighborhoods and extended households of those enrolled with severe malaria to act as community control (CC) children. Children with active or recent illness, chronic illness requiring medical care, or prior coma were excluded as CC children.

At enrollment, whole blood was collected from children with severe malaria and from CC children and stored at –80°C to be used for further testing. In children with CM, CSF was obtained to rule out bacterial meningitis or encephalitis in all children whose parents agreed to the procedure and in whom the procedure was not contraindicated. CSF samples from North American children treated for prior leukemia, in whom CSF was obtained to rule out return of malignancy, and who had no evidence of CNS disease at collection, were used to evaluate CSF levels of ST2 in a control group. Plasma from these individuals was not available. Children with severe malaria were treated at Mulago Hospital according to Ugandan national treatment guidelines at the time. Follow-up of enrolled patients was conducted at 6, 12, and 24 months after discharge.

A1.2.2 Clinical and demographic assessments

All children underwent a medical history and physical examination at enrollment. Peripheral blood smears were assessed for *Plasmodium* species by microscopy with Giemsa staining using standard protocols. Nutritional status was assessed by height-for-

age and weight-for-age z-scores (WHO Child Growth Standards), and socioeconomic status was measured using a validated scoring system published previously[183]. Duration of coma was defined as time from admission until the child regained full consciousness (Blantyre Coma Score=5 or Glasgow Coma Scale=15). Acute kidney injury (AKI) was defined as a 1.5-fold increase in creatinine from estimated baseline as described[184], using the Kidney Disease: Improving Global Outcomes guidelines based on a single-admission creatinine level[185].

A1.2.3 Biomarker assessments

The following methods were used for testing: plasma and CSF soluble ST2 (DuoSet ELISA kit, R&D Systems, Minneapolis, MN, USA), plasma diluted 1:20, CSF samples diluted 1:4), plasma IL-33 (Luminex assay, Luminex Corp, Austin, TX, USA), plasma *Plasmodium falciparum* histidine-rich protein-2 (PfHRP-2), (Malaria Ag CELISA kit, Cellabs, Brookvale, Australia), plasma soluble intracellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), vascular endothelial growth factor (VEGF), sE-selectin, sP-selectin, as well as plasma and CSF IL-1b (magnetic cytometric bead assay, R&D Systems); plasma and CSF TNF-α and IL-6 (magnetic cytometric bead assay, Millipore Sigma, Burlington, MA, USA); plasma angiopoietin-1 (Angpt-1) and -2 (Angpt-2) (DuoSet ELISA kit, R&D Systems); and plasma Von Willebrand Factor (vWF) (ELISA, Corgenix Medical Corp, Broomfield, CO, USA). Plasma and CSF albumin were quantified by the Advanced Research and Diagnostic Laboratory at the University of Minnesota using the Bromocresol Purple Albumin Assay (Sigma-Aldrich, St. Louis, MO). The CSF/plasma albumin index ([CSF albumin concentration/plasma albumin concentration] x 1000) was used as a surrogate measure of blood brain barrier (BBB) damage. CSF tau testing was performed using the Luminex-based Human Tau (total) Singleplex Bead Kit (Invitrogen, Carlsbad, CA, USA)

and the Human Neuroscience Buffer Reagent Kit (Invitrogen). Plasma tau levels were measured via ultrasensitive biomarker detection at Quanterix (Billerica, MA, USA).

A1.2.4 Neurologic and cognitive assessments

Neurologic and cognitive assessments were performed at discharge (neurologic testing) or 1 week after discharge (cognitive testing), and at 6, 12, and 24 months after discharge. A neurologic deficit (ND) was defined as presence of motor or cranial nerve deficit, ataxia, a movement disorder, or clinically detectable behavioral, speech, or visual disorders. The tests used to assess cognition were validated in cohorts of Ugandan children and described in detail in prior publications[16,17,186]. In brief, for children <5 years of age, cognitive ability was assessed by the Mullen Scales of Early Learning[187], attention by the Early Childhood Vigilance Test[188], and associative memory by the Color Object Association Test[189]. In children ≥5 years, overall cognitive ability was assessed by the summary mental processing index of the Kaufman Assessment Battery for Children, second edition (KABC-II)[190], attention by the Test of Variables of Attention (D prime measure primary outcome)[191], and working memory by the sequential processing subtest of the K-ABC-II (44). To account for differences in child age, we converted each raw score into a z-score using scores of the CC children. The z-scores were computed as (actual score–mean score for a child's age)/standard deviation (SD), where the mean score for a child's age and SD were computed by fitting a quadratic mixed effects model, including a random intercept for the child and where correlations within a child were based on time between visits, to data for all visits for all CC children.

A1.2.5 Statistical analysis

Analyses were conducted using Stata/SE14 (StataCorp, College Station, Texas, USA). For continuous variables, Wilcoxon Rank Sum tests were performed to compare differences between two groups, and Kruskal–Wallis tests were used to compare differences among all three groups. Categorical or ordinal variables, such as sex and

preschool education, were analyzed using Chi-Square tests. Spearman's correlation was used to assess the association between plasma and CSF levels of sST2. Linear, logistic, and negative binomial regression analyses were used to compare sST2 levels (log_{10}-transformed) to continuous, dichotomous, and count outcomes, respectively. These comparisons were corrected for multiplicity using the Bonferroni correction.

For cognitive outcomes, linear mixed-effects models were used to allow for comparison of plasma and CSF sST2 concentrations to test over all testing time points in the 2-year follow-up. The models were adjusted for factors that could impact cognitive outcomes, including age, sex, height-for-age z-score, weight-for-age z-score, plasma levels of HRP2, and preschool education of study participants. In the models, within-subject observations were correlated using a subject-specific intercept and timepoints were treated as categorical variables. A banded diagonal covariance matrix was assumed to model within-subject variance–covariance errors, the mixed models were fitted by restricted maximum likelihood, and Kenward–Roger approximations were used to estimate the denominator degrees of freedom.

A1.2.6 Ethical review

Written informed consent was obtained from parents or guardians of study participants. Ethical approval was granted by the institutional review boards for human studies at the Makerere University School of Medicine, the Uganda National Council for Science and Technology, and the University of Minnesota Medical School.

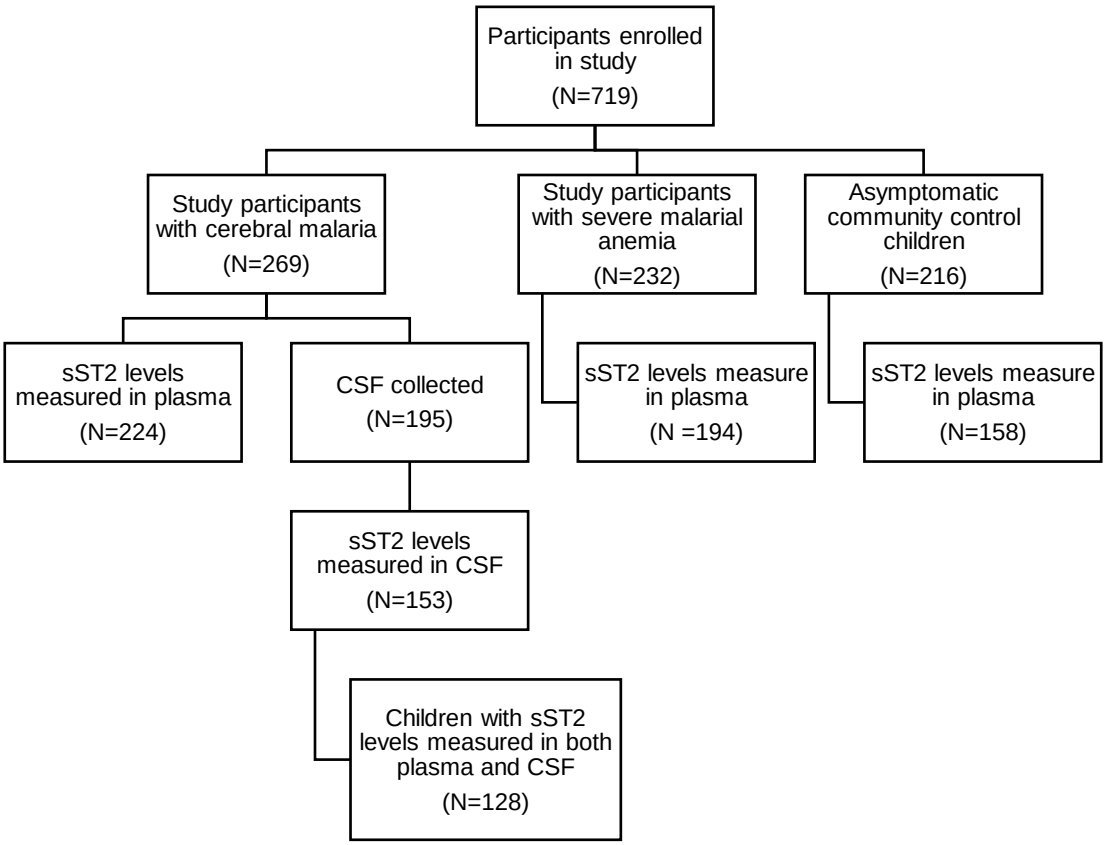
A1.3 Results

A1.3.1 Study cohort demographic characteristics

Soluble ST2 (sST2) was measured in the plasma of 224 children with cerebral malaria (CM), 194 children with severe malarial anemia (SMA), and 158 asymptomatic community control (CC) children (Figure A1.1). Of the 269 children enrolled with CM,

CSF was collected in 195 children; sST2 was measured in 153 children due to limited sample volume (Figure A1.1). Measurement of both plasma and CSF levels of sST2 was performed in 128 of the children with CM (Figure A1.1).

Figure A1.1. Diagram of Study Participants.



Loss of sample size generally caused by insufficient volume of plasma or CSF samples for testing.

Children with CM and CC children were older than children with SMA (median age, years [interquartile range] CM 3.49 [2.48, 4.88], CC 3.50 [2.63, 4.61], SMA 2.79 [2.04, 4.35], P<0.001), and there was a greater proportion of male children in the CM and SMA groups compared with CC children (male %, CM, 59.4%, SMA, 60.1%, CC, 44.9%, P=0.006).

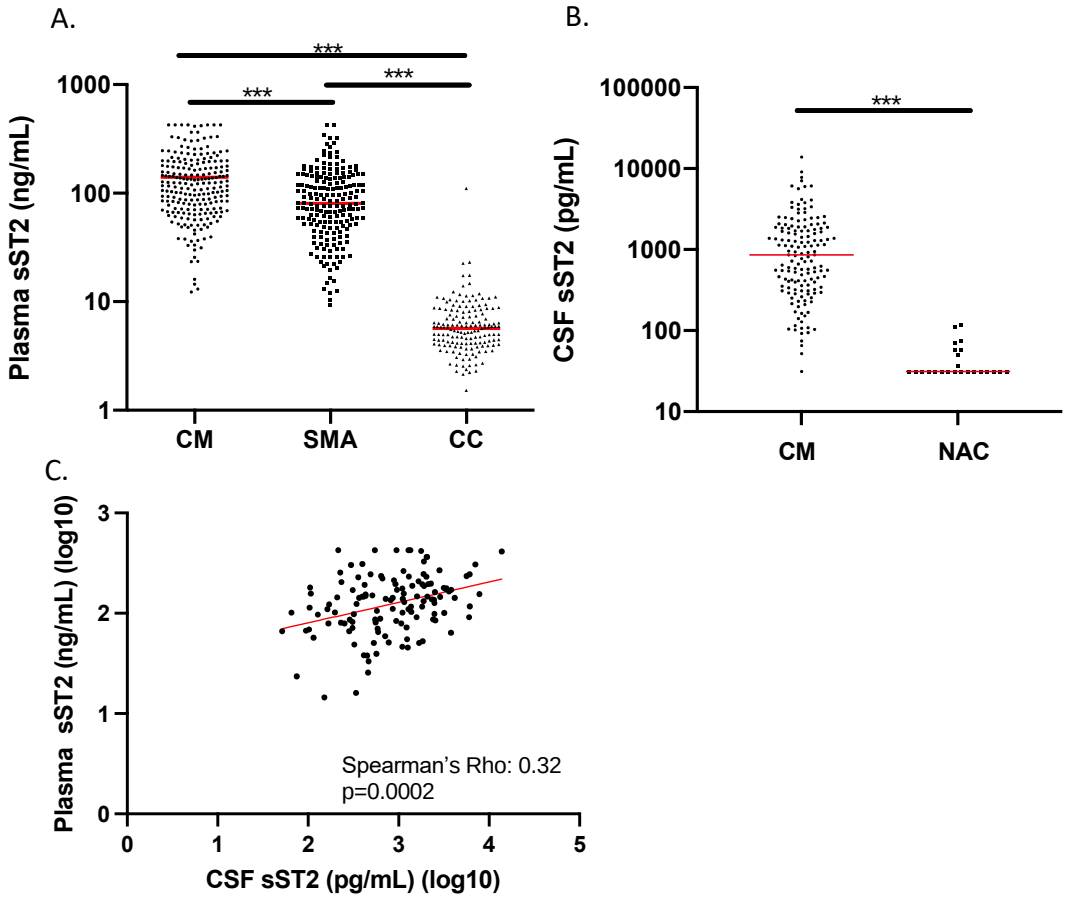
A1.3.2 Plasma IL-33 is rarely detected in children with severe malaria or in community children

IL-33 was tested for in 405 plasma samples of children with CM (n=170), children with SMA (n=169), or CC children (n=66). Levels were below the limit of detection (2.67 ng/ml) in 362 samples (89.4%), so we did not test IL-33 in further samples. IL-33 was not measured in CSF due to limited CSF sample volume.

A1.3.3 Plasma sST2 levels are elevated in children with CM and children with SMA compared with CC

Plasma sST2 levels were higher in children with CM (median (ng/mL) [interquartile range: IQR] 116.7 [70.8–178.5]) than in children with SMA (81.0 [50.2–139.4]), and both were higher than levels in CC children (5.7 [4.1–8.2]) (all $P<0.0001$; Figure A1.2A).

Figure A1.2. Plasma and CSF levels of sST2 in Ugandan children with CM or SMA and CC.



(A) Levels of sST2 were elevated in children with CM (n=224) and SMA (n=193) compared with asymptomatic community control children (CC, n=156). (B) Concentration of sST2 in the CSF of children with CM (n=153) was higher compared to that of healthy North American Control (NAC) children (n=24). (A, B) Wilcoxon Rank Sum Test was used to compare each group. ***P<0.0001. (Circles) Plasma sST2 concentration in individual children, (horizonal bar) median sST2 concentration for each group. (C) Plasma and CSF levels of sST2 in were correlated using Spearman's correlation (rho=0.32, P=0.0002, n=128). (Red line) Line of best fit based on linear regression model.

A1.3.4 CSF sST2 levels are elevated in children with CM and correlate with plasma sST2 levels

CSF sST2 levels were significantly higher in children with CM than in control samples from North American children (median (pg/mL) [IQR], CM, 860.8 [342.5–1826.2]; North American children, 31.2, [31.2, 35.1]. P<0.0001; Figure A1.2B). CSF sST2 levels correlated positively with plasma sST2 levels in children with CM

(Spearman's rho, 0.32, P=0.0002, n=128; Figure A1.2C). The CSF/plasma albumin index, a marker of BBB damage, correlated with CSF sST2 levels (beta coefficient, 95% CI, 0.71 [0.47,0.95], P<0.001; Table A1.1).

Table A1.1. Associations between plasma and CSF levels of sST2 and parasite and host response factors in children with cerebral malaria (CM) or severe malarial anemia (SMA).

	CM CSF ST2 level			CM plasma ST2 level			SMA plasma ST2 level		
	n	**beta coefficient [95% CI]**	**P value**	**n**	**beta coefficient [95% CI]**	**P value**	**n**	**beta coefficient [95% CI]**	**P value**
Parasite burden									
Peripheral blood parasite density	147	-0.01 [-0.09, 0.07]	0.81	218	0.05 [0.01, 0.09]	**0.02**	193	0.03 [-0.02, 0.07]	0.29
Plasma *Pf*HRP-2 level	153	0.21 [0.08, 0.35]	**0.003**	224	0.14 [0.07, 0.20]	**<0.001**	193	0.08 [0.02, 0.14]	**0.009**
Plasma markers of endothelial activation									
sICAM-1	140	-0.10 [-0.24, 0.04]	0.17	172	0.12 [0.05, 0.20]	**0.002**	157	0.07 [-0.00, 0.14]	0.06
sVCAM-1	140	0.68 [0.37, 0.99]	**<0.001**	172	0.34 [0.16, 0.52]	**<0.001**	157	0.50 [0.29, 0.70]	**<0.001**
Angiopoietin 1	135	-0.19 [-0.35, -0.02]	0.02	189	-0.07 [-0.15, 0.01]	0.07	154	-0.11 [-0.22, -0.01]	0.03
Angiopoietin 2	135	0.16 [-0.05, 0.37]	0.13	189	0.27 [0.17, 0.37]	**<0.001**	154	0.26 [0.16, 0.36]	**<0.001**
Ratio Angpt1:Angpt2	135	0.20 [0.07, 0.34]	**0.003**	189	0.15 [0.09, 0.21]	**<0.001**	154	0.21 [0.13, 0.28]	**<0.001**
VEGF	140	0.10 [-0.10, 0.30]	0.32	195	-0.06 [-0.16, 0.04]	0.24	157	-0.09 [-0.23, 0.05]	0.21
PDGF	140	-0.15 [-0.34, 0.04]	0.12	195	-0.11 [-0.20, -0.02]	0.02	157	-0.06 [-0.17, 0.06]	0.32
E-selectin	140	0.33 [-0.10, 0.75]	0.13	172	0.61 [0.39, 0.84]	**<0.001**	157	0.59 [0.35, 0.82]	**<0.001**
P-selectin	136	-0.03 [-0.41, 0.34]	0.86	164	0.55 [0.34, 0.76]	**<0.001**	90	0.27 [0.01, 0.54]	0.04
Von Willebrand Factor	124	-0.14 [-0.42, 0.14]	0.33	157	0.12 [-0.02, 0.27]	0.10	135	0.20 [0.04, 0.36]	0.02

Markers of neuronal injury									
Plasma tau level	106	0.15 [-0.07, 0.38]	0.18	177	0.16 [0.06, 0.27]	**0.003**	153	0.32 [0.18, 0.47]	**<0.001**
CSF tau level	122	0.12 [-0.08, 0.32]	0.24	114	0.21 [0.09, 0.33]	**<0.001**			
CSF:plasma albumin index	131	0.71 [0.47, 0.95]	**<0.001**	121	0.18 [0.02, 0.35]	0.03			

All factors are continuous variables and were log transformed (base 10) prior to linear regression analysis. Data presented as beta coefficient and 95% CI. Corrected for multiple comparisons using Bonferroni correction within each type of markers, P<0.025 for parasite factors, P<0.005 for markers of endothelial activation, and P<0.025 for markers of neuronal injury.

A1.3.5 Plasma and CSF levels of sST2 correlate with parasite biomass and endothelial activation in children with CM

Total parasite biomass, as assessed by plasma *Pf*HRP2 level, correlated positively with plasma sST2 levels in children with CM or SMA and correlated positively with CSF sST2 levels in children with CM (Table A1.1). Peripheral blood parasite density correlated positively with plasma sST2 level in children with CM but not in children with SMA (Table A1.1).

Plasma levels of sST2 in children with CM and in children with SMA were associated with increased levels of multiple markers of endothelial activation, including sVCAM-1, Angpt-2, the ratio of Angpt-2 to Angpt-1, E-selectin, and P-selectin (Table A1.1). Among these markers, sVCAM-1 and the ratio of Angpt-2 to Angpt-1 were associated with increased CSF concentrations of sST2 in children with CM (Table A1.1).

A1.3.6 Plasma levels of sST2 correlate with plasma and CSF tau levels in children with CM

Plasma sST2 levels correlated strongly with both plasma and CSF levels of tau, a marker of neuronal injury, in children with CM, and with plasma tau levels in children with SMA (Table A1.1).

A1.3.7 Plasma sST2 levels correlate with plasma TNF-α levels in CM and SMA, while CSF sST2 levels correlate with CSF IL-6 and TNF-α levels in CM

In ECM, absence of the ST2/IL-33 pathways was associated with decreased expression of the pro-inflammatory cytokines IL-1b, TNF-α, and IL-6 in the brain[178,179]. For this reason, we compared these levels in children with CM or SMA. Plasma sST2 levels in children with CM or SMA were associated with increased plasma levels of the pro-inflammatory cytokine TNF-α (Table A1.2). In children with CM, where CSF levels of these cytokines could be measured, CSF sST2 levels were positively associated with

CSF levels of IL-6 and TNF-α (Table A1.2). Plasma and CSF sST2 levels were not associated with either plasma or CSF IL-1b levels in children with CM (Table A1.2).

Table A1.2. Association between plasma and CSF levels of sST2 to plasma and CSF pro-inflammatory cytokine levels.

	CM CSF ST2 level			CM plasma ST2 level			SMA plasma ST2 level		
	n	**beta coefficient [95% CI]**	**P value**	**n**	**beta coefficient [95% CI]**	**P value**	**n**	**beta coefficient [95% CI]**	**P value**
Cytokines in Plasma									
IL-6	142	-0.04 [-0.13, 0.05]	0.42	206	0.06 [0.01, 0.10]	0.02	183	0.18 [0.11, 0.25]	**<0.001**
TNF-α	142	0.01 [-0.12, 0.15]	0.84	206	0.13 [0.05, 0.20]	**0.001**	183	0.20 [0.09, 0.31]	**<0.001**
IL-1b	140	0.15 [-0.06, 0.35]	0.16	195	-0.03 [-0.14, 0.08]	0.57	157	-0.03 [-0.15, 0.09]	0.62
Cytokines in CSF									
IL-6	153	0.24 [0.14, 0.35]	**<0.001**	147	0.02 [-0.04, 0.09]	0.49			
TNF-α	153	0.30 [0.22, 0.39]	**<0.001**	147	0.05 [-0.01, 0.11]	0.14			
IL-1b	122	0.10 [-0.14, 0.35]	0.40	114	0.09 [-0.10, 0.28]	0.35			

All factors are continuous variables and were log transformed (base 10) prior to linear regression analysis. Data presented as beta coefficient and 95% CI. Corrected for multiple comparisons using Bonferroni correction within each type of markers, P<0.013 for cytokines in plasma and CSF.

A1.3.8 Plasma sST2 levels are associated with acute kidney injury and thrombocytopenia but not mortality in children with CM

To evaluate the clinical significance of elevated plasma and CSF sST2 levels, we compared them to mortality in children with CM, and to risk of acute kidney injury, a major complication of CM and SMA, and thrombocytopenia, which occurs more often in children with severe malaria than uncomplicated malaria. Plasma and CSF sST2 levels were not associated with mortality in children with CM (Table A1.3). However, plasma sST2 levels were associated with an increased risk of acute kidney injury (AKI) and elevated blood urea nitrogen (BUN) in children with CM and in children with SMA (Table A1.4), and with a greater risk of thrombocytopenia, another marker of disease severity, in children with CM and in children with SMA (Table A1.4).

Table A1.3 Associations of admission plasma and CSF levels of sST2 with in-hospital outcomes and neurologic deficits in children with CM.

		CM plasma			CM CSF		
Clinical outcome	**Value**	**n**	**OR, beta, IRR [95% CI]**	**P value**	**n**	**OR, beta, IRR [95% CI]**	**P value**
Mortality	OR	224	1.56 [0.42, 5.80]	0.51	152	3.57 [0.82, 15.62]	0.09
Coma duration[a]	ß	195	0.13 [-0.02, 0.29]	0.10	144	0.03 [-0.07, 0.14]	0.55
No. of seizures in hospital	IRR	224	0.67 [0.09, 1.24]	**0.02**	153	-0.10 [-0.52, 0.33]	0.66
Presence of neurological deficits at:							
Discharge	OR	190	0.66 [0.25, 1.73]	0.40	143	1.11 [0.56, 2.20]	0.76
6-month follow-up	OR	186	3.27 [0.37, 28.56]	0.28	139	1.66 [0.38, 7.35]	0.50
12-month follow-up	OR	185	8.28 [0.56, 123.0]	0.13	139	2.31 [0.35, 15.35]	0.29
24-month follow-up	OR	183	16.80 [0.82, 344.93]	0.07	137	2.31 [0.28, 19.0]	0.44

Corrected for multiple comparisons using Bonferroni correction, P<0.017 for clinical outcome markers and P<0.013 for presence of neurological deficits.
[a]Coma duration log-transformed (base 10)

Table A1.4. Association between plasma and CSF levels of sST2 to clinical risk factors in children with cerebral malaria(CM) or severe malarial anemia (SMA).

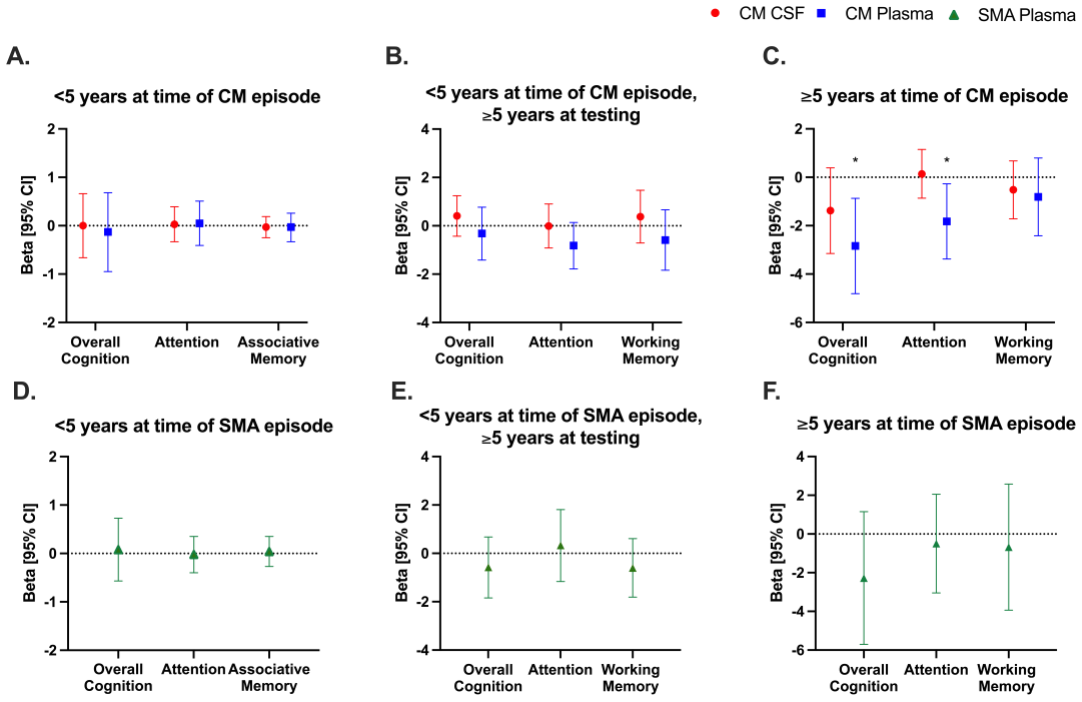
	CM CSF			CM Plasma			SMA Plasma		
	n	**OR [95% CI]**	**P value**	**n**	**OR [95% CI]**	**P value**	**n**	**OR [95% CI]**	**P value**
Clinical Risk Factors[a]									
Lactic acidosis	145	1.32 [0.66, 2.63]	0.43	207	3.23 [1.19, 8.77]	0.02	178	1.57 [0.63, 3.93]	0.33
Acute kidney injury	149	1.81 [0.94, 3.50]	0.08	215	7.61 [2.73, 21.21]	**<0.001**	187	10.50 [2.98, 37.04]	**<0.001**
Elevated BUN	153	2.38 [1.21, 4.68]	**0.01**	224	43.13 [12.25, 151.87]	**<0.001**	193	63.66 [13.95, 290.64]	**<0.001**
Thrombocytopenia	153	2.73 [1.06, 7.08]	0.04	219	7.77 [2.08, 29.05]	**0.002**	192	3.52 [1.42, 8.71]	**0.007**

Associations among levels of sST2 in children with severe malaria with clinical risk factors and clinical lab tests. Plasma and CSF levels of sST2 were log transformed (base 10). Odds ratios (OR) and 95% confidence intervals (CI) are presented. Corrected for multiple comparisons using Bonferroni correction, P<0.013.
Definitions: lactic acidosis, lactate >5 nmol/L, uremia, plasma urea nitrogen >20 mg/dL; thrombocytopenia, platelet count <150,000.
[a]Clinical risk factors are binary outcomes and were analyzed using logistic regression models.

A1.3.9 Elevated levels of sST2 in plasma are associated with long-term neurocognitive impairment in children with CM

In children with CM, the odds of neurological deficit with elevated admission plasma sST2 increased over time, but there was no significant association between plasma or CSF sST2 levels and neurological deficits at any time point (Table A1.5). Additionally, in children with CM who were <5 years of age at the time of their CM episode and at the time of cognitive testing, or who were <5 years of age at the time of CM episode but ≥5 years at testing, risk factor–adjusted plasma or CSF sST2 levels were not significantly associated with any cognitive outcome (Figure A1.3A,B). However, in children with CM who were ≥5 years, yet younger than 12 years old, at the time of the CM episode, risk factor–adjusted plasma sST2 concentrations were associated with worse overall cognitive ability (beta coefficient [95% confidence interval]; –2.84 [–4.81, –0.87] (n=46), P=0.01) and attention (–1.82 [–3.39, –0.27], P=0.02; Figure A1.3A–C; Table A1.6).

Figure A1.3. Association of risk-factor adjusted log(10)-transformed plasma and CSF sST2 levels with cognitive outcome z-scores in children with CM or SMA.



Associations of plasma and CSF sST2 concentration with cognitive outcomes in children with cerebral malaria (CM) or severe malarial anemia (SMA). Associations between CSF and plasma levels of sST2 (log10 transformed) with cognitive outcomes in children with CM <5 years old at the time of CM episode (A) and throughout the 24-month follow-up period, children that turned 5 years old during follow-up and underwent cognitive testing using tests for children over 5 years old (B), and children that were over 5 years old at time of CM episode and throughout follow-up (C). (D–F) Associations between CSF and plasma levels of sST2 (log10 transformed) with cognitive outcomes in children with SMA for same age groups. Data are presented as ß coefficient and 95% confidence interval from a longitudinal mixed effects model. (Red) sST2 concentration in CSF from children with CM, (blue) sST2 concentration in plasma from children with CM, (green) sST2 concentration in plasma from children with SMA. The model is adjusted for age, sex, height-for-age z-score, weight-for-age z-score, plasma levels of HRP2, and preschool education of study participants. See Table A1.5 for ß coefficients and 95% confidence intervals for unadjusted analysis and sample sizes. *P<0.05.

In children with SMA, there were no significant associations in unadjusted or adjusted models with any cognitive outcome in any age group (Figure A1.3D–F; Table A1.5). Children with CM or SMA who had plasma or CSF sST2 levels tested did not

differ significantly from those who did not have plasma or CSF sST2 testing in age, sex, or cognitive outcome scores (Table A1.6).

Table A1.5. Plasma and CSF sST2 concentration associations with cognitive outcomes in children with cerebral malaria (CM) and severe malarial anemia (SMA).

	N(obs), n[a]	Unadjusted beta [95% CI]	Unadjusted P value	N(obs), n[b]	Adjusted beta[c] [95% CI]	Adjusted P value
CSF sST2 levels compared to cognitive outcomes in children with CM						
<5 years at CM episode						
Overall Cognition	336, 114	0.15 [-0.49, 0.79]	0.65	334, 112	0.00 [-0.66, 0.66]	1.00
Attention	355, 114	0.16 [-0.18, 0.50]	0.35	353, 112	0.03 [-0.33, 0.39]	0.88
Associative Memory	355, 115	0.07 [-0.14, 0.28]	0.49	353, 113	-0.03 [-0.25, 0.19]	0.76
<5 years at CM episode; ≥5 years at time of testing						
Overall Cognition	77, 57	**0.93 [0.02, 1.83]**	**0.04**	77, 57	0.41 [-0.43, 1.24]	0.33
Attention	79, 57	0.69 [-0.28, 1.65]	0.16	79, 57	-0.01 [-0.92, 0.90]	0.99
Working Memory	79, 57	0.98 [-0.15, 2.11]	0.09	79, 57	0.38 [-0.71, 1.47]	0.49
≥5 years at time of CM episode						
Overall Cognition	111, 29	**-1.68 [-3.03, -0.33]**	**0.02**	107, 28	-1.38 [-3.15, 0.39]	0.12
Attention	111, 29	-0.20 [-1.07, 0.66]	0.63	107, 28	0.14 [-0.86, 1.15]	0.77
Working Memory	112, 29	-0.89 [-1.90, 0.12]	0.08	108, 28	-0.52 [-1.72, 0.68]	0.37
Plasma sST2 levels compared to cognitive outcomes in children with CM						
<5 years at CM episode						
Overall Cognition	423, 143	0.08 [-0.80, 0.95]	0.86	421, 141	-0.13 [-0.95, 0.68]	0.75
Attention	443, 144	0.15 [-0.30, 0.59]	0.51	441, 142	0.05 [-0.41, 0.51]	0.84
Associative Memory	440, 144	-0.01 [-0.30, 0.29]	0.97	438, 142	-0.03 [-0.33, 0.26]	0.82
<5 years at CM episode; ≥5 years at time of testing						
Overall Cognition	105, 76	-0.28 [-1.62, 1.05]	0.67	105, 76	-0.32 [-1.42, 0.77]	0.56
Attention	106, 76	-0.66 [-1.75, 0.43]	0.23	106, 76	-0.82 [-1.78, 0.14]	0.09

Working Memory	106, 76	-0.38 [-1.69, 0.93]	0.57	106, 76	-0.59 [-1.84, 0.66]	0.35
≥5 years at time of CM episode						
Overall Cognition	188, 49	**-2.55 [-4.47, -0.62]**	**0.01**	179, 46	**-2.84 [-4.81, -0.87]**	**0.01**
Attention	186, 49	-1.35 [-2.77, 0.07]	0.06	177, 46	**-1.82 [-3.38, -0.27]**	**0.02**
Working Memory	189, 49	-0.75 [-2.29, 0.78]	0.33	180, 46	-0.81 [-2.42, 0.80]	0.32
	Plasma sST2 levels compared to cognitive outcomes in children with SMA					
<5 years at SMA episode						
Overall Cognition	473, 156	0.11 [-0.57, 0.80]	0.75	464, 153	0.08 [-0.57, 0.73]	0.81
Attention	509, 157	-0.06 [-0.42, 0.30]	0.75	500, 154	-0.02 [-0.40, 0.35]	0.90
Associative Memory	489, 154	-0.05 [-0.35, 0.25]	0.73	480, 151	0.04 [-0.27, 0.35]	0.80
<5 years at SMA episode; ≥5 years at time of testing						
Overall Cognition	78, 50	-0.57 [-2.00, 0.86]	0.43	78, 50	-0.58 [-1.84, 0.68]	0.36
Attention	81, 53	0.06 [-1.26, 1.38]	0.93	81, 53	0.33 [-1.16, 1.81]	0.66
Working Memory	81, 53	-0.55 [-1.74, 0.63]	0.35	81, 53	-0.60 [-1.81, 0.61]	0.32
≥5 years at time of SMA episode						
Overall Cognition	128, 34	-1.02 [-3.21, 1.18]	0.35	120, 32	-2.28 [-5.70, 1.15]	0.18
Attention	129, 34	0.33 [-1.26, 1.93]	0.67	122, 32	-0.50 [-3.05, 2.05]	0.69
Working Memory	132, 34	-0.49 [-2.40, 1.42]	0.61	124, 32	-0.68 [-3.94, 2.58]	0.67

Linear mixed effects model. Cognitive testing conducted at discharge, 6, 12, and 24 months. Data are presented as beta coefficient and 95% confidence interval.

[a]N(obs) represents number of events; N represents the number of participants. sST2 levels log10 transformed.
[b]Decrease number in adjusted model on account of missing data in adjusting variables.
[c]Model adjusted for age, sex, height for age z-score, weight for age z-score, plasma levels of HRP2, and preschool education of study participants.

Table A1.6. Age, sex, neurologic deficits, and cognitive outcome scores in children with CM or SMA who had CSF or plasma sST2 testing performed compared to those who did not.

Cerebral Malaria	sST2 Measured (n=249)	Not in sST2 (n=20)	P value
Age, years, median (IQR)	3.50 [2.52, 4.87]	3.41 [2.58, 4.42]	0.64
Sex, % male (n)	60.6 (151)	40.0 (8)	0.07
Z-score for cognitive outcome at 12 months			
Under 5 years tests			
Overall cognition	-0.86 [-2.17, 0.12] (119)	-1.26 [-2.01, -0.42] (12)	0.68
Attention	-0.50 [-1.29, 0.20] (124)	-0.03 [-0.58, 0.55] (12)	0.20
Associative memory	-0.46 [-0.80, -0.04] (122)	-0.42 [-0.87, -0.17] (12)	0.94
Over 5 years tests			
Overall cognition	-0.22 [-1.89, 1.03] (83)	3.96 [2.06, 4.22] (3)	**0.01**
Attention	0.19 [-0.84, 1.16] (83)	0.36 [0.20, 1.43] (3)	0.46
Working memory	-0.24 [-1.25, 0.75] (83)	0.63 [0.26, 1.38] (3)	0.16
Z-score for cognitive outcome at 24 months			
Under 5 years tests			
Overall cognition	-1.05 [-2.23, 0.21] (68)	-0.11 [-1.42, -0.02] (5)	0.60
Attention	-0.49 [-1.13, 0.18] (68)	-0.22 [-0.31, 0.00] (5)	0.65
Associative memory	-0.81 [-1.10, -0.25] (69)	-0.84 [-0.91, -0.63] (5)	0.75
Over 5 years tests			
Overall cognition	-0.71 [-1.99, 0.42] (136)	-0.29 [-0.60, 1.17] (10)	0.12
Attention	0.03 [-0.90, 0.78] (138)	0.48 [-0.14, 1.10] (10)	0.18
Working memory	-0.28 [-1.42, 0.80] (138)	-0.45 [-0.75, 0.72] (10)	0.75
Percent of neurological deficits at:			
Discharge	36.6% (78/213)	29.4 (5/17)	0.55
6-month follow-up	5.3% (11/209)	0% (0/14)	0.38
12-month follow-up	3.4% (7/208)	0% (0/15)	0.47
24-month follow-up	2.9% (6/205)	0% (0/15)	0.50
Severe Malarial Anemia	**sST2 Measured (n=193)**	**Not in sST2 (n=39)**	**P value**
Age, years, median (IQR)	2.79 [2.04, 4.35]	2.96 [2.16, 4.53]	0.91
Sex, % male (n)	60.1 (116)	61.5 (24)	0.87
Z-score for cognitive outcome at 12 months			
Under 5 years tests			
Overall cognition	-1.25 [-2.20, -0.03] (117)	-1.32 [-2.31, -0.47] (23)	0.64
Attention	-0.10 [-0.95, 0.45] (125)	-0.25 [-0.80, 0.47] (23)	0.74

Associative memory	-0.24 [-0.57, 0.33] (120)	-0.43 [-0.76, 0.17] (23)	0.13
Over 5 years tests			
Overall cognition	-0.24 [-0.98, 1.26] (52)	0.19 [-0.96, 0.94] (10)	0.70
Attention	0.23 [-1.04, 0.79] (54)	0.87 [-0.21, 2.24] (11)	0.29
Working memory	-0.39 [-1.53, 0.58] (54)	-0.04 [-1.37, 0.82] (10)	0.51
Z-score for cognitive outcome at 24 months			
Under 5 years tests			
Overall cognition	-1.12 [-2.36, 0.14] (90)	-1.60 [-2.86, -1.03] (15)	0.14
Attention	-0.53 [-1.22, 0.06] (93)	-0.51 [-1.05, 0.03] (15)	0.75
Associative memory	-0.72 [-1.04, -0.12] (92)	-0.96 [-1.08, -0.22] (15)	0.19
Over 5 years tests			
Overall cognition	-0.20 [-1.40, 0.38] (81)	-0.31 [-1.49, 1.18] (18)	0.62
Attention	0.01 [-0.97, 0.63] (83)	0.66 [-0.95, 1.73] (18)	0.23
Working memory	-0.64 [-1.34, 0.04] (84)	-0.17 [-1.00, 0.91] (18)	0.26

For continuous variables, data presented as median [IQR] (n). Wilcoxon rank sum test used to compare age and z-scores for cognitive outcomes between children with CM or SMA who had sST2 levels measured in plasma or CSF. Chi-squared test used to compare sex and presence of neurological deficits.

A1.4 Discussion

In the present study, we provide the first evidence that sST2 is a marker of disease severity in children with severe malaria and that elevated plasma sST2 levels predict long-term cognitive impairment in children ≥5 years of age with cerebral malaria (CM). The study associations suggest a potential pathway for neuronal injury: activation of the IL-33/ST2 pathway by increased parasite biomass in children with CM; leading to release of sST2, release of pro-inflammatory cytokines, endothelial activation, and BBB dysfunction; which in turn contribute to acute neuronal injury and long-term cognitive impairment. Our study samples, taken at an individual point in time, show associations, but the pathway outlined is consistent with experimental cerebral malaria (ECM) studies in which the IL-33/ST2 pathway initiated oligodendrocyte and microglial responses in early infection, resulting in greater neuroinflammation, neuronal damage, and associated neurological and cognitive deficits[179].

Endogenous and exogenous IL-33 appear to have differing effects in murine models of severe malaria. Endogenous IL-33 leads to an increase in Th1 responses[179,192], while exogenous IL-33 induces a Th2 response through induction of innate lymphoid cells, M2 macrophages, and regulatory T cells[180], and through inhibition of the NLP3 inflammasome[181]. In murine ECM studies, exogenous IL-33 prevented ECM if given early[180] and reduced mortality and neurocognitive effects of ECM if given at the first sign of neurologic deficit[181]. These studies suggest that exogenous IL-33 could be of benefit as adjunctive therapy early in cerebral malaria. The only prior study of IL-33 in human malaria showed high plasma levels of IL-33 in children with severe malaria[193]. In the present study, few children had detectable IL-33 in plasma. The association of elevated plasma sST2 levels with endothelial activation, inflammation, neuronal injury, and long-term neurocognitive impairment in children with CM supports further

investigation of exogenous IL-33 or IL-33 analogs to suppress the pro-inflammatory response elicited by sST2 in murine or non-human primate models, to evaluate how the timing and dose of administration impact disease outcomes and host response in a model. Further research is required before these agents could be considered for human studies.

A key strength of the present study was the measurement of levels of sST2 and cytokines in CSF, providing a measure of CNS sST2 activity. Our findings revealed that CSF sST2 levels are elevated, that much of this elevation is likely to due to plasma sST2 crossing the impaired BBB, and that direct effects of CSF sST2 do not appear to be a major component in CNS neuronal injury, since plasma but not CSF sST2 levels correlated with CSF tau levels. The association of plasma sST2 levels with levels of plasma tau in children with CM[194] was of particular interest, because it provides a potential pathway through which IL-33/sST2 might lead to long-term NCI. CSF sST2 levels correlated with levels of CSF pro-inflammatory (IL-6, TNF-α) cytokines, suggesting that IL-33/sST2 may produce neuronal injury through induction of inflammation. In addition, plasma sST2 correlated with plasma TNF-α, which can cause direct and indirect neuronal injury through ischemia[195], spontaneous demyelination[196], and inducing the deaths of neurons and oligodendrocytes[197]. Thus, sST2 could potentially play a direct role in neuronal injury. Alternatively, as is seen in stroke, plasma sST2 levels may serve as a marker of brain injury, elevated as a specific response to cerebral ischemia and serving as a marker of neuroinflammation [170]. The link between sST2 and tau in this study, along with the associations with cognitive impairment in this population, and similar findings in an ECM model with IL-33 upregulation, suggest a connection between the ST2/IL-33 pathway and neuronal damage.

Elevated plasma sST2 levels were associated with worse cognitive outcome scores only in children with CM ≥5 years of age. The lack of association of plasma sST2 in children who were <5 years at CM episode and ≥5 years at time of testing suggests that this association was not due solely to effects of plasma sST2 elevation on cognitive pathways only measurable once the child is 5 years of age or older. Instead, the role of IL-33/sST2 in cognitive impairment may be greater in older children with less neural plasticity. Similarly, the association with NCI in children in CM but not SMA suggests either that this process is more important in CM or that the sST2 levels seen in CM, which were significantly higher than those in SMA, are needed to cause long-term brain injury.

Elevated plasma levels of sST2 were associated not only with inflammation and NCI in children with CM, but also with clinical findings associated with development of severe malaria (thrombocytopenia[33]) or with mortality in severe malaria (AKI[184,198], and uremia[199,200]). In AKI, the roles of IL-33 and sST2 are even less clear: in a study of myocardial infarction, elevated sST2 predicted AKI[201], but murine studies have shown IL-33-dependent endothelial activation, a process that should be dampened by elevated sST2 levels, leading to AKI after infection[202]. Additionally, in mice, exogenous IL-33 leads to further kidney damage, but exogenous sST2 leads to renal protection in mice after cisplatin-induced AKI[203]. In the present study, elevated sST2 levels were strongly associated with AKI in children with CM or SMA, suggesting a potential role for sST2 in malaria-associated AKI through endothelial activation.

Study strengths include a large sample size for a study of this kind; inclusion of community controls; longitudinal follow-up of study participants; rigorous assessment of clinical complications and assessment of multiple pathways implicated in severe malaria pathogenesis; evaluation of key biological factors in the potential disease pathway,

including measures of parasite biomass, endothelial activation, inflammation, and neuronal injury; and in-depth evaluation of long-term cognitive outcomes. A study limitation is that, as in all human studies, pathways could not be manipulated to determine directly whether changes in the ST2 pathway resulted in different clinical or neurocognitive outcomes. In addition, sST2 levels were measured at a single timepoint (hospital admission), rather than over multiple timepoints before and after admission. However, this measurement is probably the most relevant for interventions, which would be given at the time of admission for severe disease.

In conclusion, we found that plasma sST2 levels are elevated in children with CM and in children with SMA, with the greatest elevation seen in children with CM; that CSF sST2 levels are also elevated in children with CM, likely due to leakage of plasma sST2 across an impaired BBB; that elevated plasma sST2 is associated with an increase in parasite biomass, endothelial activation, and levels of the neuronal injury marker tau; and that in children with CM who are ≥5 years of age at the time of CM, elevated plasma sST2 is associated with long-term neurocognitive impairment. Together, the study findings suggest that sST2 may be a useful marker for disease severity in malaria, and in conjunction with prior ECM studies suggest a potential role for sST2 in induction of endothelial activation, neuroinflammation, neuronal injury, and long-term neurocognitive impairment in children with CM. Further research will be required to determine if factors that affect the ST2 pathway may be used safely as adjunctive treatment in severe malaria.

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Curriculum vitae

Elizabeth M. Fernander

Education

- Ph.D., Indiana University, Indianapolis, IN 2022
- M.D., Indiana University, Indianapolis, IN, pursuing, 2024
- B.S., Purdue University, West Lafayette, IN, 2016

Professional Memberships

- 2018-present; American Society of Tropical Medicine and Hygiene, Member

Honors and Awards

- 2020-2022, T32 Institutional Training Grant, Immunology and Infectious Disease Training Program

Abstracts

- Antibodies to PfEMP1 EPCR-binding antigens but not CD36-binding antigens remain elevated for 12 months in children hospitalized with severe malaria", poster session at ASTMH National Meeting 2021, National Harbor, MD, November 2021
- "The persistence of antibody response to the primary source of antigenic variation in *Plasmodium falciparum* in children with severe malaria", MD/PhD Student Conference, virtual, July 2021
- "The EPCR-binding *P. falciparum* erythrocyte protein 1 (PfEMP1) domains of Domain Cassette 8 are associated with attention deficits in Ugandan children with cerebral malaria", Poster session at ASTMH National Meeting 2020, virtual, November 2020

- “Elevated levels of soluble ST2 are associated with cognitive impairment in Ugandan children with cerebral malaria”, poster session at ASTMH National Meeting 2019, National Harbor, MD, November 2019

Meetings attended

- American Society of Tropical Medicine and Hygiene National Meeting 2021, National Harbor, MD, November 17th-21st, 2021
- MD/PhD National Student Conference 2021, virtual, July 9th-11th, 2021
- American Society of Tropical Medicine and Hygiene National Meeting 2020, virtual, November 15th-19th, 2020
- American Society of Tropical Medicine and Hygiene National Meeting 2019, National Harbor, MD, November 20th-24th, 2019
- Global Excellence Research Symposium 1, PfEMP1-From var genes to malaria vaccines, Center for Medical Parasitology, University of Copenhagen, Copenhagen, Denmark, June 27th-29th, 2018

Publications

- Fernander, E. M. et. al. Elevated plasma ST2 levels are associated with neuronal injury and neurocognitive impairment in cerebral malaria. (manuscript submitted for publication). *Pathogens and Immunity*. 2022.
- Fernander, E. M. et. al. ICAM-1-binding and C-terminal domains of PfEMP1 are elevated in children with cerebral malaria compared to severe malarial anemia and related to thrombocytopenia. (manuscript in prep)
- Fernander, E. M. et. al. Antibodies to PfEMP1 and other *P. falciparum* antigens are maintained in children with asymptomatic parasitemia but not in children with severe malaria. (manuscript in prep)

- Fernander, E. M. et. al. EPCR-binding domains of PfEMP1 are expressed in different manifestations of severe malaria and associated with anemia and thrombocytopenia. (manuscript in prep)
- Fernander, E. M. et. al. The presence of antibodies to PfEMP1 CIDR domains is highest in children with severe malarial anemia compared to other forms of severe malaria. (manuscript in prep)

Laboratory Skills

Conventional PCR, RT-qPCR, standard ELISA, and Luminex Multiplex Assays