Identifying Parasite Virulence Factors and Host Genetic and Immunologic Factors that Contribute to Severe Malarial Outcomes in Ugandan Children

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Dedication

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Abstract

Cerebral malaria (CM) and severe malarial anemia (SMA) remain drivers of morbidity and mortality due to *Plasmodium falciparum* infection in children in Sub-Saharan Africa. There are currently no adjunctive therapies for severe malaria (SM), suggesting that we need a better understanding of both host and pathogen factors that contribute to SM. This dissertation attempted to identify both host and parasite factors that contribute to disease severity in malaria, factors that differentiate between CM and SMA, and those associated with mortality and neurocognitive outcomes in CM.

Children between 18 months and 12 years of age, meeting the WHO definition for CM (n=269) or SMA (n=232), were recruited from the Acute Care Unit at Mulago Hospital in Kampala, Uganda. Healthy community children (CC, n=213) in the same age-range were recruited from the neighborhoods and extended households of children with SM. Whole blood was collected at enrollment and was either processed immediately for plasma or was preserved and stored accordingly for future RNA and DNA isolation. We performed genotyping for endothelial protein C receptor (EPCR) polymorphisms, quantitative reverse-transcriptase PCR to estimate transcript levels of *var* genes encoding *P.falciparu*m erythrocyte membrane protein 1 (PfEMP1), and used plasma to quantify a number of cytokines, chemokines, angiogenic growth factors, soluble EPCR and erythropoietin with ELISA-based assays.

The work presented in this dissertation identified both cytoadhesion of infected erythrocytes (IEs) and host immune factors as important contributors to SM pathogenesis. We have shown that polymorphisms associated with less bound and more soluble EPCR are associated with reduced risk of SM; that EPCR-binding PfEMP1 are important in SM and that their transcript levels are higher in CM than SMA; that the immune profile, while quite similar in CM and SMA, is differentiated especially by elevated levels of chemokines and IL-10 in CM. Lastly, our studies on the association of TNF-α and EPO with disease severity in CM highlight the importance of understanding both systemic and local effects of host mediators when considering targets for adjunctive therapies, and the importance of selectively inhibiting the pathogenic effects without compromising the beneficial roles of that target.

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ACT	Artemisinin combination therapy	PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
AP	Asymptomatic parasitemia	*Pf*HRP-2	*P.falciparum* histidine rich protein-2
BBB	Blood brain barrier	PC	Protein C
BCS	Blantyre coma score	aPC	Activated protein C
CC	Community controls	RBC	Red blood cells
CIDR	Cysteine rich-interdomain region	RN	Retinopathy negative
CM	Cerebral malaria	RP	Retinopathy positive
CNS	Central nervous system	Rosettes	Structures of bound infected to uninfected erythrocytes
CSF	Cerebral spinal fluid	SM	Severe malaria
DBL	Duffy binding like domain	SMA	Severe malarial anemia
DC	Domain cassette, conserved tandem arrangements of DBL and CIDR	TACE	Tumor necrosis factor-alpha converting enzyme
ECM	Experimental cerebral malaria	TM	Thrombomodulin
EPCR	Endothelial protein C receptor	UE	Uninfected erythrocyte
sEPCR	Soluble endothelial protein C receptor	Var	Gene encoding PfEMP1
rHuEPO	Recombinant human erythropoietin	VSA	Variant surface antigen
GCS	Glasgow coma score		
IE	Infected erythrocyte		
IRS	Indoor residual spraying		
ITN	Insecticide treated bed nets		

Chapter 1

Introduction

1.1 Malaria overview: epidemiology and life cycle

Although 633 million malaria cases were averted between 2000-2015 due to interventions such as the use of insecticide treated bed nets (ITNs), indoor residual spraying (IRS) and the use of artemisinin combination therapies (ACT)[1], malaria remains an important public health issue, especially in Sub-Saharan Africa (Figure 1.1)[2]. Of the 214 million malaria cases in 2015, 438,000 resulted in death, 70% of which affected children under the age of 5[1]. Malaria was responsible for 5% of deaths in children under 5 worldwide and for 10% of deaths in the same age-group in Sub-Saharan Africa[1,3]. Severe malaria, especially cerebral malaria, remains a driver of malaria mortality in children.

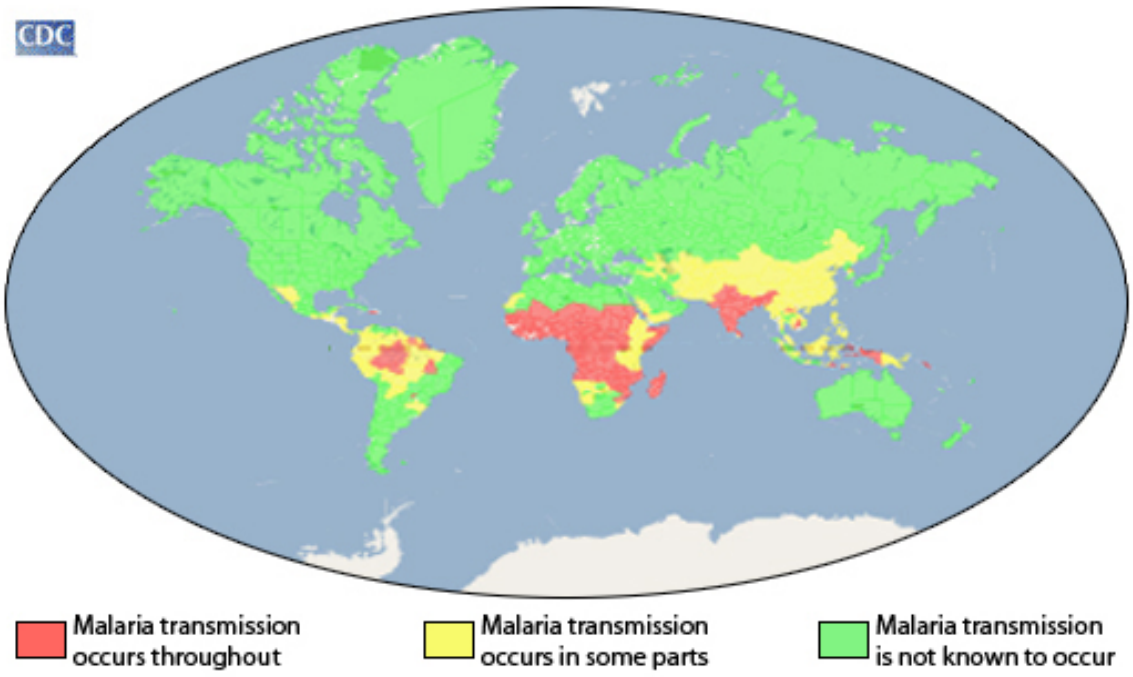


Figure 1.1. Geographical distribution of malaria transmission.

Source: Center for Disease Control and Prevention[2]

Malaria is caused by the Apicomplexa parasite *Plasmodium*. There are five *Plasmodium* species that can infect humans: *P.falciparum*, *P.vivax*, *P.malariae*, *P.ovale* and *P.knowlesi*. However, *P.falciparum* is the deadliest and accounts for the majority of severe malaria cases. An individual is infected when a female *Anopheles* mosquito carrying *P.falciparum* takes a blood meal and injects a small number of sporozoites into the skin of an individual. In minutes, these sporozoites make it to the liver, infect hepatocytes, and initiate intracellular replication. This is known as the liver stage and is clinically silent. This stage lasts for 10-12 days and is followed by the release of merozoites into the blood stream, where they invade red blood cells (RBCs) and initiate the blood stage of the infection. Upon invasion, the merozoites develop either through the asexual or sexual cycle. A small number of infected erythrocytes (IEs), under stimuli that

are not well understood, commit to the sexual cycle and develop into gametocytes, which can be picked up by another mosquito to restart the transmission cycle.

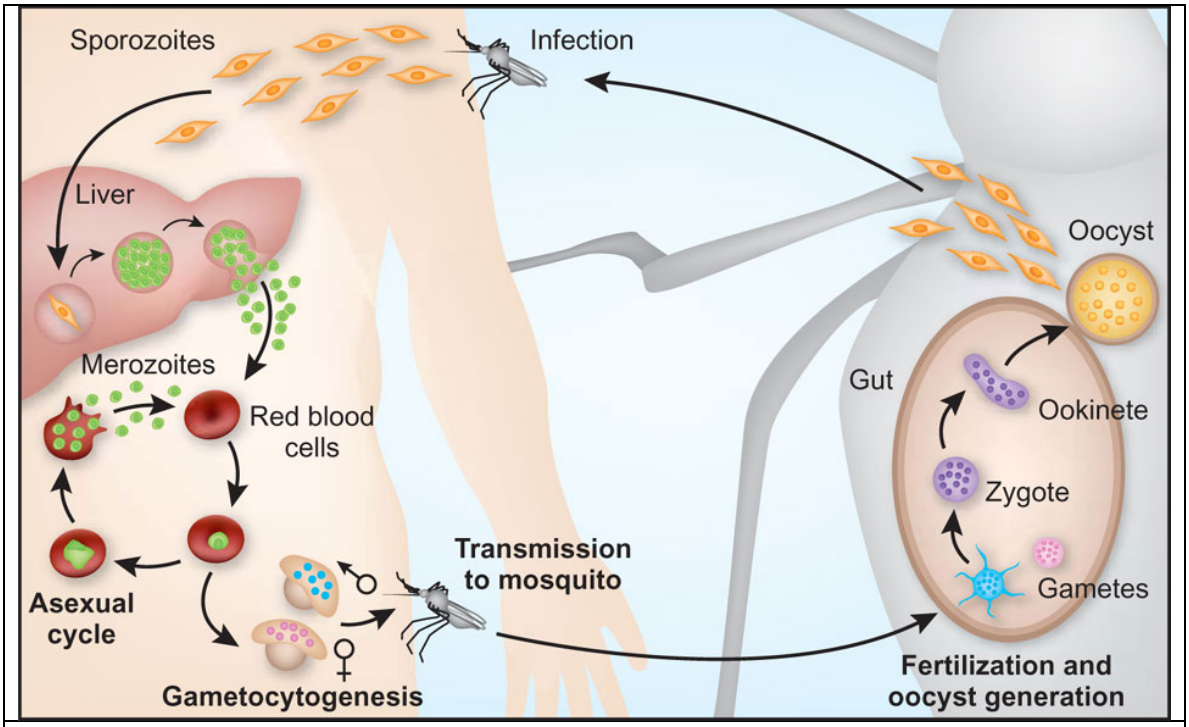


Figure 1.2. *Plasmodium falciparum* development

Source: Pasvol et al. 2010[4]

The majority of merozoites commit to the asexual blood stage, where the parasites develop within the RBC, going from early ring stage to trophozoites and finally to schizonts within 48-hours (Figure 1.2). At the end of the 48-hour cycle, the IEs burst to release more merozoites into the circulation and infect more RBCs. The rupture of the IEs induces periodic waves of fever in patients. The asexual blood stage is the clinically active stage of the disease and is responsible for the spectrum of disease manifestations in malaria from asymptomatic to life-threatening disease.

1.2 Exported *P.falciparum* variant surface proteins

During the asexual blood stage development, *P.falciparum* remodels the RBC to promote its own survival. The parasite develops within the parasitophorous vacuole (PV) and digests hemoglobin as the main source of amino acids. In addition, the parasite sets up a complicated protein transport system to allow the export of its own proteins to the surface of the RBC[5]. These alterations make the IE less flexible and more adhesive, which allows the parasite to evade spleen clearance.

At ~16-hours post-invasion, knob-like structures appear on the surface of the IE (Figure 1.3) and these structures contain a number of variant surface antigens (VSA) including *P.falciparum* erythrocyte membrane protein 1 (PfEMP1), repetitive interspersed family proteins (RIFINs), subtelomeric open reading frame proteins (STEVORs), and surface-associated interspersed gene family proteins (SURFINs)[6,7]. These antigens are encoded by large gene families and are highly polymorphic. PfEMP1 is the most well studied VSA and an important target of the immune response against asexual blood stage antigens[8]. PfEMP1 allows IE binding to various host receptors such as CD36, ICAM-1, chondroitin sulphate A (CSA), complement receptor 1 (CR1) and endothelial protein C receptor (EPCR)[9]. As a result, PfEMP1 is involved in various aspects associated with disease severity and complications in malaria such as antigenic diversity[10-13], cytoadherence to various host-receptors[9], rosetting[14] and evasion of the immune response[15]. The only other VSAs known to have a functional role to date are RIFINs

which were shown to bind to blood group A and mediate binding of IEs to UEs in structures known as rosettes, which are important in severe disease[16].

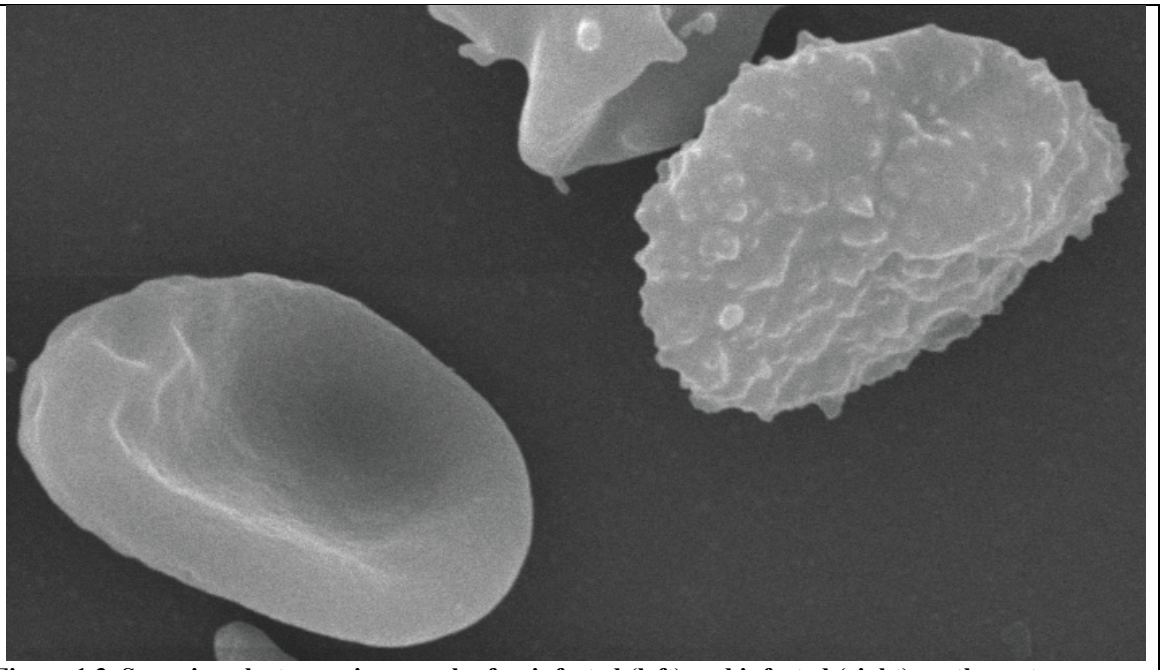


Figure 1.3. Scanning electron micrograph of uninfected (left) and infected (right) erythrocyte

Source: Spillman 2015[7]

1.3 Severe malaria manifestations

Severe malarial episodes account for only 1-2% of *P.falciparum* infections[17] but have an 18.5% mortality rate in adults[18] and 9.7% in children[19]. In low transmission areas adults are more commonly affected by severe malaria, whereas in stable transmission settings such as Sub-Saharan Africa, severe malaria is mainly a childhood disease. The most common forms of severe malaria in children are cerebral malaria, severe malarial anemia and metabolic acidosis[17]. These complications can manifest separately or overlap.

Pediatric severe malaria is characterized by sequestration of IEs in various organs; local and systemic production of pro- and anti-inflammatory cytokines and chemokines in response to parasite products, release of parasite toxins, and endothelial activation and dysfunction[17,20]. How these factors, combined with host and parasite genetic factors, influence the type of complications seen in pediatric severe malaria is not entirely clear.

1.4 Cerebral malaria: diagnosis, treatment and etiology

The World Health Organization (WHO) defines CM as impaired consciousness in the context of *P.falciparum* infection and no other identifiable cause for the coma. Impaired consciousness is determined as a *Blantyre coma score* (BCS) of less than 3 in children under the age of 5 and a *Glasgow coma score* (GCS) of less than 11 in children older than 5 years old[20]. GCS and BCS consist of 3 categories: eye, verbal and motor response. While GCS can be given a score of 1-6 for each of these categories, where 1 is the least responsive, BCS is given a score from 0-2 in each category with 0 being the least responsive. In most studies, including ours, the CM definition is restricted to children who remain unconscious at least one hour after convulsions and after receiving a bolus of glucose. This is to avoid enrolling children with impaired consciousness due to febrile convulsions or hypoglycemia. Cerebrospinal fluid (CSF) gram stain and cultures are performed on CM children to rule out bacterial meningitis.

CM is responsible for roughly 9% of malaria admissions[21] and has a mortality rate of 13-15% [22-25]. Survivors of CM are at high risk of short-[26] and long-term[27] neurocognitive

impairment. To date, no adjunctive treatment for CM in humans has decreased mortality or neurologic complications[28,29]. As a result, patients who are identified with CM are started on antimalarials and treated for their symptoms such as seizures, dehydration, hypotension or lactic acidosis[20].

In CM, sequestration of IEs, leukocytes and platelets to the blood-brain barrier (BBB) endothelium, combined with an imbalanced immune response and endothelium activation are thought to lead to BBB dysfunction and adverse clinical outcomes[17,30-33]. However the relative contribution of each of these factors and the order of events are not well understood, because of the limitations of human studies and the lack of a mouse model that reproduces all aspects of human CM[34]. Moreover, the small sample size in studies looking to understand CM pathogenesis has made it hard to identify host and parasite factors associated specifically with mortality or neurologic outcomes in CM.

The majority of our knowledge on important factors in human CM comes from autopsies. Despite the pathologic diversity that is observed in fatal CM pediatric patients, one common theme is sequestration of IEs in the BBB microvasculature[35-37]. IE binding has been associated with endothelial activation, BBB breakdown, hemorrhages, fibrin accumulation, as well as brain inflammation, demyelination and axonal damage[23,36,37]. The ability of IEs to sequester has been linked to parasite variant surface antigens[38,39] of which *P.falciparum* erythrocyte membrane protein 1 (PfEMP1) is the most studied. However, there is no direct proof that IE sequestration is sufficient to cause CM or death

in these patients. In addition, cytoadhesion of *P.falciparum* infected erythrocytes occurs in asymptomatic and mild malaria also, suggesting that parasite expression of certain PfEMP1 proteins or other parasite virulence factors could lead to CM. Unfortunately, this cannot be studied in the animal model of CM since the parasites used in these models do not express a homologue of PfEMP1.

Despite the limitations of CM studies, both experimental CM models (ECM) and clinical studies agree on the importance of the host's immune response in both controlling the infection and contributing to the pathogenesis of CM. An imbalanced pro- and anti-inflammatory response is characteristic of childhood severe malaria with elevated IFN-γ, TNF-α, IL-6, IL-10, IL-1β, IL-1ra [40-44], elevated levels of IL-8, IP-10 and reduced levels of RANTES as compared to uncomplicated or mild malaria[43-45]. The importance of the immune response in human CM is also indicated by recruitment of neutrophils, other leukocytes, and platelets to the sites of endothelium activation in the brain of fatal CM patients[46,47]. A role for the pathogenic aspect of the immune response is also seen in ECM where nude mice or mice deficient in α/β TCR, IFN-γ or IFN-γ receptor are resistant to CM[32]. While ECM is mostly an immunologically driven phenomenon, that does not seem to be the case in human CM. The use of general anti-inflammatory therapies (dexamethasone) or TNF-α neutralizing antibodies have not been successful as adjunctive therapies in CM[28], suggesting that a better understanding of both systemic and local immune mediators specifically associated with coma and mortality is needed.

Markers of endothelial activation and barrier damage are commonly observed in CM patients[17,31,33]. As mentioned above, IE sequestration has been associated with thrombosis and hemorrhages[36,37]. In addition, IE binding also triggers endothelial activation[48-50] and coagulation processes by inducing tissue factor and activating thrombin[51]. Moreover, PfEMP1 binding to endothelial protein C receptor (EPCR) inhibits the activation of protein C (aPC) and promotes endothelial inflammation and increased endothelial barrier permeability[52-56]. Inflammation, as well as consumption of nitric oxide by free hemoglobin and hemolysis contribute further to endothelium activation, as indicated by elevated levels of soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), angiopoietin 2 (Ang2) and von Willebrand Factor (VWF) in CM patients[31]. Despite systemic inflammation and the ability of IEs to bind to various microvasculature beds, it is not clear why there is only brain pathology in CM patients. Understanding host and parasite factors that make the brain microvasculature more sensitized to the infection could highlight important novel factors to target with adjunctive therapies.

1.5 Severe malarial anemia: diagnosis, treatment and etiology

SMA is defined by WHO guidelines as hemoglobin levels ≤5g/dL with detectable *P.falciparum* in peripheral blood[20]. Normal hemoglobin levels for children are 11-13g/dL highlighting the severity of anemia in the children with SMA. Severe anemia in African children is associated not only with malaria but also HIV-1 infection, bacteremia, hookworm infections, vitamin A and B_{12} deficiencies[57]. Therefore, in many studies,

including ours, the enrollment into the SMA group is limited to children with no known chronic illness or malnutrition that required medical care or hospitalization in the past or any known developmental delay.

SMA children tend to be younger than children with CM and the risk of SMA peaks in the first year of life in high transmission areas and in two year-olds in moderate and low transmission areas[58]. SMA poses a substantial burden in Sub-Saharan Africa causing about 20% of all *P.falciparum* hospitalizations[21]. In Tanzania, SMA mortality was 8.9%[58]. However, in urban settings such Kampala, where there is easier access to blood transfusions, the mortality is low. There have not been many adjunctive treatments tried in SMA patients, since a rapid blood transfusion in combination with antimalarials usually reverses the severe anemia episode and reduces mortality. In addition, SMA patients are treated for their other presenting symptoms such as lactic acidosis or deep breathing whenever present[20].

Severe anemia in malaria is thought to be multifactorial and a result of destruction of infected (IEs) and uninfected erythrocytes (UEs), dyserythropoiesis, and suppression of erythropoiesis. However, the relative contribution of these factors to SMA and disease outcomes, as well as the contribution of other host and parasite factors specifically to SMA are not well understood.

The destruction of IEs at the end of the 48-hour cycle of parasite development or as a result of phagocytosis contributes to the reduction of hematocrit during a malarial infection. However, peripheral parasite density or total parasite load is not always associated with malarial anemia, and often the highest parasite loads are observed in uncomplicated or mild forms of malaria[59]. In animal models of SMA, which rely entirely on parasitemia to drive anemia, the levels of parasitemia needed to reach these low hemoglobin values are way higher than any parasitemia observed in patients[32]. A birth-cohort study in Tanzania showed that a major drop in hemoglobin levels, sometimes more than 3g/dl occurred during the acute malaria infection[59]. Mathematical models show that parasitemia alone cannot explain this drop, and that for every single IE destroyed there needs to be lysis of ~8.5 uninfected erythrocytes[60] . These UEs are targeted for destruction due to changes that occur on their surface such as phosphotidyl serine externalization, oxidation of the plasma membrane and reduced deformability leading to elimination via autoantibodies, immune complexes and antibody-specific clearance[32].

Dyserythropoiesis and inhibition of erythropoiesis are other factors that are thought to contribute to SMA. Dyserythropoiesis indications are seen from bone marrow of adults and children with malarial anemia where erythrophagocytosis, hemozoin deposition, parenchymal damage of bone marrow and a reduced rate of erythropoietic progenitor proliferation are observed[61,62]. Moreover, indicators of reduced erythropoiesis, such as reduced reticulocyte production index or red cell distribution width, are seen in SMA

children[63,64]. The reduction in erythropoiesis does not seem to be due to inappropriate elevation of erythropoietin (EPO), but due to reduced response to EPO itself [63]. Inflammation and oxidative stress cascades can reduce responsiveness of erythroid precursors to EPO and inhibit erythropoiesis[65]. In addition TNF-α, IFN-γ and IL-1 can directly inhibit the proliferation and differentiation of erythroid precursors[66]. *P.falciparum* hemozoin (byproduct of hemoglobin digestion) has also been associated with reduced proliferation and maturation of erythroid precursors[65,67,68]. The role of parasite virulence factors such PfEMP1 are not well understood in SMA. In addition, there is not a clear picture of host immune factors that differentiate SMA from other forms of severe disease such as CM.

1.6 Gaps in knowledge

CM and SMA drive morbidity and mortality caused by *P.falciparum* infection in children in Sub-Saharan Africa. Pediatric severe malaria (SM) is characterized by sequestration of IEs in various organs, imbalanced immune response, release of parasite toxins and endothelial dysfunction[17,20]. How these factors, combined with host and parasite genetic factors, influence the type of complications seen in pediatric SM is not clear. Moreover, the small sample size in studies of CM pathogenesis has made it hard to identify host and parasite factors associated specifically with mortality or neurologic outcomes in CM.

In this study we attempted to identify parasite virulence, host genetic or immune factors that are important in both CM and SMA as compared to healthy community children

(CC), and factors that differentiate between CM and SMA. More specifically we tackled a number of knowledge gaps:

1. Cytoadhesion of *P.falciparum* IEs occurs in asymptomatic and mild malaria also. Therefore, we asked whether certain PfEMP1 variants are associated specifically with CM, SMA, and with various disease outcomes in CM.
2. Despite the ability of IEs to bind to various microvasculature beds, it is not clear why patients with CM manifest mainly with brain pathology. We try to understand whether a specific parasite protein to host receptor combination could explain the specific brain manifestation in CM.
3. The use of general anti-inflammatory therapies or TNF-α neutralizing antibodies have not been successful as adjunctive therapies in CM[28]. Here, we study the role of local TNF-α in CM patients, and obtain a more complete picture of the immune mediators associated with CM specifically and its severe outcomes.
4. The role of PfEMP1 and the involvement of host immune factors specifically in SMA are not well understood. We made use of the large SMA group in our study to start asking some of these questions.

Chapter 2

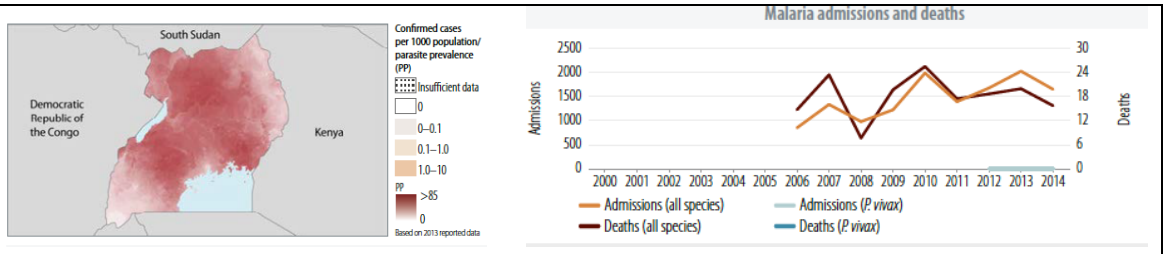
Study description

2.1 Study area location

The current study (CMR01) was conducted at Mulago National Referral and Teaching Hospital in Kampala, Uganda, which serves as the district hospital for Kampala and the surrounding areas. Uganda is located in east Africa, bordered to the east by Kenya, to the north by Sudan, to the south by Tanzania, to the southwest by Rwanda and to the west by the Democratic republic of Congo. In 2015, Uganda had a population of 39,032,000 with a gross national income of $1 per capita[69]. Agriculture is the most important sector of the economy, which employs ~80% of the population according to 1999 estimates[70]. Uganda has a tropical climate, rainy with two short dry seasons (December to February and June to August)[70]. Kampala is the capital of Uganda and the largest city in the country.

According to 2013 data, Uganda had a prevalence of more than 1 confirmed case of malaria per 1000 population (Figure 2.1a), 100% of them being due to *P.falciparum*. There were 3,631,939 reported malarial cases in 2013 with an estimate of 4,400,000–12,000,000 cases[1]. In 2013, there were 5,921 reported deaths from malaria with an estimated 5,300-17,000 deaths[1]. Numbers of admissions and deaths have remained mostly constant in Uganda from 2009-2014 (Figure 2.1b). There were fewer admissions between 2006-2009 and a sharp decrease in deaths in 2008. This decrease could be due to a number of prevention strategies and policies implemented around this time in Uganda

such as distribution of insecticide treated bed nets (ITN) free of charge since 2006, recommendation of indoor residual spraying (IRS) since 2005, as well as implementation of artemisinin combination therapies (ACT) as first line of treatment in Uganda in 2005[1]. The apparent rise in admissions and deaths between 2009-2010 could also be attributed to increased access to health care or better recording of cases in the country. Of note, hospital admission in Uganda is reserved for more complicated cases of malaria rather than for uncomplicated malaria, which is normally seen and treated in the outpatient clinics.



a. b.

Figure 2.1. a) Epidemiological profile of Uganda and b) number of admission and deaths from all Plasmodium species in Uganda

Source: World Health Organization Malaria Report 2015[1]

2.2 Study design

Ethics statement

The study was reviewed and approved by the Ugandan National Council for Science and Technology (UNCST), the Makerere University School of Medicine Research and Ethics

Committee and the University of Minnesota Institutional Review Board. Written informed consent was obtained from parents or guardians of study participants.

Selection of study population: inclusion and exclusion criteria

The CMR01 study was performed from 2008-2013 and enrolled children with cerebral malaria (CM, n=269), severe malarial anemia (SMA, n=232) and community children (CC, n=213). Children between 18 months and 12 years of age, meeting the WHO definition for CM or SMA, were recruited from the Acute Care Unit at Mulago Hospital.

Cerebral malaria was defined as: 1) coma (Blantyre Coma Score [BCS] ≤2); 2) evidence of *Plasmodium falciparum* infection on a blood smear; 3) no other known cause of coma; 4) no response to glucose one hour after administration if hypoglycemic, and 5) coma persisted at least one hour after administration of first line anticonvulsants if anticonvulsants were given. Exclusion criteria for CM included white blood cell count >5 cells/μl in cerebral spinal fluid (CSF), identification of bacteria in the CSF by Gram stain and/or culture positive for CSF. A lumbar puncture (LP) to rule out bacterial meningitis is standard of care for all children with suspected CM and was performed in all children with CM unless it was clinically contraindicated or the parents or guardians of the child would not agree to having an LP performed.

Severe malarial anemia was defined as the presence of *Plasmodium falciparum* on a blood smear in children with hemoglobin ≤ 5 g/dL. Children were excluded from SMA if

they had any signs of impaired consciousness (GCS<15 for children older than 5 years old and BCS<5 for children ≤ 5 years old) or had repeated seizures prior to admission. Some children presented with both CM and SMA (22% of CM): these children were assigned to the CM group at enrollment.

Community children were recruited from the extended family or nearby neighborhood of children with CM or SMA. Eligible CC were age 18 months to 12 years and currently healthy. CC were matched by age group, not to an individual level, but using the age distribution from the first 45 children with CM or SMA enrolled. CC were excluded if they had an active illness or had an illness in the past 4 weeks requiring medical attention. A blood smear was prepared from children with CC at the time of enrollment and those with any density of *P.falciparum* on smear are indicated here as asymptomatic parasitemic (AP). AP were sent home with antimalarials.

Other exclusion criteria for all children included: 1) known chronic illness requiring medical care; 2) known developmental delay; or 3) prior history of coma, head trauma, cerebral palsy, or hospitalization for malnutrition.

All children were followed up for 2 years, and were asked to return to Mulago hospital for any illness. Over 2 years of follow-up, 14 children with CM, 26 children with SMA, and 3 CC were admitted to the hospital, and 13 children with CM, 18 children with SMA and 1 CC were admitted to the hospital with severe malaria. These data are only for

children that were not part of a sub-study looking at the role of iron supplementation in long-term severe malaria outcomes (CM (n=164), SMA (n=155), CC (n=132)).

Malarial retinopathy diagnosis

Children were assessed for malarial retinopathy by indirect and direct ophthalmoscopy. Ophthalmoscopy was done by medical officers in all CM patients on admission, unless the patient was too clinically unstable, and was repeated every 24 hours while they remained comatose. Before each examination, pupils were dilated with sequential instillation of cyclopentolate 1% and tropicamide 1%. Using a binocular indirect ophthalmoscope, an eye exam was performed 30-60 minutes later. Direct ophthalmoscopy was also performed in many though not all patients. The medical officers were trained by an ophthalmologist experienced in the evaluation of malarial retinopathy. The study investigators and the ophthalmologist performed repeat training and assessment of accuracy of the study medical officers one year into the study.

Clinical treatment

All children underwent a medical history and physical examination. Children with severe malaria were managed according to the Ugandan Ministry of Health treatment guidelines at the time, which included quinine treatment until the patient was alert and then oral quinine for hospitalized patients; and artemether combination therapy, usually with artemether-lumefantrine, for outpatients. Since there are currently no recommended

adjunctive treatments for severe malaria, these children received supportive and symptomatic care as needed, which in most cases for CM included anticonvulsants. All children with SMA received a blood transfusion. The blood units provided to hospitals in Uganda are checked for HIV-1, syphilis, hepatitis B and C[71].

Standard laboratory and clinical testing

Thick and thin blood smears were prepared and analyzed by Giemsa staining according to a standard protocol[72]. Parasite density was estimated based on two independent readers, and whenever the two readings were >20% different from each other, a third reader counted independently. The final parasite density was estimated as the median of the three readings, or the average of the two initial readings. However, parasite density gives only an estimation of the parasites that are circulating in a patient. Considering that *P.falciparum* IEs can sequester in the microsvaculature of various organs, we also measured the levels of *P.falciparum* histidine rich protein-2 (*Pf*HRP-2), a parasite protein released in the circulation upon bursting of IEs at the end of the 48-hour asexual cycle. *Pf*HRP-2 quantification was performed using the Malaria Ag CELISA (Cellabs, Brookvale, Australia), and sequestered parasite biomass was calculated as previously described [73]. Briefly, sequestered parasite biomass is estimated by subtracting circulating parasite biomass (based on parasite density and weight of each child) from total parasite biomass (based on levels of *Pf*HRP-2, weight and hematocrit of each child).

A complete blood count (CBC) was performed on all enrolled children using a COULTER® Ac·T™ 5diff CP (Cap Pierce) hematology analyzer. Blood glucose, hemoglobin and lactate levels were estimated immediately upon admission by hand held devices (glucometer, hemocontrol and the lactate monitor, respectively), which require only a couple of drops of blood. These results were then included in the study after consent was obtained from the caregiver. HIV-1 testing was performed whenever the parents or guardians of the child approved. Three immunochromatographic tests (Determine, STAT-PAK and Uni-Gold) were used and the decision was made based on the Uganda National HIV testing algorithm. Stool was examined by microscopy for the presence of red blood cells, motile trophozoites or protozoa, protozoan cysts, and helminthic ova or larva. Blood culture was initially performed with the Bactec 9050 Blood Culture System and for the negative samples they were further examined by microscopy, blood agar or chocolate agar cultures to completely rule out any infections.

Specimen collection and storage

Blood was collected in EDTA tubes at enrollment and at 6-month and 12-month follow-up visits. Plasma was prepared from whole blood tubes and was separated in 1-1.5ml aliquots, which were stored at -80 ^{o}C. Another tube of whole blood was collected at 24 hours from patients with severe malaria. Those tubes were also stored -80^{o}C. For all patients, we also collected dried blood spots at enrollment, 6-month, and 12-month follow-up, which were stored at 4^{o}C. Specific methodology and sample collection for the

work presented in each chapter are highlighted in the methods section of the respective chapters.

Neurologic assessment and follow-up

A detailed neurologic examination was performed at discharge and six months later. A neurologic deficit was defined as the presence of motor deficits, ataxia, movement disorder, behavior, or speech or visual disorders, in a child with no known prior deficits. Children that had only hypereflexia were not considered to have a neurologic deficit.

Cognitive assessment and follow-up

Children had cognitive assessment a week after discharge (or at enrollment for CC) and then at 6 and 12 months after enrollment. For children younger than 5 years old, the Mullen Scales of Early Learning[74] were used to measure cognitive ability. Scores from fine motor, visual reception, receptive language, and expressive language scales were summed to give the early learning composite score, a measure of overall cognitive ability. Attention was assessed using the Early Childhood Vigilance Test (ECVT)[75], in which a child was required to focus his/her gaze on cartoons screened on a computer for about 7 minutes. The measure of attention is the percent time the child spent gazing at the screen. Associative memory was assessed using the Color Object Association Test[76], in which children are required to associate toys with specific color-coded boxes and scored on the total number of toys placed in the correct boxes. In children 5 years and older, the

Kaufman Assessment Battery for Children (second edition) was used to measure overall cognitive ability[77]. Lauria's model was used to obtain a composite score including sequential processing, simultaneous processing, learning ability and planning ability. Attention in these children was assessed using the Test of Variables of Attention (TOVA) to measure attention and impulse control in four main areas: response time variability, response time, impulse control (commission errors), and inattention (omission errors)[78]. Neuropsychology testers were blinded to the study groups (CM, SMA, or CC) being tested.

2.3 Dissertation focus

There are currently no adjunctive therapies for severe malaria[28]. Adjunctive therapies, which have mostly targeted one host factor at a time have not shown success in controlled clinical trials[28], suggesting that we need a better understanding of both host and pathogen factors that contribute to severe malaria, and that successful adjunctive therapies may need to target both host and pathogen pathways simultaneously. This dissertation takes a more comprehensive approach in identifying both host and parasite factors that contribute to disease severity in malaria. More importantly, the sample size and study design allow this work to identify factors that specifically differentiate between cerebral malaria and severe malarial anemia, and to identify markers of mortality and neurocognitive deficits in cerebral malaria.

Chapter 3 *The endothelial protein C receptor rs867186-GG genotype is associated with increased soluble EPCR and could mediate protection against severe malaria.* This chapter determines the prevalence of a known functional polymorphisms in the gene encoding the endothelial protein C receptor (EPCR), a host receptor for infected erythrocytes, in both disease groups and community controls. This chapter also looks at the levels of soluble EPCR at enrollment and 6-months follow-up in order to understand the availability and role of EPCR in malaria as compared to other infectious and inflammatory processes without malaria.

Chapter 4 *EPCR-binding PfEMP1 variants differ in variant type and expression in cerebral malaria and severe malarial anemia.* This chapter assesses transcript abundance of *P.falciparum* erythrocyte membrane protein 1 (PfEMP1), an important parasite virulence factor, and determines whether the extent of PfEMP1 expression contributes to disease manifestation in severe malaria.

Chapter 5 *Cerebrospinal fluid tumor necrosis factor-alpha levels are associated with coma duration and acute and long-term neurologic deficits in Ugandan children with cerebral malaria.* This chapter investigates the role of systemic and local TNF-α in CM, and emphasizes the need to further study and target local inflammatory pathways in the central nervous system.

Chapter 6 *Systemic immunologic markers of malarial disease severity in Ugandan children*. This chapter assesses the levels of 18 different cytokines, chemokines and growth factors in severe malaria patients with the goal of identifying immune response pathways that differentiate between cerebral malaria and severe malarial anemia. In addition, this chapter looks at whether certain cytokines or chemokines are associated specifically with mortality and neurologic deficits in children with CM.

Chapter 7 *High plasma erythropoietin levels are associated with prolonged coma duration and increased mortality in children with cerebral malaria.* This chapter suggests that recombinant erythropoietin (EPO), which has been tried as an adjunctive therapy in small phase I trial in CM, may not be safe in children with CM. By looking at the association of EPO with coma and mortality in CM, we emphasize that host factors with multifunctional roles systemically and locally in the CNS do not represent optimal targets for adjunctive therapies.

Chapter 3

The endothelial protein C receptor rs867186-GG genotype is associated with increased soluble EPCR and could mediate protection against severe malaria[79]

3.1 Objectives

- ✓ Determine the prevalence of rs867186-G, an EPCR polymorphism associated with less bound and more sEPCR, in the Ugandan population with and without severe malaria
- ✓ Evaluate the association of genotype with phenotype for rs867186-G
- ✓ Determine the availability and role of EPCR in severe malaria as compared to other infectious and inflammatory processes

3.2 Introduction

Binding of infected erythrocytes (IEs) to host endothelium via *P.falciparum* erythrocyte membrane protein1 (PfEMP1) is an important driver of CM as it prevents parasite clearance, and is associated with increased local vasoconstriction, hypoxia and acidosis [23,35-37,46,80]. Binding of IEs and sequestration are also important in SMA [81,82] together with other factors such as erythrocyte lysis and suppression of hematopoiesis [65]. PfEMP1 can

bind to various host-receptors [83,84] and recently, PfEMP1 variants associated with severe malaria [85-87] were shown to bind EPCR [52], suggesting an important role for this receptor in pathogenesis of severe malaria.

EPCR regulates coagulation by enhancing activation of protein C (PC) [88-90], and has cytoprotective functions when bound to activated PC (aPC) (Figure 3.1) [91]. EPCR is cleaved into its soluble form (sEPCR) by tumor necrosis factor-α converting enzyme (TACE) [92]. TACE's activity is increased, by TNF-α, IL-1β and thrombin generation [93]. EPCR gene (*PROCR*) variations can also affect sEPCR levels. The rs867186-G variant in exon 4 of *PROCR* causes a serine-to-glycine substitution in the transmembrane region, making bound EPCR more susceptible to shedding [94,95].

The evidence that PfEMP1 binds to EPCR at the binding site of PC and aPC [52,54], reducing the production and cytoprotective effects of aPC (Figure 3.1) [55] makes EPCR a potential important link between sequestration, coagulation defects and endothelial activation in severe malaria. sEPCR can bind to IEs and inhibit their adhesion to human brain microvasculature endothelial cells [55]. Reduced EPCR was observed in autopsy samples from pediatric CM patients, which coincided with sequestration of IEs and fibrin accumulation [96]. Also, a study from Thailand found that rs867186-GG genotype was protective against severe malaria [97].

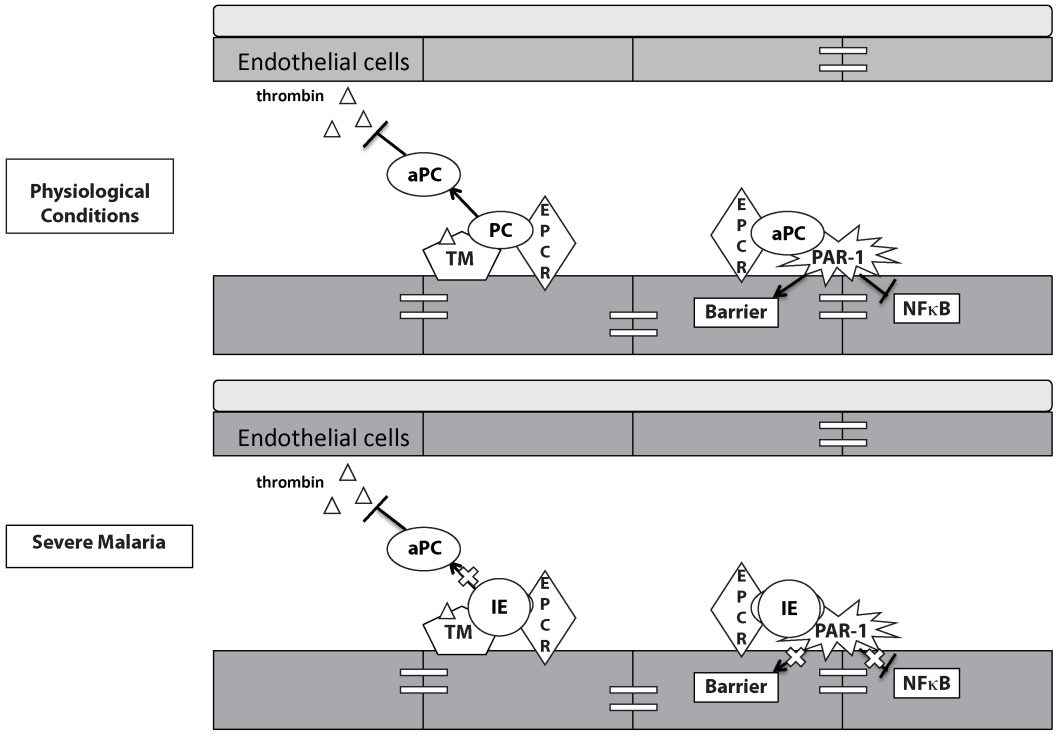


Figure 3.1. Physiological function of EPCR and the effect of binding of infected erythrocytes on these functions in the context of a *P.falciparum* infection.

EPCR promotes activation of protein C (PC) into activated PC (aPC) by thrombomodulin (TM), which is important in controlling thrombin production. In addition, binding of EPCR to aPC promotes cleaving of protease activated receptor-1 (PAR-1), resulting in endothelial cytoprotective and anti-inflammatory functions. Binding of infected erythrocytes (IEs) to EPCR at the binding site of PC and aPC inhibits these physiological functions of EPCR.

However, other studies, including studies in African children showing no association between the rs867186-G variant and severe disease [98,99] and conflicting studies showing an increase [100] or decrease [99] in sEPCR levels in severe malaria suggest that the

contributions of the rs867186-GG genotype and sEPCR levels in severe malaria are still unclear. These unresolved questions about the association of severe malaria with the rs867186-G variant and changes in sEPCR levels led us to investigate these associations in a cohort of Ugandan children with severe malaria (cerebral malaria or severe malarial anemia), uncomplicated malaria, and otherwise healthy Ugandan children.

3.3 Methods

Study design

In addition to the main study described in section 2.2, the work presented in this chapter also includes samples obtained from a smaller study that preceded the CMR01. This smaller study was performed during 2003-2005 and enrolled children with cerebral malaria (CM), uncomplicated malaria (UM) and community controls (CC) between the ages of 3-12 years old. This study was reviewed and approved by the Ugandan National Council for Science and Technology (UNCST), the Makerere University School of Medicine Research and Ethics Committee and Case Western Reserve University. Written informed consent was obtained from parents or guardians of study participants.

CM and CC were enrolled as described in section 2.2. Children with UM (fever, *P. falciparum* on blood smear, no criteria for severe malaria, not admitted) were enrolled from the Mulago Hospital outpatient malaria clinic.

Importantly, none of the community children were readmitted for severe malaria in the 6-month follow-up period, while 5.3% of the children with severe malaria were readmitted for severe malaria, demonstrating that the CC group did have protection against severe malaria as compared to the severe malaria group.

PROCR genotyping was done on samples with sufficient DNA quality and volume (551 SM (325 CM and 226 SMA), 71 UM, 172 CC). Plasma sEPCR levels were tested in children at baseline and 6-month follow-up if a sufficient volume was collected (Figure 3.1). Cerebrospinal fluid (CSF) sEPCR levels were measured in CM children who had adequate CSF volume for testing (n=76). Control CSF samples were obtained from North American children successfully treated for prior leukemia who had CSF obtained after treatment to rule out return of malignancy (ruled out in all).

DNA extraction and *PROCR* rs867186 genotyping

Genomic DNA was isolated from whole blood samples of severe malaria patients using the DNeasy Blood and tissue kit (Qiagen, Valencia, CA) and from filter papers for UM patients and CC using QIAamp 96 DNA Blood Kit (Qiagen, Valencia, CA). E4F (5'-GCTTCAGTCAGTTGGTAAAC-3') and E4R (5'- TCTGGCTTCACAGTGAGCTG-3')[101] were used to amplify the region of the *PROCR* gene containing rs867186 and rs9574. Genotyping of rs867186 and rs9574 was done by initially amplifying the region of interest using HotStar Taq plus master mix (Qiagen, Valencia, CA), followed by Sanger Sequencing (ABI 3730xl, University of Minnesota Genomics Center).

Laboratory testing

Soluble EPCR in plasma, serum and CSF were quantified using Asserachrom® sEPCR immunoassay (Stago, France). Plasma and serum were diluted according to manufacturer's instructions (1:51); CSF was diluted 1:2. The Asserachrom® sEPCR immunoassay uses antibodies directed against the PC binding site of sEPCR.

Plasma soluble intercellular adhesion molecule-1 (sICAM-1), vascular cellular adhesion molecule-1 (sVCAM-1), and TNF-α levels were measured by magnetic cytometric bead assay (R&D Systems, Minneapolis, MN and EMD-Millipore, Billerica, MA, respectively) according to manufacturers' instructions with a BioPlex-200 system (Bio-Rad, Hercules, CA). Plasma angiopoietin-2 (Ang-2) and von Willebrand Factor (VWF) levels were quantified using the human angiopoietin 2 DUO ELISA kit (R&D Systems, Minneapolis, MN) and REAADS von Willebrand Factor activity ELISA kit (Corgenix, Broomfield, CO), respectively. Plasma and CSF albumin were quantified by the Advanced Research and Diagnostic Laboratory at the University of Minnesota.

Statistical analysis

Fisher's exact test for 2x2 tables was used to compare prevalence of *PROCR* variants between the control and malaria groups, when considering a dominant or recessive

model. Fisher's exact test for 2x3 tables was used for the additive model. To control for multiple comparisons, only $P<0.008$ was considered statistically significant in both tests.

Measures with skewed distributions, including sEPCR levels, were replaced by their common logs (log to base 10) for ANOVA or regression analyses. sEPCR levels were compared between groups or between genotypes using ANOVA, followed by Tukey's post-hoc test to control for multiple comparisons. Clinical and laboratory findings for children in the different disease groups were compared using the chi-squared test if categorical and if continuous, ANOVA followed by Tukey's post-hoc test. Regression analyses used linear regression for continuous outcomes and logistic regression for categorical outcomes. All regression analyses were adjusted for age.

3.4 Results

Baseline characteristics

Of the 794 children who were genotyped for rs867186, sEPCR levels at enrollment were quantified in 484 SM (277 CM and 207 SMA), 38 UM and 110 CC (see Methods, Figure 3.2). Children with SM were younger than children with UM or CC (Table 3.1). sEPCR level was associated with age in children with SM (Spearman's rho -0.10, $P=0.03$) but not in children with UM or healthy controls ($P>0.64$ for all). sEPCR level was not associated with sex in any group ($P>0.17$ for all).

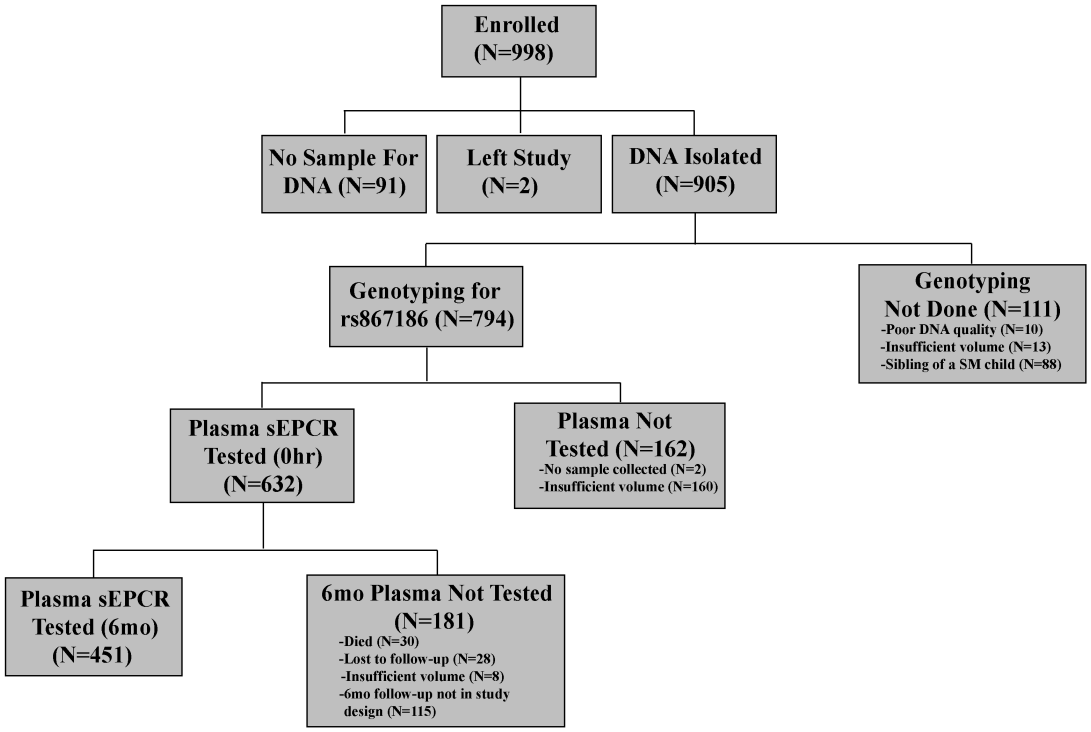


Figure 3.2. Study profile.

Abbreviations: sEPCR, soluble endothelial protein C receptor; SM, severe malaria

Table 3.1. Age and sex of children with severe or uncomplicated malaria and community children

	Severe Malaria (SM, n=551)	Uncomplicated Malaria (UM, n=71)	Community Children (CC, n=172)	P[a]
Age (months) median (IQR)	41.7 (28.1-59.3)	78.0 (58.6-108)	55.5 (36.1-84.9)	<0.0001[b]
Sex, male n (%)	329 (59.7)	31 (43.7)	85 (49.4)	0.005[c]
P. falciparum peripheral blood density (parasites/µl)[e], median (IQR)	39660 (9900-191380)	33420 (7860-116580)	0 (0-0)	<0.0001[d]

[a]ANOVA, Tukey post-hoc test adjustment for multiple comparisons with log10 transformed values for variables with no normal distribution. Chi-squared test was used for categorical variables, with $P<0.017$ considered significant to control for multiple comparisons.
[b]In post-hoc testing, all pairs of groups differ significantly
[c]SM significantly different from UM
[d]In post-hoc testing, CC differ from SM and UM
[e] n=540 for SM, n=69 for UM and n=131 for CC

Prevalence of rs867186-G EPCR variant in children with severe malaria, uncomplicated malaria and healthy community children

The prevalence of rs867186-G was higher in healthy controls than severe malaria children in an additive model (P=0.006, Table 3.2). A recessive model looking at the prevalence of GG genotype vs. AG+AA showed that healthy community children had a higher prevalence of the GG genotype (4.1%) compared to children with SM (0.6%, P=0.002). The GG genotype was associated with an 87% reduced rate of severe malaria (odds ratio (OR) 0.13, 95% CI 0.03-0.50, P=0.003). The prevalence of AA vs. GG+AG did not differ significantly between the disease groups and CC in a dominant model (P>0.37 Table 3.2).

Table 3.2. Prevalence of rs867186-G variant in malaria disease groups and community children						
	rs867186 (A4600G)			P[a] Additive model	P[a] Recessive model	P[a] Dominant model
	AA, N (%)	AG, N (%)	GG, N (%)		GG vs. AG+AA	GG+AG vs. AA
SM (N=551)	446 (80.9)	102 (18.5)	3 (0.6)	**0.006**[b]	**0.002**[b]	0.38[b]
UM (N=71)	57 (80.3)	14 (19.7)	0 (0)	0.28[c]	0.11[c]	0.73[c]
CC (N=172)	134 (77.9)	31 (18.0)	7 (4.1)	Reference	Reference	Reference

SM, severe malaria (cerebral malaria or severe malarial anemia); UM, uncomplicated malaria; CC, community children
[a]Fisher's exact test is used. $P<0.008$ considered significant to control for multiple comparisons
[b]SM vs. CC
[c]UM vs. CC

The rs867186-G variant tags haplotype 3 of *PROCR.* We also assessed the prevalence of haplotype 1, tagged by rs9574-C, as it has been associated with increased risk of thromboembolism in some [101] but not all [102] studies, and one study associated the presence of both these haplotypes with protection from severe sepsis [103]. In our cohort, the prevalence of rs9574-C did not differ significantly between malaria groups and CC under a recessive, dominant or additive model ($P>0.13$ for all comparisons, Supplemental Table 3.1). Moreover, children who had both variants were not less likely to have severe malaria ($P>0.98$, Supplemental Table 3.2).

Levels of soluble EPCR in children with severe malaria were lower at enrollment but normal at six-months follow-up

Plasma sEPCR levels at enrollment were significantly lower in children with SM (n=484, median, ng/ml [25th percentile, 75th percentile], 91.8ng/ml [69.4,118]) compared to CC (n=110, 117ng/ml [94.9, 189], $P<0.001$, Figure 3.3a). sEPCR levels in children with

uncomplicated malaria (UM, n=38, 114ng/ml [82.4,156]) were lower than CC (P=0.03), and higher than children with SM (Figure 3.3a), but the latter comparison did not reach statistical significance (P=0.07), potentially due to the small sample size of the UM group. When controlling for age, the (log-transformed) sEPCR level was significantly lower in the SM group compared to CC (P<0.001). The difference between the UM and CC group was modest (p=0.055).

At six months post-discharge, sEPCR levels in children with SM (n=378, 118ng/ml [94.7,176]) did not differ significantly from CC (n=73, 118ng/ml [94.8, 163], P=0.77, Figure 3.3b), and were similar to the CC levels at enrollment. These results suggest that lower plasma sEPCR levels in children with SM occur most notably during the disease processes of severe malaria.

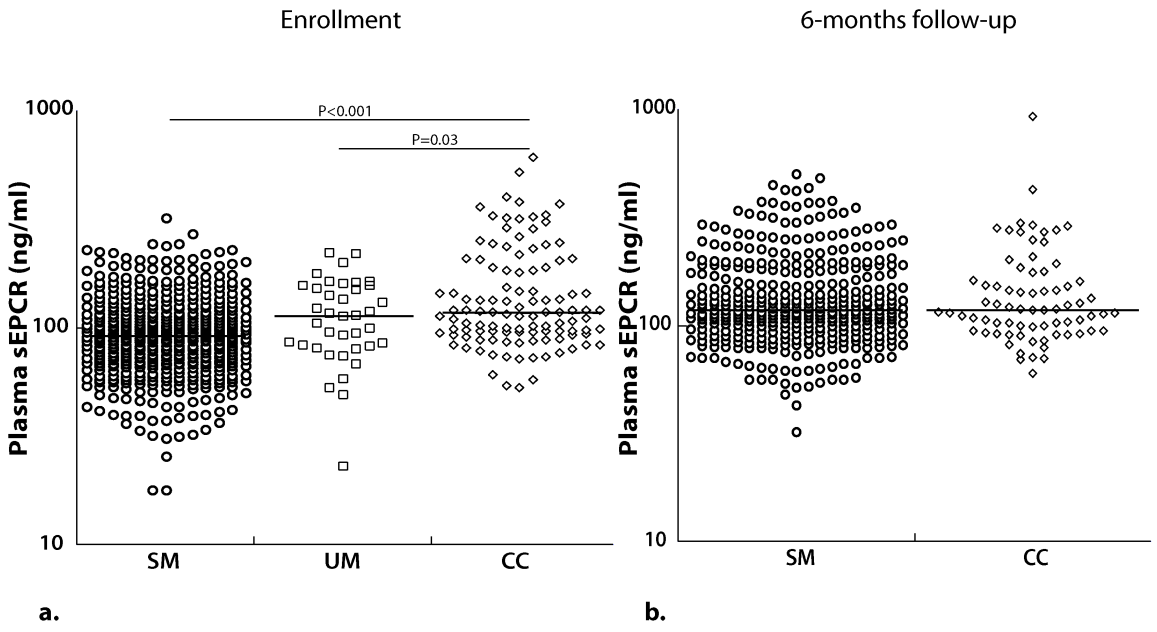


Figure 3.3. Plasma sEPCR levels are lower with increased disease severity at enrollment, but normal at 6-months follow-up

(a) sEPCR levels (on a logarithmic scale) at enrollment and (b) at 6-months follow-up. The horizontal line represents median values. Severe malaria (SM), uncomplicated malaria (UM), community controls (CC).

Plasma sEPCR levels at enrollment and 6-month follow-up trend lower in children with repeated SM

Readmission rates for severe malaria were assessed in the children from the CM/SMA study who were not enrolled in a subsequent nested study of iron treatment and who did not leave the study (301 children: CM, n=156, SMA, n=145), as iron could change risk of readmission. We compared plasma sEPCR levels at enrollment in children with severe malaria that were readmitted with severe malaria within 6-months of discharge versus

sEPCR levels in children that were not readmitted with severe malaria. sEPCR levels at enrollment trended lower in children who were readmitted with severe malaria as compared to not readmitted (readmitted with severe malaria within 6-months of discharge n=16, median, [25th percentile, 75th percentile] ng/ml, 72.2ng/ml [60.5, 102] vs. not readmitted n=244, 95.0ng/ml [72.8, 123], *P*=0.06, Figure 3.4). Readmitted children also tended to have lower sEPCR levels at 6-month follow-up (readmitted n=15, median, [25th percentile, 75th percentile] ng/ml, 101ng/ml [87.6, 116] vs. not readmitted n=226, 121ng/ml [94.1, 176], *P*=0.06).

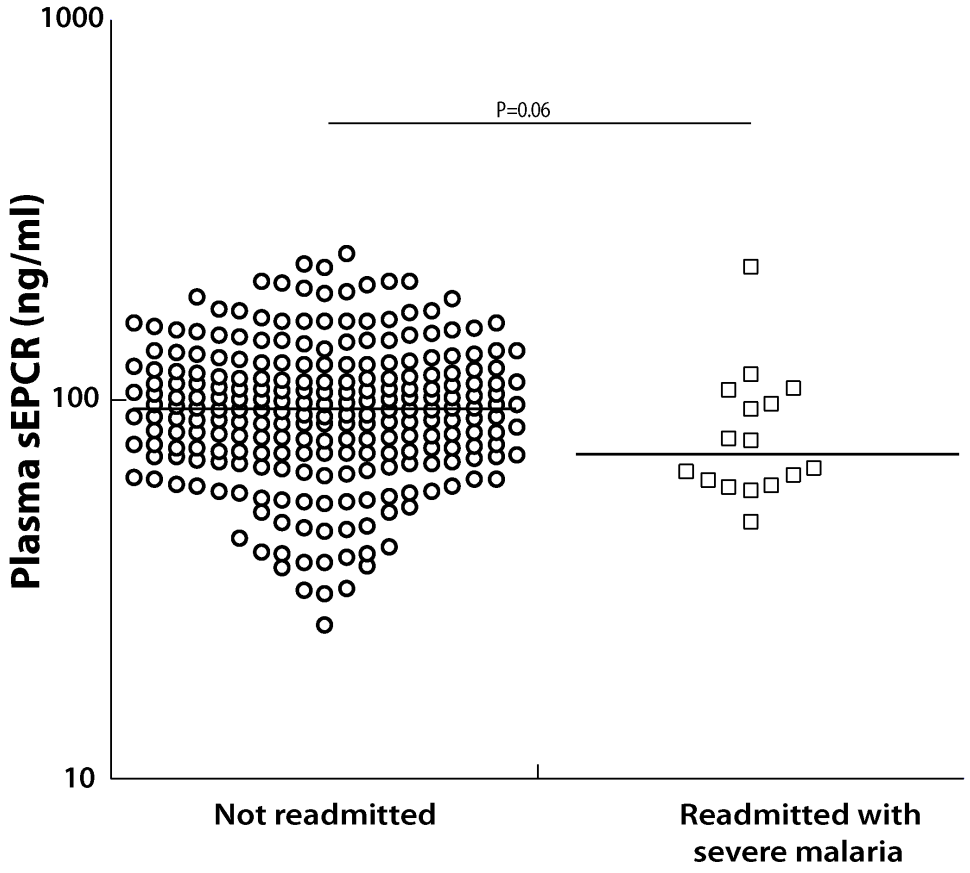


Figure 3.4. Plasma sEPCR levels trended lower in the children readmitted with severe malaria

sEPCR levels (on a logarithmic scale) at enrollment in children with SM separated by whether they were readmitted within 6-mo of discharge for severe malaria. The horizontal line represents median values.

Association of rs867186-G variant with higher sEPCR levels

In our cohort, rs867186-G variant and sEPCR levels were strongly associated in each disease group, with AG and GG genotypes having higher sEPCR levels than AA (Figure 3.5). Children with SM who had genotype AG (n=91, median, ng/ml [25th, 75th percentile], 131ng/ml [107,170]) had significantly higher levels than children with

genotype AA (n=390, 84.5ng/ml [65.7, 104], P<0.001). Only three SM children had the GG genotype, and they had higher sEPCR level than the children with AA (n=3, 194ng/ml [104, 211], P=0.007) but not AG genotypes (P=0.71, Figure 3.5). Similarly, children with UM with the AG genotype (n=13, 161ng/ml [142, 164]) had higher plasma sEPCR levels than those with AA (n=25, 86.5ng/ml [75.4, 113], P<0.001). The effect of rs867186-G variant was clearest in healthy CC children. Plasma sEPCR levels were higher with increasing presence of the G variant (AA (n=79, 98.4ng/ml [87.8, 121]; AG, n=25, 241ng/ml [203, 288]); GG, n=6, 350ng/ml [319, 380], P<0.006 for all comparisons, Figure 3.5). The rs867186-G variant was similarly associated with sEPCR level at 6-months follow-up (Supplemental Figure 3.1).

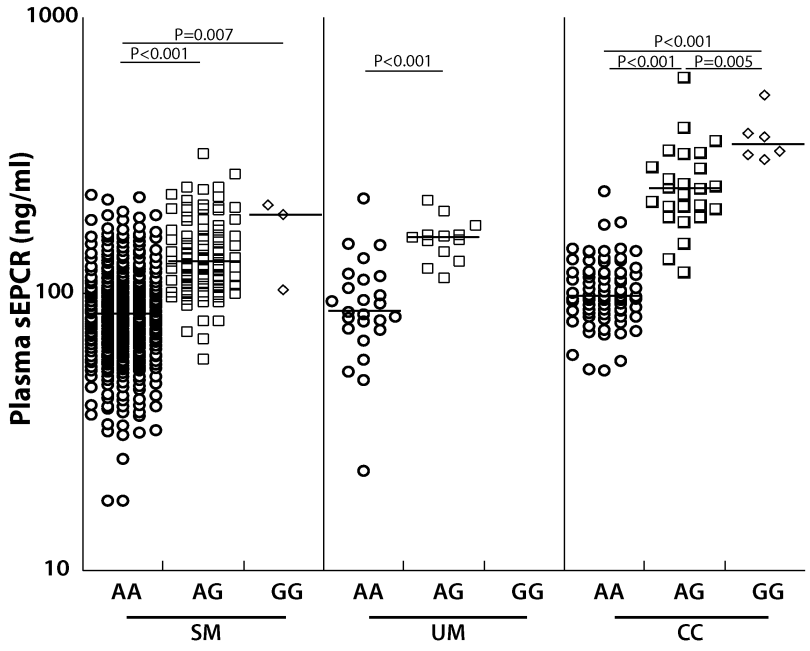


Figure 3.5. rs867186-G is associated with higher sEPCR level

sEPCR levels are represented on a logarithmic scale and each disease group is separated by rs867186 genotype: AA, AG or GG. The horizontal line represents median values. Severe malaria (SM), uncomplicated malaria (UM), community controls (CC).

For the AA, AG and GG genotypes, sEPCR levels were higher with decreasing disease severity (Figure 3.6). Thus, sEPCR levels were lower in children with severe malaria even after controlling for the rs867186-G variant.

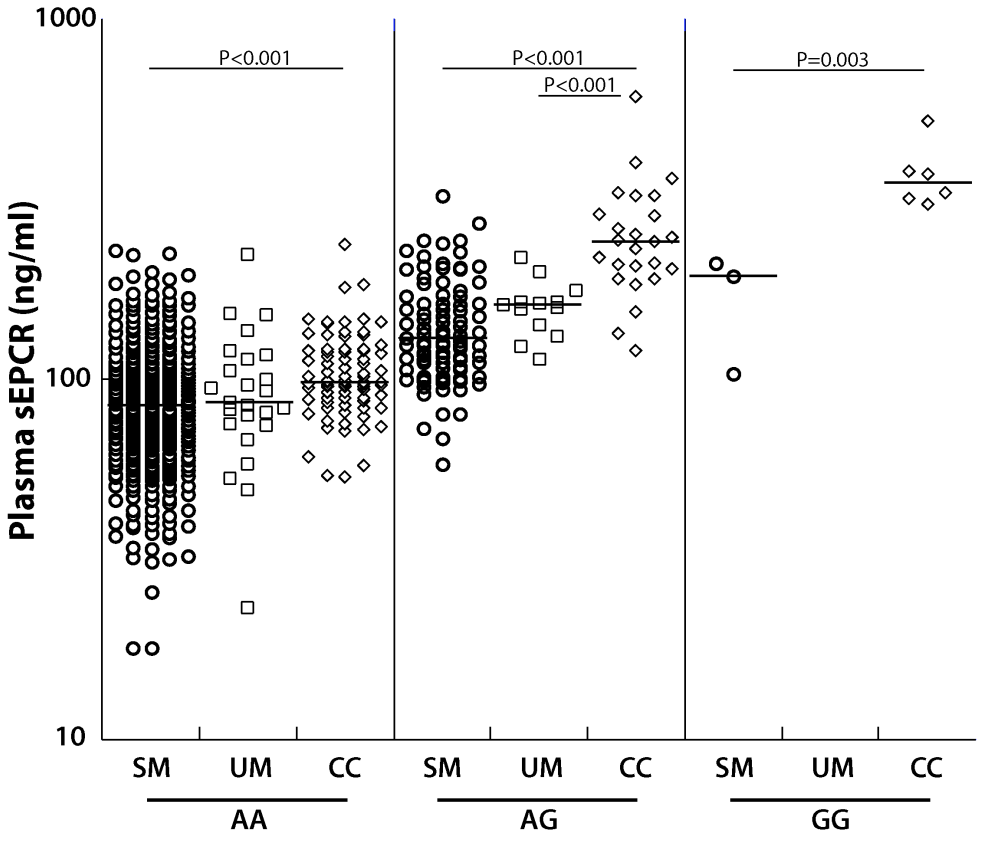


Figure 3.6. Plasma sEPCR levels are lower with increased disease severity when controlling for rs867186-G variant

For each genotype (AA, AG or GG) the median plasma sEPCR levels are represented for each group. The horizontal line represents median values. Severe malaria (SM), uncomplicated malaria (UM), community controls (CC).

Relationships between inflammation, parasite biomass and endothelial activation and plasma sEPCR levels in children with severe malaria

Inflammation and parasite biomass can affect sEPCR levels, while EPCR can in turn affect endothelial activation. When comparing levels of markers of inflammation, endothelial activation and parasite biomass to sEPCR levels, all levels were log transformed (log base 10) because of their skewed distribution, so β-coefficients represent comparisons of log 10 increase in one factor to a log 10 increase in the other factor. After adjustment for age, plasma TNF-α levels correlated positively with sEPCR levels in children with severe malaria (β-coefficient 0.03, 95% CI 0.002-0.06, P=0.04, Table 3.3). Plasma *Pf*HRP-2 levels in the full study cohort had a negative but non-significant correlation with plasma sEPCR levels (β coefficient -0.01, 95% CI -0.03-0.007, P=0.24). However, among children with severe malaria, sEPCR levels were positively associated with total (β-coefficient 0.05, 95%CI 0.03-0.08, P<0.001) and sequestered parasite load (β-coefficient 0.04, 95% CI 0.02-0.07, P=0.002, Table 3.3), after adjusting for age.

Among markers of endothelial activation, including von Willebrand Factor (VWF), angiopoietin 2 (Ang-2), intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), sEPCR levels were associated with increased levels of soluble ICAM-1 (β-coefficient 0.51, 95% CI 0.20-0.82, P=0.001), but not with VWF, VCAM-1 and Ang-2 levels (Table 3.3).

Table 3.3. Association of plasma sEPCR levels with endothelial activation markers and PfHRP-2 levels in children with severe malaria

	Plasma sEPCR (ng/ml)	
	β coefficient (95% CI)	P
TNF-α (pg/ml)[a]	0.03 (0.002-0.06)	0.04
*Pf*HRP-2 (ng/ml)[a]	0.05 (0.03-0.08)	<0.001
Sequestered biomass[a]	0.04 (0.02-0.07)	0.002
VWF (% of normal)[b]	0.11 (-0.10-0.33)	0.29
Plasma Ang-2 (ng/ml)[b]	0.08 (-0.22-0.38)	0.60
sICAM-1 (ng/ml)[b]	0.51 (0.20-0.82)	0.001
sVCAM-1 (ng/ml)[b]	0.07 (-0.06-0.20)	0.31

[a]Models adjusted for age
[b]Models adjusted for age, and systemic TNF-α levels
All values were log-transformed (log10).

sEPCR levels in the cerebrospinal fluid of children with CM

EPCR is also important in the central nervous system (CNS) as it transports aPC across the blood brain barrier (BBB) [104] and facilitates neuroprotective effects of aPC [104-106]. Elevated levels of sEPCR in cerebrospinal fluid (CSF) could inhibit these neuroprotective effects by depleting available aPC. To assess the association of CSF sEPCR levels with adverse outcomes in CM, we quantified sEPCR in the CSF of children with CM. Median [25th percentile, 75th percentile] CSF sEPCR levels (ng/ml) were higher in children with CM (n=76, 4.8ng/ml [3.9, 7.3]) than in control North American children with prior neoplastic disease (n=10, 2.2ng/ml [1.8, 2.3], P<0.0001, Figure 3.7a). CSF sEPCR levels correlated positively with plasma sEPCR levels (Spearman's rho=0.34, P=0.003)

suggesting a passive diffusion due to BBB breakdown. To investigate this further, we assessed the association of CSF-to-plasma sEPCR ratio (CSF sEPCRx1000/Plasma sEPCR (ng/ml)) with CSF-to-plasma albumin ratio (CSF albumin x1000/Plasma albumin (mg/L)). The sEPCR ratio correlated positively with the albumin ratio (Spearman's rho=0.68, $P<0.0001$, Figure 3.7b), suggesting that the major source of sEPCR in the CSF of children with CM is transport from plasma across an impaired BBB.

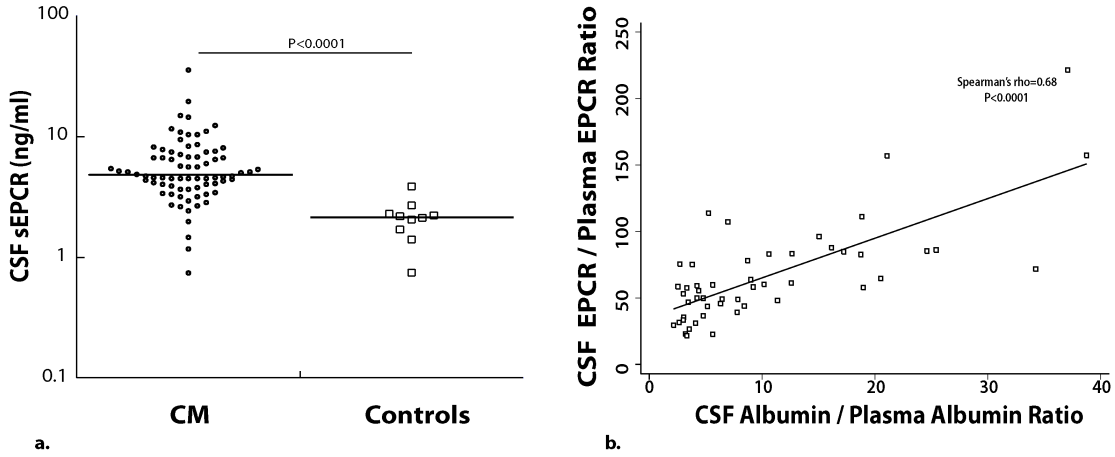


Figure 3.7. CSF sEPCR levels are elevated in children with cerebral malaria

(a) Levels of sEPCR were measured in CSF obtained in CM children who were in stable conditions for a spinal tap. Control CSF samples were obtained from asymptomatic children successfully treated for prior leukemia who had CSF obtained after treatment to rule out return of malignancy. (b) Spearman correlation of CSF-to-plasma albumin ratio vs. CSF to plasma sEPCR ratio for children with CM.

Association of plasma and CSF sEPCR with disease severity markers in cerebral malaria

Among cerebral malaria (CM) children that had plasma sEPCR quantified (n=277), 30 died and of the children who survived, 80 children were discharged with neurologic deficits and 11 had neurologic deficits at 6-months follow-up. In children with CM, neither plasma nor CSF sEPCR was associated with mortality or neurologic deficits at discharge or 6-months follow-up (P>0.10 for all, Table 3.4), adjusting for age. CSF and plasma sEPCR were also not associated with coma duration or seizure number during admission (data not shown). sEPCR levels were also not associated with neurocognitive outcomes (overall cognitive ability, associative memory, or attention) in children with CM under 5 years of age (P>0.10 for all, Table 3.5).

Table 3.4. Relationship of plasma and CSF sEPCR levels to mortality and neurologic morbidity in children with cerebral malaria

	Mortality		Neurologic deficit (discharge)		Neurologic deficit (6mo)	
	OR (95% CI) n	P	OR (95% CI) n	P	OR (95% CI) n	P
Plasma sEPCR (ng/ml)	3.13 (0.36-27.39)[a] n=277	0.30	3.53 (0.76-16.35)[b] n=243	0.11	3.87 (0.12-128)[c] n=233	0.45
CSF sEPCR (ng/ml)	4.30 (0.17-110)[d] n=76	0.38	0.99 (0.13-7.65)[e] n=70	0.99	0.09 (0.0005-16.14)[f] n=67	0.37

All models were adjusted for age. Plasma and CSF sEPCR levels were log transformed (log 10)
[a] Survived (n=247), died (n=30)
[b] Discharged with neurologic deficits (n=80) vs. without (n=163)
[c] Neurologic deficits at 6-months follow-up (n=11) vs. not (n=222)
[d] Survived (n=70), died (n=6)
[e] Discharged with neurologic deficits (n=26) vs. without (n=44)
[f] Neurologic deficits at 6-months follow-up (n=4) vs. not (n=63)

Table 3.5. Relationship of plasma and CSF sEPCR levels with cognitive outcomes at 12 months follow-up in children with cerebral malaria

	Overall cognition		Associative memory		Attention	
	β coefficient (95% CI),n	P	β coefficient (95% CI), n	P	β coefficient (95% CI), n	P
Plasma sEPCR (ng/ml)	-1.26 (-2.90-0.38) n=120	0.13	-0.65 (-1.48-0.18) n=120	0.12	-0.82 (-1.83-0.18) n=123	0.11
CSF sEPCR (ng/ml)	-0.45 (-3.18-2.28) n=47	0.74	-0.33 (-0.99-0.33) n=47	0.31	1.09 (-0.24-2.41) n=47	0.11

All models were adjusted for age. Plasma and CSF sEPCR log transformed (log 10).

Finally, we compared sEPCR levels in the children with CM who were malaria retinopathy positive versus negative. Children who were retinopathy positive had lower sEPCR levels, and difference approached statistical significance (n, median, ng/ml [25th percentile, 75th percentile] levels in retinopathy positive, n=153, 88.7ng/ml [71.0,115] vs. retinopathy negative, n=72, 98.9ng/ml [72.8, 141], *P*=0.07).

3.5 Discussion

The present study found that in Ugandan children, the rs867186-GG genotype is more prevalent in healthy community children than in SM and is associated with increased sEPCR levels; that healthy community children have higher sEPCR levels than children with SM, and that among children with an initial episode of SM, those with repeated episodes of SM tended to have lower sEPCR levels during the initial admission and at 6-month follow-up than those without repeated SM. Since sEPCR levels in other infectious and inflammatory processes are almost uniformly elevated, the present study's findings suggest a distinctive role for sEPCR in severe malaria as compared to other infectious

diseases, and support the idea that the rs867186-GG genotype might mediate protection from severe malaria through increased sEPCR levels.

The reduced prevalence of the rs867186-GG genotype in severe malaria is similar to the findings of a study of Thai adults [97], but differs from studies in Ghanaian [98] and Tanzanian children [99], which found no association between the prevalence of rs867186-G variant and severe malaria [98,99]. In all these studies, rs867186-GG was uncommon, occurring in <5% of the population, suggesting that the benefits are either modest or counterbalanced by deleterious effects, such as the association of this variant with an increased risk of thrombotic disorders [107]. The inconsistencies between findings could arise from host and parasite genetic factors, diseases, or co-infections that differ between these study populations. Large multi-center studies including areas of differing malaria transmission are needed to understand the selection pressure, if any, on this gene and others involved in the aPC/EPCR system in Sub-Saharan Africa.

The present study also found that sEPCR levels were decreased in severe malaria, in contrast to the elevated sEPCR levels typically seen in other infections and disease processes characterized by inflammation. We did not see a significant difference in sEPCR levels between children with CM and SMA (data not shown). High sEPCR levels are seen in SLE [108,109], before relapse in Wegener's granulomatosis [110], and in Behcet's disease [111]. In sepsis, the findings are more nuanced, but the majority of the studies have shown elevated[108,112,113] or similar [114,115] levels of sEPCR in sepsis patients as compared

to healthy individuals, with one study showing significantly lower sEPCR levels in patients with severe sepsis at the onset of organ failure than in healthy controls[116]. The differences in findings could be explained partially by the lack of rs867186-G genotyping, which is strongly associated with sEPCR levels. The present study's findings on low sEPCR in SM are consistent with an earlier small study of children with severe malaria [99], but contrast with a study of children from Benin in which sEPCR levels were higher in children with CM than in children with uncomplicated malaria, and in which the highest sEPCR levels were seen in children who died [100]. Differences in sample processing or testing, or differences in levels due to extremely severe disease in the Benin study (in which patients with CM had a 47% mortality rate) or differences in population genetics might have contributed to the differing findings in the Benin study. However, the present study, which has a sample size more than triple that of either previous study, clearly found that sEPCR levels are lower in severe malaria, and also showed that children readmitted with severe malaria tended to have lower sEPCR levels than children not readmitted with severe malaria, further supporting an association of low sEPCR levels with severe malaria.

While the rs867186-G variant can affect the levels of sEPCR, we showed that even when controlling for the prevalence of this variant, children with SM had lower levels of sEPCR than CC (Figure 3.6), suggesting that disease processes in SM are affecting the levels of sEPCR seen in SM. Why might plasma sEPCR levels be decreased in severe malaria? There are several potential reasons. Because sEPCR can bind to IEs [55], the IE-

bound EPCR may be cleared by the spleen or be removed during plasma processing. Binding of PfEMP1 to EPCR could also provide an immune evasion mechanism for the parasite. Moxon et al. demonstrated that loss of EPCR was associated with parasite sequestration [96], suggesting that interaction of IEs with EPCR may decrease detection of endothelial cell-bound EPCR. How this affects shedding of sEPCR is unknown; it is possible that IE binding to cell-bound EPCR could reduce EPCR shedding. Also, sEPCR could bind to activated neutrophils [117], or due to its small size, leak into damaged organs as seen in the CSF of children with CM (Figure 3.7b). Any or all of these processes could contribute to decreased systemic sEPCR in severe malaria. Determining the expression level of EPCR in subcutaneous tissues [96] or circulating endothelial cells [109] would complement our findings. Additionally, measuring sEPCR levels and parasite clearance at multiple time-points could help determine whether the changes in sEPCR are indeed due to a malaria-specific event.

In the present study, we found elevated levels of CSF sEPCR in CM children, similarly to a previous smaller study [96], but unlike Moxon et al. we did not find strong evidence for local shedding of sEPCR since sEPCR and albumin ratios strongly correlated and there was no evidence of an upward shift in sEPCR ratios more than what would be predicted from a similar increase in albumin index (Figure 3.7b). However, we could not measure the albumin index in our control samples, and so could not rule out any local production of sEPCR. Furthermore, considering the nature of our study, we cannot determine causality and order of events. It could be that BBB leakage as a result of inflammation

leads to increased sEPCR in the CSF, but it could also be that considering the cytoprotective effects of EPCR[91], increased shedding of EPCR as a result of inflammation leads to loss of BBB integrity and increased leakage of plasma proteins including sEPCR. In our study, plasma and CSF sEPCR levels were not associated with mortality, morbidity (neurologic deficits, seizure number, coma duration), or cognitive outcomes in children with CM, suggesting that a further decrease in the already low sEPCR levels of children with severe disease did not lead to increased mortality or adverse neurologic complications. However, lower sEPCR levels at enrollment showed a trend towards increased risk of readmission for malaria in children with severe malaria, suggesting that children with the lowest sEPCR levels during disease might have a greater risk of increased disease severity (requiring admission) with subsequent *P. falciparum* infection. This finding supports the idea that the ability to bind parasites with increased sEPCR might lead to protection from severe malaria, but the study numbers were small and additional studies are required to determine if this association is consistently seen.

Across all children, sEPCR levels had a non-significant but negative correlation with *Pf*HRP-2 levels, as might be expected if increased parasite load led to increased binding of sEPCR in plasma. However, among children with CM or SMA, children with higher parasite biomass also had higher sEPCR levels. Since within disease groups, TNF-α correlated strongly with *Pf*HRP-2 (Spearman's rho 0.57, $P<0.0001$), and TNF-α is known to be associated with severe disease [118-120], it is possible that this correlation between

sEPCR and *Pf*HRP-2 reflects the second phase of a biphasic response: while initially EPCR binds IEs and sEPCR could be protective against sequestration, later in the disease stage, an increase in TNF-α levels in response to an increase in parasite biomass, leads to elevated shedding of sEPCR[93]. Moreover, considering the role of EPCR in endothelial stability we hypothesized that elevated levels of sEPCR would be associated with elevated endothelial activation in SM. When adjusting for TNF-α levels, sEPCR levels were associated only with elevated sICAM-1 (Table 3.3), emphasizing the multifactorial processes that could be contributing to endothelial activation in SM.

EPCR-binding PfEMP1 are large multi-domain proteins and are likely binding to other receptors. Therefore it will be important to determine the relative importance of other receptors working in concert with EPCR in severe malaria. *In vitro* studies and clinical studies across multiple research sites could provide much additional information on what induces production of sEPCR, how it is regulated and removed from the body, and how sEPCR levels relate to endothelial cell-bound EPCR.

In summary, our study found that in Ugandan children, the rs867186-GG genotype was associated with increased sEPCR levels and was less common in severe malaria, higher sEPCR levels were seen in healthy community children than in children with severe malaria, and lower sEPCR levels during severe malaria and in follow-up were associated with readmission for malaria. The findings suggest that sEPCR has a distinctive role in malaria, probably due to its binding to IEs. The mechanisms by which sEPCR levels are

altered in severe malaria, the sequence of events, and the full consequences of decreased sEPCR levels are important areas for future studies.

Chapter 4

EPCR-binding PfEMP1 variants differ in variant type and expression in cerebral malaria and severe malarial anemia

4.1 Objectives

- ✓ Evaluate the performance of newly designed primers that target a larger diversity of *P.falciparum var* genes
- ✓ Determine whether *var* genes encoding for group A (rosetting or non-rosetting) and EPCR-binding PfEMP1 are differentially transcribed in parasites from Ugandan children with CM vs. SMA, from CM children with or without retinopathy, and from CM children that died vs. those that survived.

4.2 Introduction

Cerebral malaria (CM) and severe malarial anemia (SMA) are two distinct clinical entities, CM characterized by coma and high mortality and SMA characterized by severe anemia. In CM, adhesion of infected erythrocytes (IEs) to other uninfected erythrocytes (UEs) (rosette formation), sequestration of IEs, leukocytes and platelets to the blood-brain barrier (BBB) endothelium, combined with an imbalanced immune response and endothelium activation are thought to lead to BBB dysfunction and adverse clinical outcomes[17,30-33]. In SMA, destruction of IEs and UEs, dyserythropoiesis and suppression of erythropoiesis are considered important contributors to severe anemia. However, little

is known about how parasite virulence factors may contribute to the development of these different clinical manifestations of severe malaria.

Within CM, malarial retinopathy has been proposed to distinguish "true" CM (retinopathy positive, RP) from coma due to other causes, with incidental *P.falciparum* parasitemia (retinopathy negative, RN)[36]. However, some studies[121,122] suggest that RN CM may be part of the clinical spectrum of CM, milder than RP CM but still due to *P. falciparum* and not other causes. Assessment of parasite gene expression could help determine whether parasite virulence factors expressed in RP CM are also expressed in RN CM.

The best-studied parasite virulence factor is *P.falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is involved in various aspects associated with disease severity and complications in malaria such as antigenic diversity[10-13], cytoadherence to various host-receptors[9], rosetting[14] and evasion of the immune response[15]. PfEMP1 is encoded by the diverse *var* gene family[12,123,124]. *Var* genes can be classified into group A, B, C, B/A and B/C based on their 5' upstream sequence, chromosome location and direction of transcription[125,126]. The extracellular portion of PfEMP1 varies in organization and length but comprises a combination of Duffy binding like domains (DBLα-ζ) and cysteine rich-interdomain regions (CIDR α-δ)[127,128], which can be found in conserved tandem arrangements known as domain cassettes (DC)[128].

The observation that immunity to severe malaria arises rapidly after only a few episodes[129] together with studies showing increased recognition of IE surface antigens with age[130] have led to the hypothesis that severe malaria is associated with a small number of PfEMP1 variants to which antibodies are acquired early in life. Expression of group A[131-135] *var* genes and *var* genes encoding DC8 (*var* B/A) and DC13 (*var* A) PfEMP1[85] have been associated with severe malaria in some, but not all, studies[136-138]. Antibodies against *var* A and B/A PfEMP1 are gained earlier in life[139] and a broader reactivity of antibodies to group A and B *var* domains has been associated with protection against severe malaria[140].

Endothelial protein C receptor (EPCR) binding appears particularly important for the PfEMP1 variants associated with severe malaria. DC8 (*var* B/A) and DC13 (*var* A) PfEMP1 mediate binding of IEs to various microvasculature beds[86,141] *via* EPCR[52], thus reducing the production and cytoprotective effects of aPC[53,55,56]. As a result, the extent of PfEMP1-EPCR binding could determine the amount of sequestration, coagulation defects, endothelial activation and permeability, which in turn could define the outcomes of severe malaria. Whether group A *var*, and EPCR-binding *var* genes (group A or B) are differentially expressed in the different manifestations of severe malaria is not well characterized. In a study of Kenyan children, non-rosetting *var* A-like genes were found more commonly in parasites infecting children with impaired consciousness (BCS<4), whereas rosetting *var* A types were associated with respiratory distress[142]. Comparable *var* transcript levels were seen between CM and SMA, however transcript levels of group

A *var* genes (DC13 and DC5) trended higher in CM[85]. A recent study did not see a significant difference in transcript levels of *var* genes encoding DC8 and DC13 PfEMP1 between RP and RN CM children[143], suggesting that parasites infecting RN express the *var* genes associated with severe malaria and IE-binding to the same extent as RP. However, a comparison of RN with another form of severe malaria was not performed, which could help in further placing RN CM in the disease spectrum of malarial severity.

Despite the evidence that EPCR-binding PfEMP1 variants are important in development of severe malaria, there is limited data on how these variants may contribute to development of CM or SMA, or whether they differ in RP vs. RN CM. In the current study we addressed whether *var* genes encoding group A (rosetting or non-rosetting) and EPCR-binding PfEMP1 are differentially transcribed in parasites from Ugandan children with CM vs. SMA, from CM children with or without retinopathy, and from CM children that died vs. those that survived. We have re-designed the qRT-PCR primers based on the comparison of 226 *var* sequences. The *var* profile that leads to the most severe malaria manifestations could help identify the binding characteristics that can be targeted to reduce morbidity and mortality in severe malaria.

4.3 Methods

Sample collection and 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. The samples were stored long-term at -80^{o}C. RNA was isolated using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland).

Quantification of var transcription by qRT-PCR

Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA). cDNA was synthesized using random hexamers and the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. qRT-PCR was performed in 20µl reactions using KiCqStart® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, St. Louis, MO) with the 7500 Real Time PCR System (Applied Biosystem, Foster City, CA). Amplification was performed following the previously published conditions[85] and data was collected at the final elongation step. No reverse transcriptase and no template controls for both housekeeping genes were included in each plate tested to rule out DNA contamination in the RNA samples and any nucleic acid contamination in reagents, respectively. Gene expression was normalized to the average of two housekeeping genes: seryl t RNA synthetase and fructose-bisphosphate aldolase ($\Delta Ct_{var\_primer} = Ct_{var\_primer} - Ct_{average\_control\ primers}$). Only samples that had a $Ct_{average\_control}$ below 25 were included in the analysis, to allow analysis only of those samples that fell well within the linear range of the standard curves for these two genes. ΔCt_{var_primer} was transformed into arbitrary units using $T_u=2^{(5-\Delta Ct)}$. Any time ΔCt_{var_primer} was 5 or higher, it was given an arbitrary unit of 1. As an example, if a domain was expressed 5 fold lower than the housekeeping genes ($\Delta Ct_{var\_primer}=5$), the arbitrary units would be 1. This cutoff of was set to allow for analysis of only the samples that had Ct values, which fell within

the linear range of the standard curves generated from the dilution of 3D7 gDNA for each domain. Melting temperature analysis was performed for each target and only samples with T_m within 1.7^oC of median T_m were analyzed. If only primer dimers or non-specific larger targets were detected, T_u for that target was assigned as 1.

Statistical analysis

Data was analyzed using Stata/SE 12.1 (StataCorp, College Station, Texas). Transcript levels of *var* genes were compared between disease groups using Mann-Whitney U test. Clinical and laboratory findings for children in the different disease groups were compared using the chi-squared test if categorical and using t-tests for continuous measures. Median T_u for group A EPCR-binders was determined as median of CIDRα1.4, CIDRα1.5a, CIDRα1.5b, CIDRα1.6b and CIDRα1.7 T_u; median T_u for group B EPCR-binders was determined as median of CIDRα1.1, CIDRα1.8a and CIDRα1.8b T_u; median of CIDRα1 EPCR-binders was calculated as median of CIDRα1.1-CIDRα1.8b T_u.

4.4 Results

Characteristics of study population

We had RNA with sufficient volume and quality to quantify *P.falciparum var* transcript levels from 159 patients (98 cerebral malaria [CM], 47 severe malarial anemia [SMA], and 14 asymptomatic parasitemic [AP]). Median age of this population was 40.0 months

[25th percentile, 75th percentile], [28.7, 54.6]). Age and sex did not significantly differ between disease groups (Table 4.1). As expected, parasite biomass, indicated by *P.falciparum* histidine rich protein-2 (*Pf*HRP-2) levels differed between disease groups (P<0.0001, Table 4.1), being higher in CM than SMA than AP. Sequestered biomass followed the same trend (P<0.0001, Table 4.1) confirming that while sequestration occurs commonly in *P.falciparum* infections, its magnitude and contribution to disease severity differs among various manifestations of malaria.

Table 4.1. Study population characteristics

	CM (n=98)	**SMA (n=47)**	**AP (N=14)**	**P[a]**
Age (months), median (IQR)	41.5 (30.9-54.6)	33.4 (24.9-52.4)	48.5 (31.0-71.0)	0.14
Sex (male), n (%)	59 (60.2)	35 (74.5)	7 (50.0)	0.14
Weight for age z-score, mean (SD)	-1.11 (1.49) n=97	-1.98 (1.39)	-0.31 (1.17)	0.0001[b]
Hemoglobin (g/dL), mean (SD)	7.07 (2.30)	3.81 (0.74)	11.2 (2.15)	<0.0001[c]
Parasite density (/µl), median (IQR)	67010 (18030-347010) n=96	43880 (11940-156040) n=46	2170 (520-11880)	<0.0001[d]
Parasite load (*Pf*HRP-2, ng/ml), median (IQR)	2648 (883-5150)	862 (288-2033) n=46	88.8 (4.80-158) n=13	<0.0001[c]
Sequestered biomass (x10^8), median (IQR)	17928 (5323-39891) n=96	6249 (1303-15839) n=45	469 (0-1309) n=13	<0.0001[c]

[a] ANOVA, Tukey post-hoc test adjustment for multiple comparisons with log10 transformed values for variables with no normal distribution. Chi-squared test was used for sex, with *P*<0.017 considered significant to control for multiple comparisons.
[b] In post-hoc testing, SMA differed from CM and AP
[c] In post-hoc testing, all groups differed from each other
[d] In post-hoc testing, CM and SMA differed from AP

Children with asymptomatic *P. falciparum* parasitemia have low transcript levels of *var* genes encoding group A and EPCR-binding PfEMP1 variants

Previous studies have shown higher transcript levels of group A and B PfEMP1 *var* genes in children with severe malaria (SM) when compared to children with uncomplicated malaria[85,131,133] or asymptomatic parasitemia[133,138]. To confirm these findings in a cohort of Ugandan children, we used newly designed primers to compare transcript levels of a number of group A and B PfEMP1 *var* genes between children with SM (CM or SMA) and children from the same extended household or neighborhood of the children with SM who had asymptomatic parasitemia (AP). Prior comparisons assessed either only DBLα domains and classified them based on cysteine residues[138] or quantified transcript levels of group A, B or C *var* genes based on the 5' upstream sequence without being able to determine specifically the *var* A, B or C domains and their binding phenotype[133]. In the present study, we used degenerate primers to quantify transcript levels of a number of EPCR-binding PfEMP1 variants and group A non-EPCR binders that have not been previously assessed in children with AP. The domains that were quantified include: DBLα1ALL, targeting the head structure of all group A *var* genes; DBLα1.5/6/8 type domains, targeting the head structure of group A genes that do not normally bind to EPCR and have some rosetting ability[144]; CIDR1δ domain which is normally preceded by a DBLα1.5 and is associated with rosetting[144]; as well as a number of EPCR-binding domains (Figure 4.1, Supplemental Table 4.1). The EPCR-binding PfEMP1 domains included DBLα2/1.1/2/4/7/9 types, group A EPCR-binders (median of CIDRα1.4, CIDRα1.5a, CIDRα1.5b, CIDRα1.6b and CIDRα1.7) and group B-EPCR binders

(median of CIDRα1.1, CIDRα1.8a and CIDRα1.8b) (Figure 4.1, Supplemental Table 4.1). To provide an overall idea of the transcript levels of EPCR-binding CIDRα1 domains, we also determined median transcript levels of CIDRα1 EPCR-binding PfEMP1 (median of CIDRα1.1-CIDRα1.8b). Since CIDRα1.1 and CIDRα1.4 represent the largest CIDRα1 families for DC8 and DC13, respectively we have reported these domains separately.

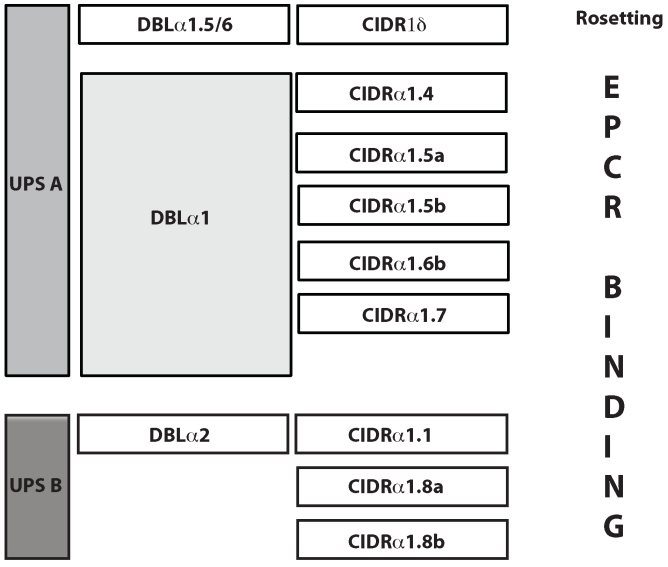


Figure 4.1. Schematics of the extracellular PfEMP1 domains, whose transcript levels are quantified in the study and their known binding phenotype

In the current study, median transcript levels of all *var* genes quantified were higher in parasites infecting children with SM compared to AP ($P \leq 0.05$ for all, Figure 4.2). Only DBLα2/1.1/2/4/7/9 types showed a range of transcript abundance in AP. These domains

normally precede CIDRα1.1 in DC8, however CIDRα1.1 showed mostly basal levels of transcription in AP. Since DBLα2 is a DBLα0/DBLα1 hybrid, it could be that the primers targeting DBLα2 are quantifying some DBLα0, which are normally followed by CIDRα2-6 of group B *var* genes, not quantified here. All AP samples included in the analysis had average C_t values for the two housekeeping genes below 25, which fell within the linear portion of the gDNA standard curves for both housekeeping genes (Supplemental Figure 4.1), suggesting that the observed basal expression for the rest of the *var* genes was not due to lack of sensitivity.

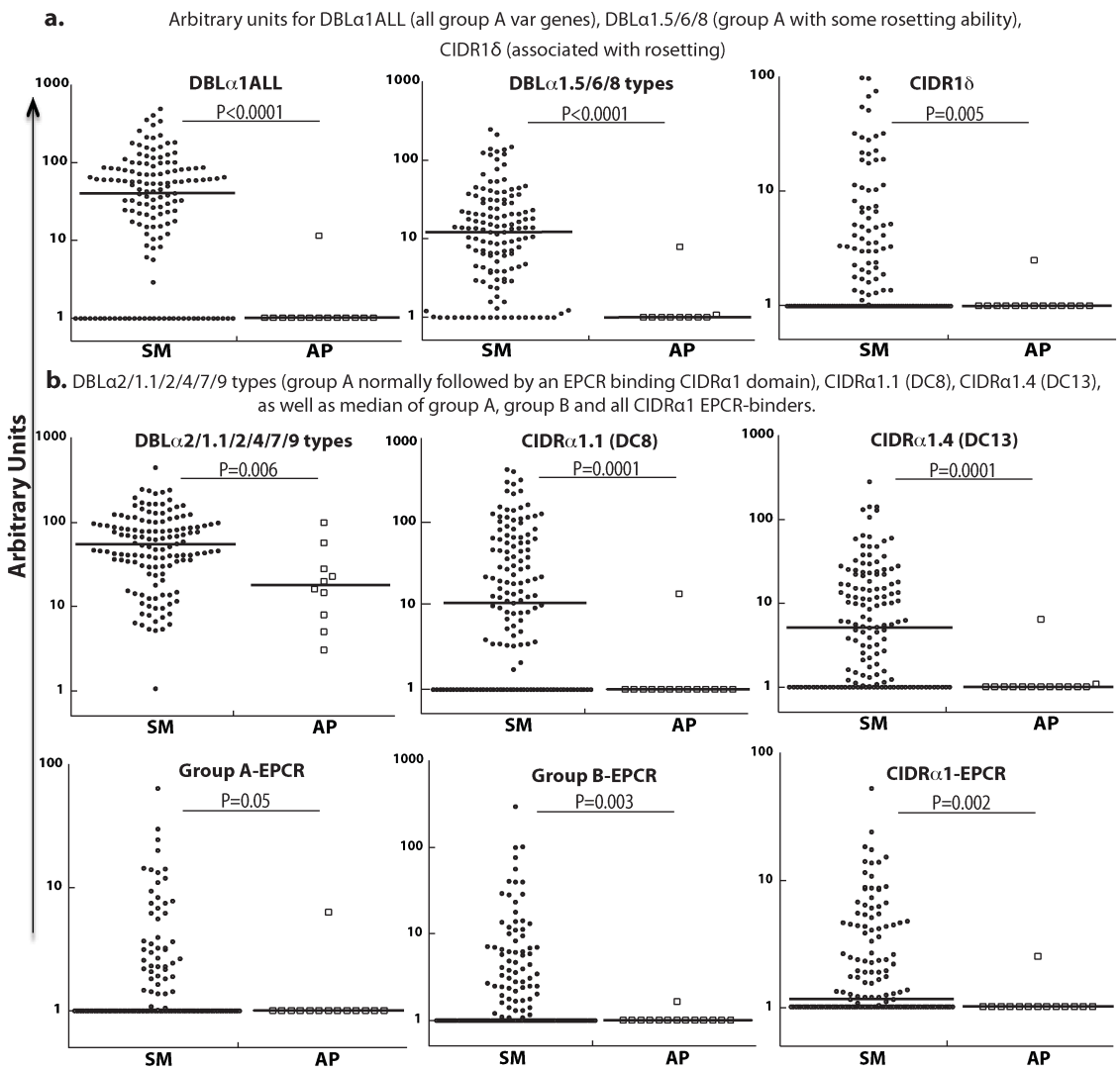


Figure 4.2. Group A and EPCR-binding PfEMP1 transcript levels are higher in severe malaria than asymptomatic controls

(a) Arbitrary unit values for DBLα1ALL (all group A var genes), DBLα1.5/6/8 types (group A with some rosetting ability), CIDR1δ (associated with rosetting) and b) DBLα2/1.1/2/4/7/9 types (group A normally followed by an EPCR binding CIDRα1 domain), CIDRα1.1 (DC8), CIDRα1.4 (DC13), as well as median of group A, group B and all CIDRα1 EPCR-binders. Arbitrary units of expression are shown on a logarithmic scale. The horizontal line represents median values. Medians are compared by Mann-Whitney test. Severe malaria (SM), asymptomatic parasitemia (AP).

Transcript levels of EPCR-binding PfEMP1 variants are higher in children with CM compared to SMA

To determine whether different *var* genes associated with severe malaria contribute differently to the various manifestations of severe malaria we compared their median transcript levels between CM and SMA.

P.falciparum parasites infecting children with CM had similar transcript level of group A *var* genes compared to SMA (P>0.10 for all, Figure 4.3a). However, the median transcript level of *var* genes encoding EPCR-binding PfEMP1 trended higher in CM than SMA (Figure 4.3b), and reached statistical significance for DBLα2/1.1/2/4/7/9 types (CM, n=73, median, arbitrary units [25th percentile, 75th percentile], 43.5 [24.5,60.5] vs. SMA, n=43, 27.3 [10.1, 45.7], *P*=0.01), CIDRα1.1 (DC8) (CM, n=77, 10.4 [1, 43.4] vs. SMA, n=47, 3.11 [1, 21.4], *P*=0.04) and group A EPCR-binders (CM, n=77, 1 [1, 2.38] vs. SMA, n=47, 1 [1, 1], *P*=0.02). In a multiple regression model including DBLα2/1.1/2/4/7/9, CIDRα1.1 (DC8) and group A EPCR binders adjusted for *Pf*HRP-2 levels, age, sex and weight for age z-score, log base 10-transformed DBLα2/1.1/2/4/7/9 transcript levels were independently associated with increased risk of CM (odds ratio (OR) 5.46, 95% CI 1.60-18.6, *P*=0.007). Combined with the clinical characteristics showing that children with CM have higher total parasite biomass and sequestered parasite biomass than SMA, these data suggest that EPCR-binding and parasite load can independently increase the risk of CM.

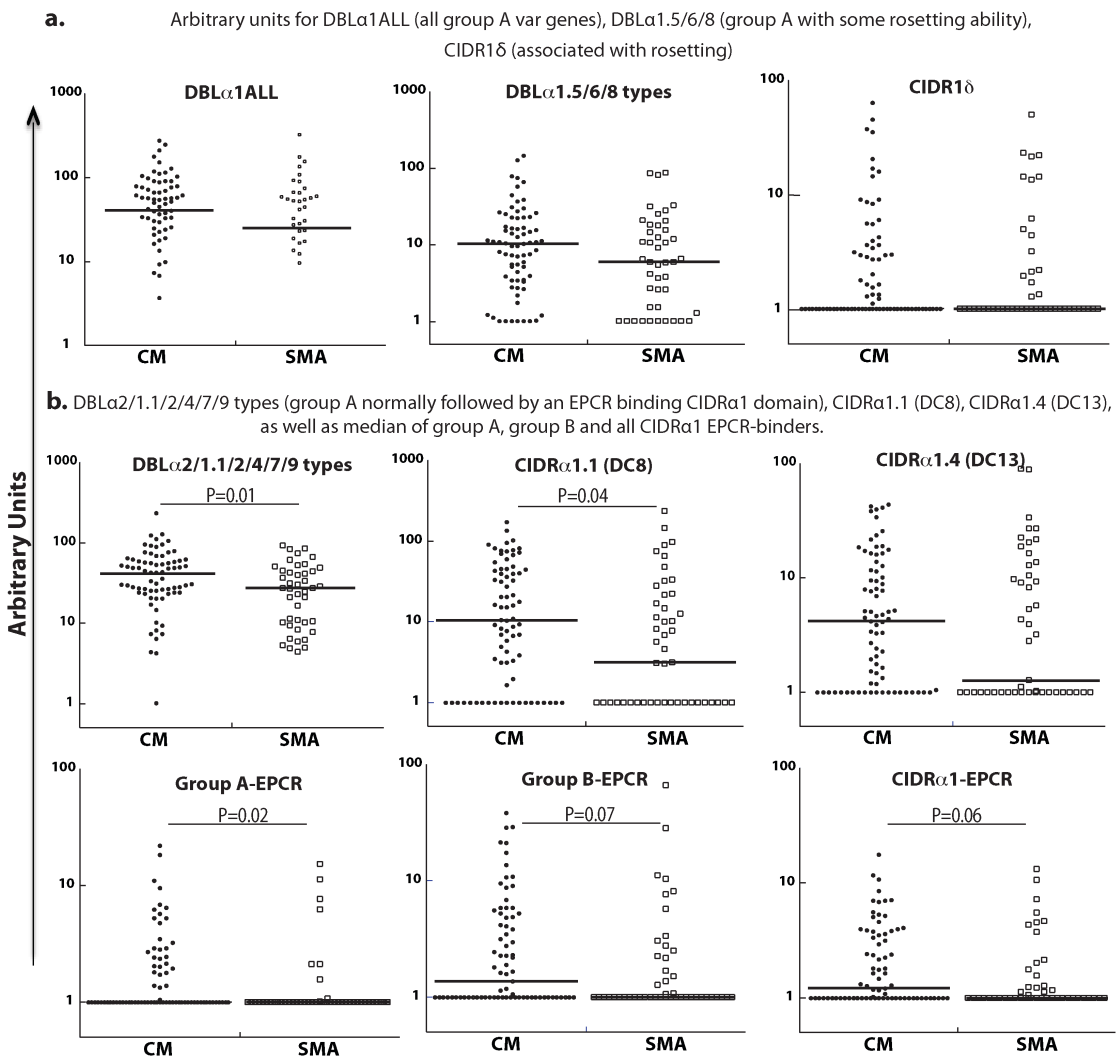


Figure 4.3. Transcript abundance of EPCR-binding PfEMP1 is higher in parasites from children with cerebral malaria than severe malarial anemia

(a) Arbitrary unit values for DBLα1ALL (all group A var genes), DBLα1.5/6/8 types (group A with some rosetting ability), CIDR1δ (associated with rosetting) and b) DBLα2/1.1/2/4/7/9 types (group A normally followed by an EPCR binding CIDRα1 domain), CIDRα1.1 (DC8), CIDRα1.4 (DC13), as well as median of group A, group B and all CIDRα1 EPCR-binders. Arbitrary units of expression are shown on a logarithmic scale. The horizontal line represents median values. Medians are compared by Mann-Whitney test. Cerebral malaria (CM, hemoglobin >5g/dL), severe malarial anemia (SMA).

In our study, 21 of 98 CM children (21.4%) had both cerebral malaria and severe malarial anemia (CM/SMA). These children were not included in the analysis above, which focused on children with CM alone or SMA alone, but were compared separately to the children with CM to assess how they differed from this primary group. Children with CM/SMA had higher DC13 transcript levels (n=21, median, arbitrary units [25th percentile, 75th percentile], 11.1 [2.85, 20.3]) than children with CM only (n=77, 4.16 [1, 11.7], *P*=0.02, Supplemental Table 4.2). DBLα1ALL transcript levels also trended higher in CM/SMA compared to CM only (*P*=0.09, Supplemental Table 4.2), suggesting that the extent of *var* group A transcription could contribute to the type of severe manifestation seen in children with SM.

PfEMP1 transcript levels differ by presence of retinopathy in children with CM only for DC13, and are similar in retinopathy negative CM and SMA

Malarial retinopathy (MR) during hospitalization was found to be a good predictor of brain sequestration post-mortem in children classified as CM by WHO definitions[36]. As a result, MR is used to qualify retinopathy positive (RP) CM as "true" CM and retinopathy negative (RN) CM as incidental parasitemia with another cause for the coma. Indirect ophthalmoscopy in our study was performed by trained medical officers and represents a real-life setting for retinopathy diagnosis in the field (see Methods). In our study, RN children have the characteristics of a less severe form of CM[122] , however children with RN still have high parasite loads and estimated sequestered biomass compared to

SMA[121], suggesting a potential contribution of the parasite load and sequestration to the clinical manifestations of RN CM.

In order to determine whether parasites infecting RN CM express *var* genes associated with severe disease and with IE binding to host endothelium, we compared transcript abundance of group A and EPCR-binding PfEMP1 between RP and RN CM, as well as between RN CM and SMA. In these patients, which represent a subset of those assessed for *Pf*HRP-2 levels[121], sequestered parasite biomass trended lower in RN CM compared to RP CM (P=0.08, Table 4.2) but was similar to SMA. Transcript levels of DBLα1ALL, DBLα1.5/6/8, DBLα2/1.1/2/4/7/9 and CIDRα1.1 (DC8) were significantly higher in RP CM vs. SMA (P<0.05 for all, Figure 4.4). However, only DC13 transcripts were higher in RP (n=50, 8.74 [2.33, 18.6]) vs. RN CM (n=47, 3.28 [1, 8.88], P=0.02, Figure 4.4b), and all *var* transcript levels were similar between RN CM and SMA (P>0.05 for all). Altogether, the data suggest that rosetting and IE cytoadhesion may also be important in RN CM pathogenesis.

Table 4.2. Clinical characteristics of CM children with and without malarial retinopathy

	RP (n=50)	**RN (n=47)**	**SMA (n=47)**	**P[a]**
Age (months), median (IQR)	40.1 (29.6-50.2)	42.0 (31.7-59.4)	33.4 (24.9-52.4)	0.23
Sex (male), n (%)	29 (58.0)	29 (61.7)	35 (74.5)	0.21
Weight for age z-score, mean (SD)	-1.30 (1.26) n=49	-0.92 (1.71)	-1.98 (1.39)	0.002[b]
Hemoglobin (g/dL), mean (SD)	6.34 (2.17)	7.80 (2.21)	3.81 (0.74)	<0.0001[c]
Parasite density (/µl), median (IQR)	100260 (21830-415920) n=48	50690 (10780-273100)	43880 (11940-156040) n=46	0.17
Parasite load (*Pf*HRP-2, ng/ml), median (IQR)	3190 (1418-5222)	2491 (446-3900)	862 (288-2033) n=46	<0.0001[d]
Sequestered biomass (x10^8), median (IQR)	20880 (11037-44350) n=48	15766 (2450-31276) n=47	6248 (1303-15839) n=45	0.0005[e]

[a] ANOVA, Tukey post-hoc test adjustment for multiple comparisons with log10 transformed values for variables with no normal distribution. Chi-squared test was used for sex, with $P<0.017$ considered significant to control for multiple comparisons.
[b] In post-hoc testing, SMA differed from RN
[c] In post-hoc testing, all groups differed from each other
[d] In post-hoc testing, SMA differed from RP and RN
[e] In post-hoc testing, RP differed from SMA. For RP vs. RN, $P=0.08$

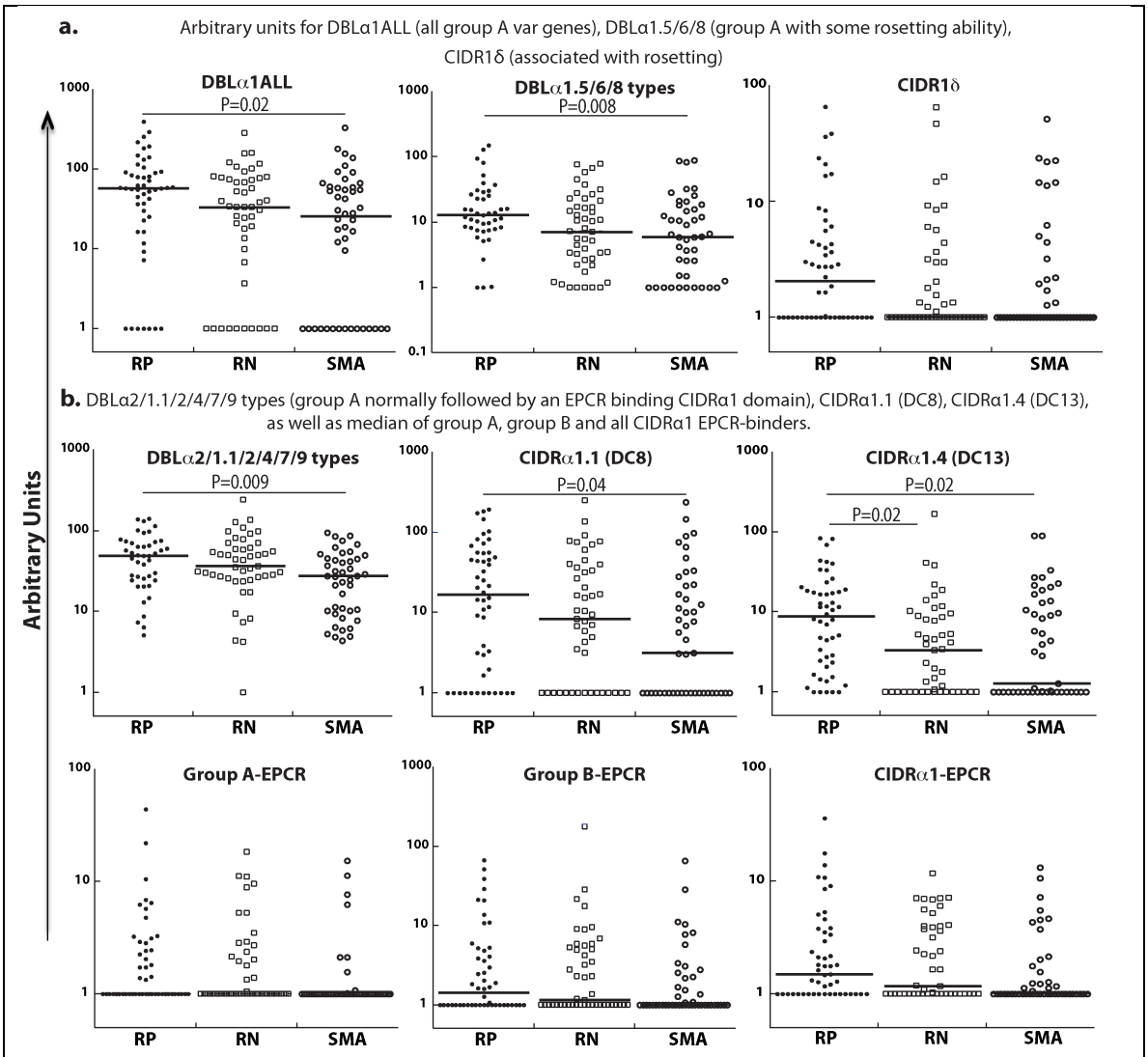


Figure 4.4. DC13 transcripts are higher in cerebral malaria patients with malarial retinopathy.

(a) Arbitrary unit values for DBLα1ALL (all group A var genes), DBLα1.5/6/8 types (group A with some rosetting ability), CIDR1δ (associated with rosetting) and b) DBLα2/1.1/2/4/7/9 types (group A normally followed by an EPCR binding CIDRα1 domain), CIDRα1.1 (DC8), CIDRα1.4 (DC13), as well as median of group A, group B and all CIDRα1 EPCR-binders. Arbitrary units of expression are shown on a logarithmic scale. The horizontal line represents median values. P values are estimated by ANOVA on log10 transformed arbitrary units followed by Tukey adjustment for multiple comparisons. Retinopathy positive CM (RP), retinopathy negative CM (RN) and severe malarial anemia (SMA).

Due to the difficulties and the expertise needed for indirect ophthalmoscopy, *Pf*HRP-2 levels have been identified as a good predictor of MR. It has previously been shown that *Pf*HRP-2 levels >1700ng/ml at enrollment had a 90% sensitivity and 87% specificity in predicting MR[145]. We used this cutoff to redefine two groups within CM: one with *Pf*HRP-2 levels higher than 1700 ng/ml (*Pf*HRP-2-high, n=62) and one with levels lower than 1700ng/ml (*Pf*HRP-2-low, n=35). Transcript levels of the *var* genes considered in this study did not differ significantly between the *Pf*HRP-2-high and *Pf*HRP-2-low groups (Supplemental Table 4.3). When considering a *Pf*HRP-2 cutoff based on the samples from all children with CM in the study (not only those who had RNA for gene expression testing), a cutoff of 1392ng/ml had the highest sensitivity and specificity in distinguishing RP from RN CM (sensitivity 78.3% and specificity 41.9%)[121]. When classifying the CM group based on this *Pf*HRP-2 cutoff, 33 CM patients had lower levels than the cut-off and 64 higher. The *var* transcript levels did not differ significantly in CM patients that were above vs. below this cutoff (data not shown). The data from the present study showing similar transcript levels of all PfEMP1 variants associated in multiple studies with severe malaria suggest that use of a *Pf*HRP-2 cutoff could lead to misdiagnosis of a substantial proportion cases of true CM as coma due to other causes.

P.falciparum parasites infecting CM children that died have lower transcript levels of group A *var* genes compared to those that survived

To further assess the association of group A and EPCR-binding PfEMP1 transcript levels with malarial disease severity, we compared *var* transcripts between CM children that died vs. those that survived. DBLα1ALL which targets all group A *var* genes had higher transcript level in children that survived (n=87, 55.0 [19.0, 83.4]) vs. those that died (n=11, 1 [1, 45.0], *P*=0.005, Figure 4.5a). There was no significant difference in the other domains (*P*>0.09 for all). A log base 10 increase in DBLα1ALL transcript levels was associated with a 74% decreased risk of mortality in CM patients (OR 0.26, 95% CI 0.11-0.62, *P*=0.003) when adjusted for *Pf*HRP-2 levels. The difference in DBLα1ALL transcript level persisted even when considering only retinopathy positive CM children (survived, n=41, 58.8 [36.9, 92.7] vs. died, n=9, 9.22 [1-45.0], *P*=0.006, Supplemental Figure 4.2), suggesting that the lower transcript abundance of DBLα1ALL in children with CM who died cannot be explained by some other cause of mortality.

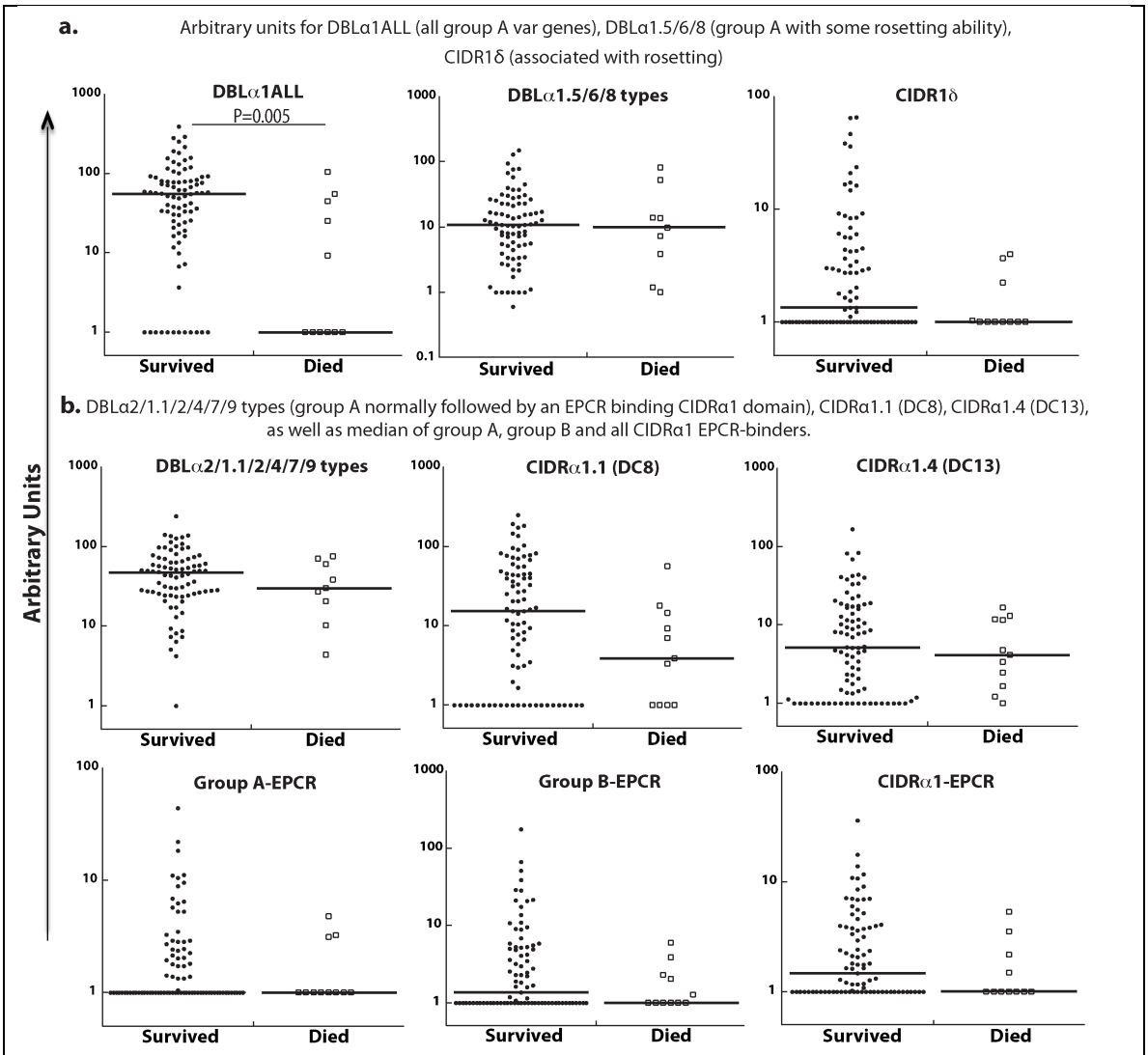


Figure 4.5. DBLα1 transcripts, targeting all group A *var* genes are lower in parasites from cerebral malaria patients that died

(a) Arbitrary unit values for DBLα1ALL (all group A var genes), DBLα1.5/6/8 types (group A with some rosetting ability), CIDR1δ (associated with rosetting) and b) DBLα2/1.1/2/4/7/9 types (group A normally followed by an EPCR binding CIDRα1 domain), CIDRα1.1 (DC8), CIDRα1.4 (DC13), as well as median of group A, group B and all CIDRα1 EPCR-binders. Arbitrary units of expression are shown on a logarithmic scale. The horizontal line represents median values. Medians are compared by Mann-Whitney test.

To assess whether time to death was associated with level of DBLα1ALL transcripts at enrollment, we looked at median DBLα1ALL transcript levels stratified by time to death. In our study, 5 children died the day of enrollment, 3 within 24 hours, 2 within 48 hours and one within 72 hours of enrollment (DBLα1ALL median T_u 9.22, 1, 23.0 and 55.3, respectively). The differences in median transcript levels at later time of death were large, but the numbers were small to be statistically significant (P for Kruskal-Wallis test for trend = 0.27).

4.5 Discussion

In the present study, we use redesigned degenerate primers that target a larger diversity of *var* genes and found that transcript levels of group A and EPCR-binding PfEMP1 were higher in parasites infecting children with severe malaria (SM) compared to asymptomatic parasitemia (AP); that transcript levels of EPCR-binding PfEMP1 were higher in CM than SMA; that PfEMP1 *var* transcript levels were similar in retinopathy negative CM and SMA, and differed between retinopathy positive and retinopathy negative CM only for DC13; and that DBLα1ALL (targeting all group A *var*) was higher in CM children that survived compared to those who died. The data provide new insights into parasite contributions to disease pathogenesis in SM and add to the ongoing debate as to what constitutes a case of "true" CM.

Group A and B *var* genes were previously found to be less prevalent in AP than SM [133,138], however our study was able to quantify a larger diversity of *var* genes and show higher transcript levels for DBLα1ALL (all group A *var* genes), DBLα1.5/6/8 types (group A with some rosetting ability), CIDR1δ (associated with rosetting), DBLα2/1.1/2/4/7/9 types (group A normally followed by an EPCR binding CIDRα1 domain), as well as group A and B CIDRα1 EPCR-binders (Supplemental Table 4.1) in SM as compared to AP (Figure 4.2). In future studies, we plan to assess transcript levels of group B and C CD36-binding PfEMP1, which have shown to be similar[133] or higher[137] in AP as compared to uncomplicated or SM in prior studies. The AP group in this study had no history of prior SM and did not experience SM over the 2 years of follow-up, despite presumably similar malaria exposure (since they lived in the same extended household as SM children). AP children appear to be protected from SM, and therefore represent a valuable comparator group to SM. We did not have access to RNA samples from patients with uncomplicated malaria, which represent another important comparison group of malaria without severe manifestations. However, parasites from patients with uncomplicated malaria could still express some of the domains associated with SM, even though these patients never reached severe disease due to early treatment.

Recently, sequencing of almost full-length *var* genes showed that CIDRα1 were the only common domains found in pediatric CM and SMA patients[146]. However, in the current study we show that EPCR-binding PfEMP1 transcript levels (DBLα2/1.1/2/4/7/9, CIDRα1.1, and overall group A EPCR) were higher in parasites from children with CM

compared to SMA (Figure 4.3b). Moreover, high DBLα2/1.1/2/4/7/9 transcript levels were associated with CM independently of *Pf*HRP-2 levels. These finding suggest that 1) not only the presence, but more importantly the transcript level and therefore the extent of EPCR binding by PfEMP1 may be important in determining the clinical manifestation of SM and that 2) both EPCR binding and total parasite biomass are important in determining the type of severe manifestation (coma or severe anemia). PfEMP1 binding to EPCR reduces the production and cytoprotective effects of aPC[53,55,56] and loss of EPCR has been associated with sequestration and fibrin deposition in CM patients[96]. In addition, EPCR expression is low in small microvasculature beds, such as the microvasculature of the brain [147]. As a result, it could be hypothesized that once a certain parasite load is reached, parasites expressing higher EPCR-binding PfEMP1 occupy more of the EPCR binding sites available in the brain microvasculature contributing to brain pathology. In SMA, EPCR binding by PfEMP1 could lead to anemia through effects on hematopoiesis since EPCR signaling in the bone marrow environment is important for retention of long-term hematopoietic stem cells and for hematopoiesis[148]. We found that DBLα2/1.1/2/4/7/9, CIDRα1.1, and group A EPCR-binders transcript levels were higher in CM than SMA, suggesting that these variants specifically contribute to the development of CM, but no variants were expressed at higher levels in SMA than CM. This does not rule out a contribution of the *var* genes seen in both CM and SMA to the development of SMA, as these genes were expressed at higher levels in SMA than AP, but it suggests other non EPCR-binding group A PfEMP1 could contribute specifically to severe anemia. Quantifying transcript levels of the secondary DBL-CIDR structure of

PfEMP1 could help determine whether domains binding to ICAM-1 or other host receptors make up the rest of group A *var* that are not accounted for by the EPCR-binding PfEMP1 in SMA.

A recent study did not find any significant difference in group A, DC8 and DC13 transcript levels between RP and RN CM in a Kenyan cohort[143]. The present study, which uses new primers that target a higher diversity of *var* genes, found only higher DC13 transcripts in RP compared to RN CM (Figure 4.4). The Kenyan study saw higher proportional expression of group A and DC8 *var* genes in RN vs. RP[143]. We did not assess proportional expression because transcript levels are not absolute values, and no study captures 100% of *var* diversity in a patient, so proportional values can be strongly influenced by outlier values. Nevertheless, both these studies suggest that IE binding *via* group A and EPCR- binding PfEMP1 is an important contributor to RN CM etiology, and therefore that coma in RN CM is at least partially due to *P. falciparum*. While assessment of retinopathy in either study may have been imperfect, these studies represent real world assessment of retinopathy, and are probably more accurate than retinopathy diagnosis in a typical low-resource clinical setting in Africa. The use *Pf*HRP-2 cutoff levels, proposed as a simpler test to distinguish "true" CM, also showed similar *var* gene expression in those above vs. below the cutoff level and discourages the use of *Pf*HRP-2 to distinguish "true" CM from coma with incidental parasitemia. Assessment of *var* transcript levels in the field is unlikely to ever be a practical diagnostic tool, but could be very useful in future research studies of CM for attributing coma to *P.falciparum* or another cause.

Evolutionary, it has always been intriguing why *P.falciparum* parasites maintain *var* genes that sustain cytoadhesion when cytoadhesion and rosetting could result in death of the host. Our study's findings suggest that host mortality is not driven entirely by these adhesion traits, but rather by a combination of host and parasite factors. Parasites infecting CM children that died had lower transcript levels of DBLα1ALL (targeting all group A *var*) than CM children that survived, despite higher *Pf*HRP-2 levels in children who died compared to survivors, and this remained true when analysis was restricted to RP CM. A similar trend towards lower *var* transcript abundance in CM children that died was observed in a previous study[85]. Possible reasons for low *var* A transcript levels in those who died include the inability of group A *var* genes to provide an advantage at this stage of the disease, possibly because coagulation and rosette formation can promote mechanical sequestration and there is less need for active sequestration *via* PfEMP1 binding to host receptors. It is also possible that in the most severe forms of disease the parasite is expressing *var* genes characterized by weaker binding or binding to CD36. Quantifying transcript levels of these *var* genes and assessing *var* gene expression over time could help test these hypotheses.

Children with SM and AP in this study are from the same households in Kampala and therefore have had similar malaria exposure. Why parasites infecting certain patients have higher group A and EPCR-binding PfEMP1 transcript levels remains an interesting question. While it is becoming more apparent that epigenetic mechanisms regulate *var*

transcription[149], it is less well-understood how host factors and host environmental signals can affect these epigenetic mechanisms. In addition, our data shows that the parasites expressing the highest levels of group A and EPCR-binding *var* genes are not always the ones causing the most severe disease, emphasizing that other parasite binding proteins such as RIFINs, host factors (genetic or immunologic) and potential co-infections and co-morbidities could be determining whether the parasites expressing these group A or EPCR-binding PfEMP1 lead to severe malaria.

In conclusion, the current study shows that transcript abundance of EPCR-binding PfEMP1 were higher in CM than SMA; that group A PfEMP1 variants and many of the EPCR-binding PfEMP1 variants had similar transcript abundance in RP CM and RN CM, and that DBLα1ALL (targeting all group A *var*) was higher in CM children that survived vs. those that died. These findings suggest that expression of specific EPCR-binding PfEMP1 variants, in combination with host factors, could contribute to disease severity and clinical manifestation of disease in severe malaria, and the disruption of this binding could therefore help reduce morbidity due to severe malaria.

Chapter 5

Cerebrospinal fluid tumor necrosis factor-alpha levels are associated with coma duration and acute and long-term neurologic deficits in Ugandan children with cerebral malaria

5.1 Objectives

- ✓ Determine TNF-α levels in plasma and CSF of children with CM as compared to controls
- ✓ Evaluate the association of systemic and local TNF-α levels with markers of disease severity in CM such as coma duration, neurologic deficits and mortality

5.2 Introduction

The incomplete understanding of host-pathogen interactions in cerebral malaria (CM) has hindered the discovery of successful adjunctive therapies. Tumor necrosis factor-alpha (TNF-α) is considered an important contributor to CM pathogenesis due to its role in promoting endothelial activation, which can further increase binding of infected erythrocytes (IE) to host endothelium[150,151], an important hallmark of CM. Treatment with anti- TNF-α monoclonal antibody did not reduce mortality and was associated with increased risk of neurologic deficits in children with CM[152], suggesting that more work is needed to understand the regulation of TNF-α systemically and especially locally in CM and its association with acute and long-term neurocognitive deficits.

TNF-α is a pro-inflammatory cytokine with a broad spectrum of biological activities. It is primarily produced by activated immune cells such as macrophages and T and B cells, and is important in promoting macrophage activation, neutrophil recruitment and production of other pro-inflammatory cytokines[151,153]. High levels of TNF-α, however can contribute to pyrexia, tissue damage and apoptosis[151,153]. TNF-α is also an important cytokine in the central nervous system (CNS) that can be locally produced by microglia, astrocytes and neurons[154-156]. TNF-α is found in healthy neurons[157] and is important in controlling synaptic strength[158], but elevated levels in the CNS can lead to activation of astrocytes and microglia and demyelination, and have been implicated in a number of CNS diseases such as ischemic stroke, multiple sclerosis and Parkinson's disease[156,159,160].

Since the finding that *P.falciparum* infected erythrocytes stimulate TNF-α production from mononuclear cells[161], a number of studies have investigated the role of this cytokine in malarial infections. In mouse models of malaria, TNF-α has been shown to reduce parasitemia and protect against the early stages of infection[162-165] but has also been associated with disease severity when elevated at later stages[163]. TNF-α was also shown to be essential in the pathogenesis of experimental cerebral malaria[166,167]. This dual effect is thought to occur during human infections as well, as lower systemic TNF-α levels are seen at enrollment in patients with uncomplicated or mild malaria but higher levels are consistently detected in patients with severe malaria[118,168-171]. The pathogenic role of

TNF-α is attributed to its ability to limit the growth of erythroid precursors *in vitro*[172] and promote erythrophagocytosis and dyserythropoiesis[173], as well as its association with endothelial activation[150,174,175], which can promote IE adhesion to the endothelium [150].

In humans, two to ten fold elevated systemic TNF-α levels have been associated with mortality in children with severe malaria[119,170] and in children with CM specifically[118] in some studies, but not in others[168]. High levels of TNF-α have also been associated with hyperparasitemia and hypoglycemia[118,119], deeper coma[120] and endothelial activation[170] in severe malaria. In addition, TNF-α polymorphisms associated with high TNF-α expression were more prevalent in patients with CM and fatal CM[176,177]. Despite all this information hinting at elevated systemic TNF-α levels being pathogenic in severe malaria in humans, the use of antibodies against TNF-α had adverse effects in children with CM[152], suggesting that more work is needed to understand the role of this cytokine not only systemically but also locally in the CNS.

In the CNS, TNF-α production by microglia and astrocytes has been associated with fatal murine cerebral malaria[178]. The data from human studies of CNS TNF-α in CM patients is more limited, but also generally suggests a role for CNS TNF-α in CM: two studies have documented elevated CSF TNF-α levels in CM patients[120,179] (another did not) [119], and autopsy studies of individuals who died of CM have shown TNF-α expression in the brain parenchyma [180-182]. Our group has previously shown that high levels of TNF-α at enrollment in the CSF but not plasma were associated with neurologic deficits at three

months post-discharge and impaired attention and working memory at six months follow-up in children five years and older [179] suggesting a role for local TNF-α in the neurologic outcomes of CM. Whether these findings would be found be in the developing brains of children with CM who are younger than 5 years old is not known.

To better assess the role of systemic and CNS TNF-α in children with CM across the typical age spectrum in which CM is seen in Africa, we investigated how plasma and CSF TNF-α levels in Ugandan children 18 months to 12 years of age correlated with mortality, coma duration and acute and long-term neurologic deficits.

5.3 Methods

Cytokine Testing

Cytokine testing was performed on plasma and CSF samples collected at enrollment from children with CM. CSF samples were obtained for children with CM in whom a lumbar puncture was not contraindicated. Control CSF samples were obtained from North American children successfully treated for prior leukemia who had CSF obtained after treatment to rule out return of malignancy (ruled out in all).

Plasma and CSF levels of TNF-α were measured by a magnetic cyometric bead assay (EMD-Millipore, Billerica, MA) according to the manufacturer's instructions with a BioPlex-200 system (Bio-Rad, Hercules, CA). Plasma soluble intracellular adhesion

molecule-1 (sICAM-1) and vascular cellular adhesion molecule-1 (sVCAM-1) were also measured by magnetic cytometric bead assay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Plasma angiopoietin-2 (Ang-2) and von Willebrand Factor (VWF) levels were quantified using the human angiopoietin 2 DUO ELISA kit (R&D Systems, Minneapolis, MN) and REAADS von Willebrand Factor activity ELISA kit (Corgenix, Broomfield, CO), respectively. Plasma and CSF albumin were quantified by the Advanced Research and Diagnostic Laboratory at the University of Minnesota.

Statistical Analysis

Demographic characteristics were compared using t-tests for continuous measures and Pearson's χ^2 test for categorical variables. Plasma and CSF TNF-α levels, endothelial activation markers, coma duration, and number of seizures had skewed distributions, so for these variables, Wilcoxon rank-sum testing was used for comparisons between groups (e.g., children with vs. without neurologic deficits), and Spearman's rank correlation (rho) was used for assessment of correlation with continuous variables.

5.4 Results

Demographic characteristics of children with cerebral malaria

248 children with CM and 199 CC had sufficient plasma for TNF-α testing, and 166 children with CM had sufficient CSF sample available for TNF-α testing.

The 19 children who did not have plasma available for testing did not differ from the 248 children with plasma available for testing in terms of age (median 65.3 vs. 41.5 months, *P*=0.07), mortality (3/19, 15.6%, vs. 30/248, 12.1%, *P*=0.64), neurologic deficits at discharge (4/16 survivors, 25.0%, 79/216 survivors, 36.6%, *P*=0.35) or coma duration (median 42 vs. 46 hours, *P*=0.41).

The 82 children who had plasma but no CSF available for testing differed from the 166 children who had CSF available for testing in mortality (21/82, 25.6%, vs. 9/166, 5.4%, *P*<0.001), neurologic deficits at discharge (16/61 survivors, 26.2%, 63/155 survivors, 40.7%, *P*=0.05) and coma duration during admission (median 36.5 vs. 56.3 hours, *P*<0.0001) but not age (median 44.7 vs. 39.8 months, *P*=0.09).

The median age of the children in the study was 42 months. Age did not differ significantly between CM (n=248, median, months [25th percentile, 75th percentile], 41.5 [30.2, 56.9]) and CC children (n=199, 43.2 [32.1, 56.5], *P*=0.39). A higher proportion of children with CM than CC were male (59.3% vs. 45.7% respectively, *P*=0.004). The median parasite density for children with CM was 47880 parasites/μl [11360, 234360]. Plasma and CSF TNF-α levels were not associated with age (Spearman's rho -0.10, *P*=0.13 and Spearman's rho -0.10, *P*=0.22, respectively), sex (*P*>0.20 for both) or weight for age z-score (Spearman's' rho 0.06, *P*=0.37 and Spearman's rho 0.07, *P*=0.34, respectively) in children with CM. Regression models adjusting for these factors were

therefore not employed when comparing plasma and CSF TNF-α levels to clinical outcomes.

Relationship between plasma and cerebrospinal fluid TNF-α levels

Median plasma TNF-α levels in children with CM (n=248, median, pg/ml [25th percentile, 75th percentile], 104pg/ml [49.4, 209]) were significantly higher than CC (n=199, 26.4pg/ml [18.0, 41.8], P<0.0001, Figure 5.1a). In addition, children with CM had significantly higher CSF levels of TNF-α (n=166, 1.35pg/ml [0.55, 3.10]) as compared to control North American children with prior neoplastic disease (n=13, 0.02pg/ml [0.02, 0.06], P<0.0001 Figure 5.1b). CSF TNF-α was only weakly correlated with plasma TNF-α levels in children with CM (Spearman's rho 0.15, P=0.06), suggesting that CSF levels of TNF-α may reflect local CNS production of TNF-α. To investigate this further, we assessed the association of CSF-to-plasma TNF-α ratio (CSF TNF-α x1000/Plasma TNF-α (pg/ml)) with CSF-to-plasma albumin ratio (CSF albumin x1000/Plasma albumin (mg/L)). The TNF-α ratio correlated positively with the albumin index (Spearman's rho=0.29, P=0.0003), suggesting that BBB leakage affects the levels of TNF-α seen in the CSF. However when looking at the absolute values for these ratios, CSF-to-plasma TNF-α ratios (n=166, median [25th percentile, 75th percentile], 13.0 [3.1, 35.9]) are higher than the values for albumin index (n=148, 5.3 [3.1, 10.4]) suggesting some local production of TNF-α in the CNS.

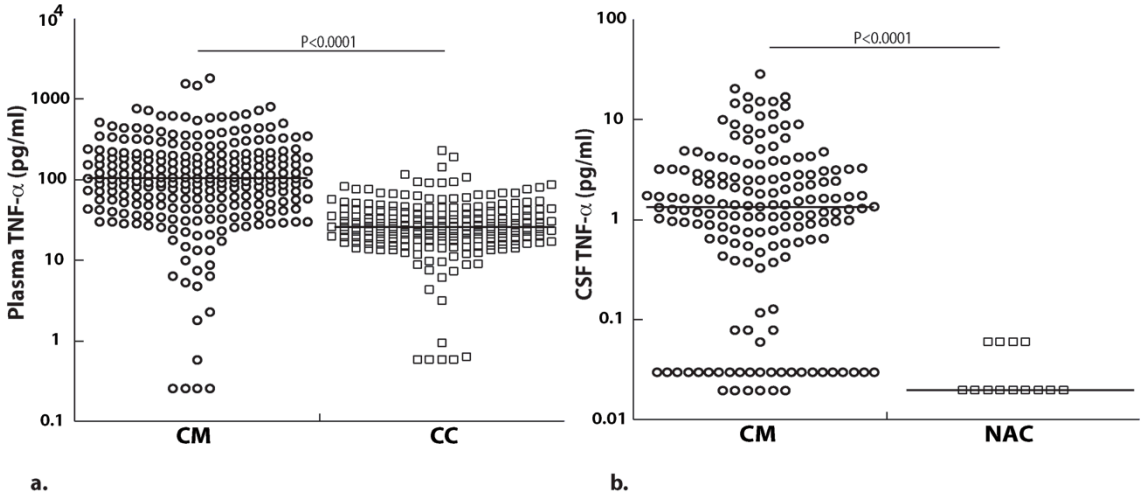


Figure 5.1. Plasma and CSF TNF-α levels at enrollment are higher in children with cerebral malaria than controls

(a) Plasma and (b) CSF TNF-α (on a logarithmic scale) at enrollment. The horizontal line represents median values. Two-sample Wilcoxon rank-sum (Mann-Whitney) test used to compare median levels between groups. Cerebral malaria (CM), Ugandan community children (CC), North American control children (NAC). NAC samples were obtained from children successfully treated for prior leukemia who had CSF obtained after treatment to rule out return of malignancy (ruled out in all).

Plasma and CSF TNF-α levels do not differ according to acute mortality

Plasma TNF-α levels did not differ significantly between children with CM who died (n=30, median, pg/ml, [25th percentile, 75th percentile], 108 [50.4, 270]) as compared to those who survived (n=218, 103 [48.7, 196], *P*=0.29, Figure 5.2a). CSF TNF-α levels also did not differ significantly between children with CM who died (n=9, 1.29 [0.97, 3.15]) and those who survived (n=157, 1.36 [0.48, 2.94], *P*=0.58, Figure 5.2b).

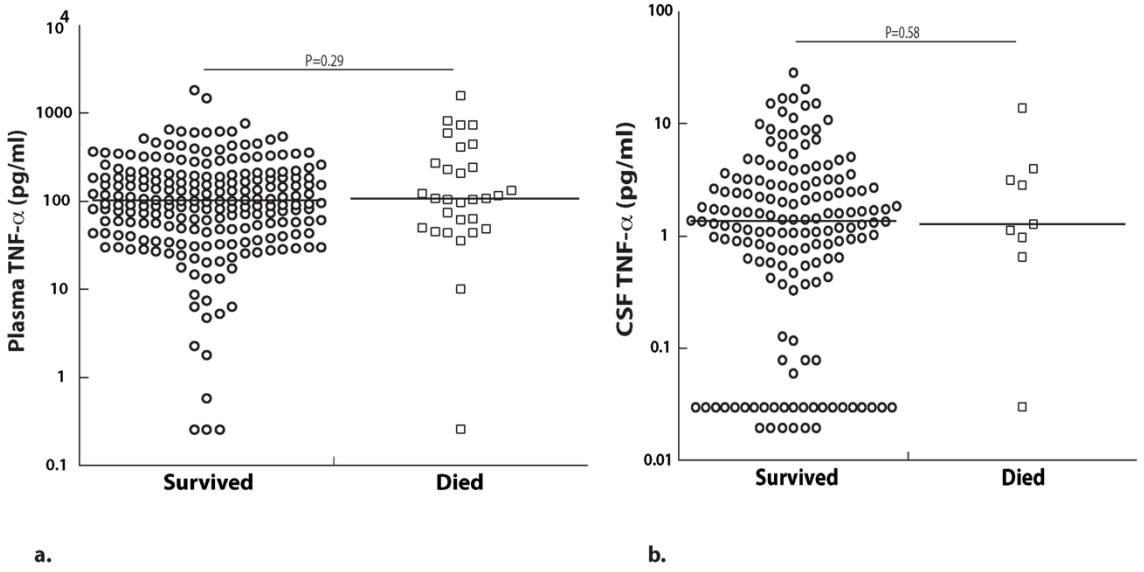


Figure 5.2. Plasma and CSF TNF-α levels at enrollment are not significantly different between cerebral malaria children that died vs. those that survived

(a) Plasma and (b) CSF TNF-α (on a logarithmic scale) at enrollment based on survival outcome. The horizontal line represents median values. Two-sample Wilcoxon rank-sum (Mann-Whitney) test used to compare median levels between groups.

Elevated CSF TNF-α levels are associated with prolonged coma during admission and neurologic deficits at discharge and 6-months follow-up

Plasma TNF-α levels did not differ significantly between children with (n=79, median, pg/ml, [25th percentile, 75th percentile], 89.9pg/ml [31.6, 213]) vs. without (n=137, 105pg/ml [54.0, 195], *P*=0.39, Figure 5.3a) neurologic deficits at discharge and between CM children with (n=11, 57.4pg/ml [26.0, 274]) or without neurologic deficits at 6-months follow-up (n=197, 103pg/ml [50.4, 191], *P*=0.62, Figure 5.3a). However, levels of TNF-α in the CSF were significantly higher in children with CM who were discharged

with neurologic deficits (n=63, 1.74pg/ml [0.86, 4.37]) as compared to those who were not (n=92, 1.16pg/ml [0.36, 2.64], *P*=0.04, Figure 5.3b). CSF TNF-α levels also differed between children with (n=10, 2.19pg/ml [1.41, 4.37]) vs. without neurologic deficits at 6-months follow-up (n=140, 1.20pg/ml [0.38, 2.74], *P*=0.05, Figure 5.3b).

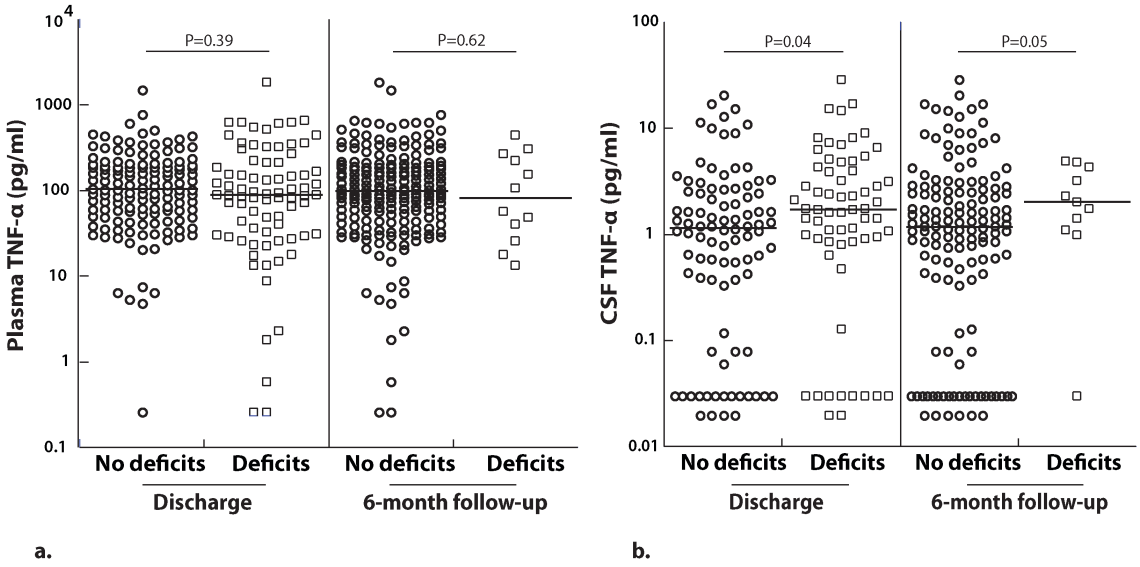


Figure 5.3. CSF TNF-α levels at enrollment are higher in cerebral malaria children discharged with neurologic deficits and those who had neurologic deficits at 6-months follow-up

(a) Plasma and (b) CSF TNF-α (on a logarithmic scale) at enrollment based on neurologic outcomes at discharge or 6-months follow-up. The horizontal line represents median values. Two-sample Wilcoxon rank-sum (Mann-Whitney) test used to compare median levels between groups.

CSF TNF-α, but not plasma TNF-α was positively associated with coma duration in children with CM (n=157, Spearman's rho 0.18, *P*=0.02 and n=217, Spearman's rho -0.05, *P*=0.43, respectively). Neither plasma nor CSF TNF-α levels were associated with

number of seizures during admission (n=248, Spearman's rho 0.02, *P*=0.81 and n=166, Spearman's rho 0.02, *P*=0.80, respectively).

Plasma and CSF TNF-α levels do not differ according to malaria retinopathy

The presence of malarial retinopathy at admission has been associated with brain sequestration post-mortem in cerebral malaria [36], but it is unclear if children with clinical CM and no retinopathy have a milder form of CM or an alternative cause of coma. To assess whether the presence of retinopathy was associated with differences in TNF-α responses, we compared TNF-α levels in children with CM with vs. without retinopathy. Plasma TNF-α levels did not differ significantly between retinopathy positive (RP, n=158, median, pg/ml, [25th percentile, 75th percentile], 104pg/ml [45.8, 190]) and retinopathy negative CM children (RN, n=80, 123pg/ml [53.8, 211], *P*=0.62, Figure 5.4a). CSF TNF-α levels also did not differ significantly between RP (n=110, 1.41pg/ml [0.44, 3.25]) and RN CM (n=52, 1.18pg/ml [0.57, 2.31], *P*=0.30, Figure 5.4b).

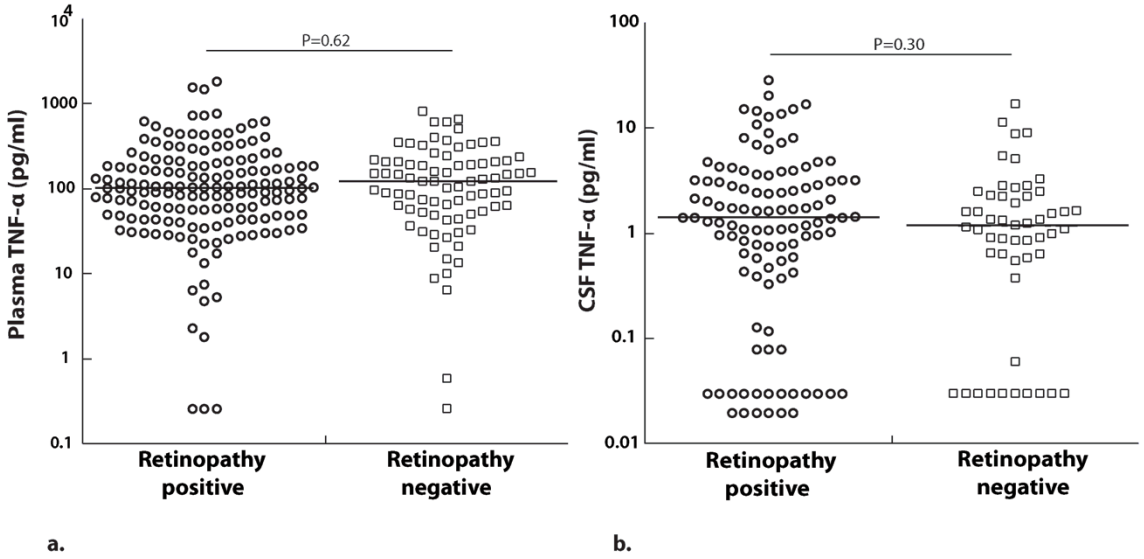


Figure 5.4. Plasma and CSF TNF-α levels at enrollment are not significantly different between children with cerebral malaria with or without retinopathy

(a) Plasma and (b) CSF TNF-α (on a logarithmic scale) at enrollment based on retinopathy characteristics. The horizontal line represents median values. Two-sample Wilcoxon rank-sum (Mann-Whitney) test used to compare median levels between groups.

Plasma TNF-α levels correlate with parasite biomass and endothelial activation

Considering the role of TNF-α in endothelium activation, and as a consequence its role in promoting parasite sequestration, we investigated how plasma TNF-α levels correlated with markers of endothelial activation such as soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble P-Selectin and E-Selectin, angiopoietin-2 (Ang-2) and von Willebrand Factor (VWF) in children with CM. We also assessed the correlation of plasma TNF-α levels with total and

sequestered parasite biomass. Plasma TNF-α levels correlated strongly and positively with all markers of endothelial activation (P<0.0004 for all, Table 5.1) except VWF. Plasma TNF-α levels were also associated with increased *Pf*HRP-2 levels, as well as total and sequestered parasite biomass (P<0.0001 for all, Table 5.1), suggesting an important role for this cytokine in endothelial activation and parasite sequestration and persistence.

Table 5.1. Association of plasma TNF-α levels with endothelial activation markers and parasite biomass in children with CM

	Plasma TNF-α		
	N	Spearman's rho	P
sP-Selectin	194	0.30	<0.0001
sE-Selectin	204	0.37	<0.0001
sICAM-1	204	0.25	0.0003
sVCAM-1	204	0.33	<0.0001
Ang2	152	0.51	<0.0001
VWF	198	0.05	0.50
*Pf*HRP-2	248	0.57	<0.0001
Total parasite biomass	248	0.55	<0.0001
Sequestered parasite biomass	241	0.49	<0.0001
Circulating parasite biomass	241	0.30	<0.0001

5.5 Discussion

In the present study, we show that CSF but not plasma TNF-α correlate with key clinical outcomes in children with CM, including coma duration and acute and long-term neurologic deficits. The study findings demonstrate the importance of CNS TNF-α in the neurologic outcomes in children with CM, and suggest that CNS TNF-α, or factors, such as sequestered parasite biomass, that appear to affect CNS TNF-α production, may still be good targets for adjunctive therapy to reduce neurologic morbidity in CM.

Plasma TNF-α levels were 4-fold higher in children with CM as compared to healthy community controls (Figure 5.1a). TNF-α is released mainly upon activation of the innate[161] and adaptive immune system[183-186] in response to *P. falciparum*-infected red blood cell proteins and toxins. Though we cannot determine the exact source of this cytokine in the current study or the specific stimulants, TNF-α levels were positively associated with parasite density at enrollment (Spearman's rho 0.31, $P<0.0001$) in children with CM supporting the role of a high infectious burden in immune activation and TNF-α production. TNF-α is also important in endothelial activation[150,175] which can promote sequestration[150] , further production of other pro-inflammatory cytokines[174,175], release of endothelial microparticles and induction of apoptosis[175]. Sequestration is an important immune evasion mechanism allowing the parasite to evade spleen clearance and persist. An association between plasma TNF-α levels, endothelial activation markers and parasite sequestration is supported in the present study by the positive correlation between plasma TNF-α levels and the endothelial activation markers, *Pf*HRP-2 levels and sequestered parasite biomass (Table 5.1).

Plasma TNF-α levels were not associated with mortality, neurologic deficits at discharge or 6-months follow-up, or coma duration and number of seizures during admission in the current study. Similar to other studies of this nature, we observe a wide range of plasma TNF-α levels in our cohort. This could be due to genetic factors that can affect TNF-α levels[176,177], or possibly to differences in malaria exposure, since it has been shown that

with increased exposure, the production of TNF-α from CD4+ T cells is reduced[184,185]. The children in the present study come from an approximately 25km radius of the hospital, and so could have some variability in malaria exposure.

In the current study, CSF TNF-α levels were elevated in CM children as compared to controls (Figure 5.1b). Elevated levels of TNF-α in the CNS have been shown in a number of disorders such as multiple sclerosis[159], Parkinson's disease[160], and murine[178] and human cerebral malaria[180-182]. However, most of the human CM studies that have looked at local TNF-α have done so in brain tissue. Despite allowing for careful assessment of areas of the brain affected by TNF-α, these types of studies are limited in sample size and do not permit assessment of the role of CNS TNF-α in children who survive CM. As a result, quantification of CSF TNF-α allows for evaluation of CNS TNF-α in survivors of CM, and the correlation of CSF TNF-α with clinical outcomes. Parasite sequestration and systemic inflammation are thought to lead to BBB damage and leakage in CM, which could expose the brain parenchyma to plasma proteins and promote astrocyte and microglial activation[187]. In the present study, the albumin index and CSF to plasma TNF-α ratio were strongly correlated, but the absolute values for TNF-α ratios were higher than the albumin index, suggesting that TNF-α found in the CSF is not entirely due to the BBB leakage but is also partially produced in the CNS. Though we cannot determine the source of CSF TNF-α in the study children, activated astrocytes and microglia are frequent sources of CSN TNF-α [154,155]. While the inflammatory pathways that occur locally are most likely complicated, our data suggests

local production of TNF-α in the CNS of CM children, possibly added to by crossing of some plasma TNF-α across an impaired BBB.

We have previously shown an association of CSF TNF-α with neurologic deficits at 3-months follow-up and impaired attention and working memory at 6-months post-discharge in children 5 years and older[179]. In the present study we present a larger cohort and have expanded the age range between 18-months to 12 years old. In the present study, median CSF TNF-α levels were higher in children that were discharged with neurologic deficits and that continued having neurologic deficits at 6-months follow-up compared to those children that did not have acute or long-term neurologic deficits (Figure 5.3). These findings expand on the adverse effects of CSF TNF-α on neurologic deficits to children younger than 5 years old [179].

We did not see a significant difference in plasma TNF-α levels in CM children with vs. without malarial retinopathy, findings that differ from one recent study [188]. In addition, CSF TNF-α levels did not differ significantly between retinopathy positive and negative CM, suggesting a role for this cytokine in the pathogenesis of CM, despite the presence of malarial retinopathy.

In the present study, we were not able to obtain CSF from all CM children. In addition, in some cases the CSF obtained was not sufficient. Children who were not tested for CSF TNF-α had higher mortality, lower chance of being discharged with neurologic deficits

and shorter duration of coma during admission than those who had CSF obtained and enough sample for TNF-α testing. Thus, a study limitation is that the associations of CSF TNF-α with disease outcomes in CM can be captured only for a subset of children.

In conclusion, our study data show that plasma and CSF TNF-α levels are elevated in children with cerebral malaria, and that elevated CSF TNF-α levels in children with CM are associated with prolonged coma and acute and long-term neurologic deficits. Our results emphasize the importance of studying both the peripheral and CNS immune responses since they do not always tell the same story, suggest that CNS TNF-α, as opposed to systemic TNF-α, is particularly important in neurologic outcomes in children with CM, and demonstrate the need for assessment of how age may alter the effects of risk factors for neurologic impairment. Further studies that aim to understand the role and regulation of CNS TNF-α in children with CM, with the goal of targeting the CNS production and effects of TNF-α, could lead to adjunctive treatments that decrease neurologic morbidity in CM.

Chapter 6

Systemic immunologic markers of malarial disease severity in Ugandan children

6.1 Objectives

- ✓ Identify immunologic markers that distinguish between cerebral malaria and severe malarial anemia
- ✓ Determine immune markers that predict the risk of mortality and neurologic deficits in children with CM.

6.2 Introduction

Identifying an immunologic profile that differentiates between various manifestations of severe malaria could help our understanding of the pathways that contribute to these forms of severe disease, as well as inform the design of better diagnostics and successful adjunctive therapies.

Blood stage infection in malaria is characterized by elevated pro-inflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12p70), which induce fever and other symptoms of malaria, but also contribute to controlling the infection[32,189,190]. IFN-γ and TNF-α are produced early in the infection[191,192] and are important in mediating

macrophage and neutrophil activation to control parasite load[193,194]. IFN-γ and TNF-α can also induce the production of IL-1β, more TNF-α and IL-6, which allows for further control of early parasite blood-stage infection and priming of an adaptive immune response[32,189,190]. Parasite factors such as hemozoin, together with inflammatory cytokines stimulate production of a number of inflammatory chemokines such as interleukin-8 (IL-8), macrophage inflammatory-1 alpha (MIP-1α), MIP-1β, and monocyte chemoattractant protein-1 (MCP-1)[45,195]. Interferon gamma inducible protein-10 (IP-10) is also elevated in malaria[196,197], whereas regulated on activation normal T cell expressed and secreted (RANTES) is usually downregulated[45,198]. While chemokines are generally important in immune cell recruitment to sites of inflammation, the exact role of these chemokines in human malaria remains understudied. Anti-inflammatory cytokines such as interleukin-10 (IL-10) are also seen elevated during a malaria infection and are important in controlling inflammation and tissue damage in malaria[32,189,190].

Severe malaria is characterized by an over-vigorous and imbalanced immune response[189,190]. More specifically, childhood severe malaria is typically characterized by elevated IFN-γ, TNF-α, IL-6, IL-10, IL-1β, IL-1ra [40-44], as well as elevated levels of IL-8, IP-10 and reduced levels of RANTES as compared to uncomplicated or mild malaria[43-45]. However, not all studies have found the same analytes elevated or downregulated across the board. This highlights the variability introduced by host genetics, sex and age in these studies, as well as the impact on the immune response of malaria endemicity, environmental factors and time in the disease progression at which patients are studied.

Importantly, a small proportion of febrile uncomplicated malaria patients progress to severe disease and the factors that lead to this progression are complex and difficult to study, since children who are treated for uncomplicated malaria rarely progress to severe disease. As a result, human studies looking at the immunologic profile in malaria patients are better designed to identify markers of disease severity and potential targets for adjunctive therapies, rather than to determine the pathways that lead from uncomplicated to severe malaria.

A number of studies have focused on identifying immunologic markers of disease severity in malaria. Elevated TNF-α levels have been associated with mortality in children with severe malaria[119,170] and in children with cerebral malaria (CM) specifically[118] in some studies, but not in others[168]. Elevated serum IP-10 and IL-1ra, along with reduced RANTES, have also been associated with mortality in pediatric severe malaria[43,44,196]. In addition, high levels of TNF-α were associated with other markers of disease severity such as hyperparasitemia and hypoglycemia[118,119], deeper coma[120], and endothelial activation[170]. The immune regulation of TNF-α by IL-10[199,200] is important for controlling immunopathology. An imbalance of these two cytokines as indicated by low IL-10 to TNF-α ratio (IL-10: TNF-α) has been associated with severe malarial anemia (SMA)[201-203]. High levels of IL-10 and TNF-α have also been associated with respiratory distress in severe malaria[42]. Moreover, low levels of RANTES were associated with severity of anemia in malaria[64] . Growth factors such as vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF)

were also shown to be important in SM. Elevated VEGF levels were associated with seizures and signs of intracranial pressure in Kenyan children with CM[204], but lower levels of VEGF have been associated with increased severity in some studies of severe malaria in adults[205,206]. There has been no comparison to date of VEGF levels between CM and another form of severe disease in pediatric patients. G-CSF levels were elevated in CM as compared to uncomplicated malaria and were higher in children that died of CM in Ugandan children[44]. These studies have provided new insights into how inflammation may lead to disease severity in malaria, but typically grouped multiple manifestations of severe malaria, and often assessed a limited number of cytokines and chemokines and had a relatively small sample size.

To better assess how pro- and anti-inflammatory cytokines and chemokines and angiogenic growth factors may contribute to development of two very different forms of severe malaria, cerebral malaria (CM) and severe malarial anemia (SMA), we assessed 18 different cytokines, chemokines and angiogenic growth factors in a large study cohort of Ugandan children with CM (n=239), SMA (n=174) or healthy community children (CC, n = 161) from the same extended household or neighborhood as the children with CM or SMA. To define how the immune response may affect disease outcomes in children with CM, we further assessed how these cytokines, chemokines or angiogenic growth factors related to mortality, neurologic deficits, coma duration and seizure number in children with CM.

6.3 Methods

Cytokine Testing

Plasma was processed from peripheral venous blood collected in EDTA tubes at enrollment and was stored long-term in -80^{o}C. North American control samples (NAC) were obtained from healthy adults who had never been to a malaria endemic country and who were healthy at the time of blood collection.

Plasma concentrations of 16 analytes: IFN-γ, IL-1β, IL-1ra, IL-4, IL-8, IL-10, IL-12p70, IP-10, MCP-1, MIP-1α, MIP-1β, G-CSF, FGF basic, PDGF-BB, RANTES and VEGF were measured using the Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad, Hercules, CA) in plasma diluted 1:4, according to manufacturer's instruction. We selected those 16 analytes from the 27-plex assay based on their importance in malaria, severe malaria and inflammatory processes in general. Plasma levels of TNF-α and IL-6 were measured by magnetic cyometric bead assay (EMD-Millipore, Billerica, MA) according to the manufacturer's instructions. All the testing was performed with a BioPlex-200 system (Bio-Rad, Hercules, CA). To assess intra-assay reproducibility, 10% of samples were randomly selected from each assay plate to re-test on subsequent plates. The mean coefficient of variance for these samples for all analytes was 25.8%.

Because concurrent parasitemia can alter peripheral blood cytokine levels[207], only cytokine/chemokine/growth factor levels in CC with no *P. falciparum* parasitemia by microscopy (n=161) were compared to the levels in children with CM or SMA.

Statistical Analysis

Measures with skewed distributions, which included all immune markers, were replaced by their common logs (log to base 10) for ANOVA or regression analyses. Cytokine and chemokine levels were compared between groups using Wilcoxon rank-sum when comparing two groups or ANOVA, followed by Tukey's post-hoc test when comparing more than two groups. Clinical and laboratory findings for children in the different disease groups were compared using the chi-squared test if categorical and if continuous, Wilcoxon rank-sum when comparing two groups, and ANOVA followed by Tukey's post-hoc test when comparing more than two groups. Linear regression analysis was used for continuous outcomes and logistic regression for categorical outcomes. Regression analyses were adjusted for potential confounding variables as indicated in the Results section and tables. Multivariate regression analysis was performed including the immune mediators that showed $P<0.10$ in the univariate regression analyses for the specific outcome, adjusting for potential confounding variables as indicated in the Results section.

6.4 Results

Distribution of cytokines and chemokines in severe malaria and community children

Levels of plasma IFN-γ, TNF-α, IL-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-12p70, IP-10, MCP-1, MIP-1α, MIP-1β, G-CSF, FGF-basic, PDGF-BB, RANTES and VEGF were

quantified in 413 children with severe malaria (SM), 239 cerebral malaria (CM) and 174 severe malarial anemia (SMA) and in 161 healthy community children enrolled from the households or neighborhood of children with severe malaria. To get an idea of the balance between pro- and anti-inflammatory cytokines in an individual, we also calculated IL-10 to TNF- α ratio (IL-10: TNF-α). IL-1β and IL-4 were on the lower end of the standard curve and had little variance in all samples studied (n=594, median pg/ml [25th percentile, 75th percentile], 3.03pg/ml [1.91, 4.84] and 2.78pg/ml [1.09, 4.79], respectively), therefore they are not included in further analysis.

As expected, children with severe malaria had elevated levels of a number of pro- and anti-inflammatory cytokines and chemokines. IL-1ra, IL-6, IL-8, IL-10, TNF-α, IL-10: TNF-α, G-CSF, IP-10, MCP-1, MIP-1β, and VEGF were higher in children with SM than CC (Table 6.1). In contrast, PDGF-BB, FGF-basic, and RANTES were lower in SM compared to CC. Surprisingly; IFN-γ levels were also lower in children with SM (n=413, median pg/ml [25th percentile, 75th percentile], 96.9pg/ml [58.4, 170]) as compared to CC (n=161, 150pg/ml [81.9, 254], P<0.0001, Table 6.1). The results were similar when comparing children with SM to community children who were negative for *P.falciparum* by PCR (Supplemental Table 6.1).

As compared to CC (n=161) children with SM (n=413) were younger (age in months, median [25th percentile, 75th percentile], SM, 38.5 [26.9, 53.9], CC, 44.2 [32.0-55.6], P=0.01), and more malnourished (weight for age z-score, mean [standard deviation], SM,

-1.46 [1.33], CC, -0.89 [1.09], P<0.0001). A higher proportion of children with SM were male (61.3% vs. 46.6%, P=0.001). In a logistic regression model that included age, sex, and weight for age z-score, a log10 increase in the cytokine value was associated with a 3- (G-CSF, VEGF), 4- (IL-6), 6- (TNF-α), 7- (MCP-1), 16- (IL-8), 40- (IL-10:TNF-α), 69- (IL-1ra), 140- (IP-10), 166- (IL-10), and 2179- (MIP-1β) fold increased risk of SM (Table 6.2). PDGF-BB and RANTES were associated with significantly reduced risk of SM (Table 6.2), however when adjusting for number of platelets, which are important source for PDGF-BB and RANTES in the body[208], only RANTES remained associated with an 80% reduced risk of severe malaria (one log10 increase in RANTES, odds ratio (OR) 0.20, 95% CI 0.08-0.48, P<0.001). Additionally IFN-γ was also associated with reduced risk of severe malaria upon adjusting for age, sex, and weight for z-score (Table 6.2). FGF-basic was no longer associated with reduced risk of SM when adjusting for platelet number, an important source of FGF-basic[208] (OR 1.04, 95% CI 0.50-2.15, P=0.92). To address our main questions of which immune markers best distinguished between CM and SMA and were associated with morbidity and mortality in CM, we focused only on the immune markers that clearly differentiated severe malaria from CC. Therefore, IL-12p70, MIP-1α, FGF-basic and PDGF-BB were not considered further.

Table 6.1. Differences in plasma cytokines and chemokines between severe malaria and community control children

	SM (n=413)	CC (n=161)	P[a]
IL-1ra (pg/ml), median (IQR)	900 (398-2840)	216 (127-320)	<0.0001
IL-8 (pg/ml), median (IQR)	36.8 (23.5-66.8)	19.7 (12.8-31.2)	<0.0001
IL-10 (pg/ml), median (IQR)	163 (55.4-560)	8.68 (4.51-14.7)	<0.0001
IL-12p70 (pg/ml), median (IQR)	20.3 (11.6-36.8)	22.1 (13.7-39.4)	0.23
FGF-basic (pg/ml), median (IQR)	32.7 (12.8-48.6)	45.4 (27.4-65.4)	<0.0001
G-CSF (pg/ml), median (IQR)	67.2 (39.5-137)	48.5 (29.3-75.2)	<0.0001
IFN-γ (pg/ml), median (IQR)	96.9 (58.4-170)	150 (81.9-254)	<0.0001
IP-10 (pg/ml), median (IQR)	3566 (1421-9540)	559 (414-816)	<0.0001
MCP-1 (pg/ml), median (IQR)	56.4 (25.6-180)	21.8 (15.0-32.7)	<0.0001
MIP-1α (pg/ml), median (IQR)	6.73 (3.93-11.4)	6.81 (4.15-10.9)	0.82
MIP-1β (pg/ml), median (IQR)	306 (185-533)	75.1 (59.5-104)	<0.0001
PDGF-BB (pg/ml), median (IQR)	730 (301-1424)	1218 (610-2287)	<0.0001
RANTES (pg/ml), median (IQR)	2563 (1412-5351)	7578 (3713-12053)	<0.0001
VEGF (pg/ml), median (IQR)	55.0 (33.0-98.1)	39.0 (22.6-69.7)	<0.0001
TNF-α (pg/ml), median (IQR)*	93.0 (49.3-175)	26.4 (18.1-41.6)	<0.0001
IL-6 (pg/ml), median (IQR)*	53.7 (18.8-216)	12.5 (2.65-39.8)	<0.0001
IL-10: TNF- α ratio, median (IQR)*	1.88 (0.97-4.11)	0.24 (0.15-0.54)	<0.0001

[a] Wilcoxon rank-sum (Mann-Whitney) test.
*SM (n=391), CC (n=153)

Table 6.2. Association of plasma immune markers with severe malaria compared to community controls

	OR[a] (95% CI)	P[b]
IL-1ra (pg/ml)	69.2 (29.8-161)	**<0.001**
IL-8 (pg/ml)	16.1 (7.95-32.6)	**<0.001**
IL-10 (pg/ml)	166 (61.4-451)	**<0.001**
IL-12p70 (pg/ml)	0.79 (0.51-1.22)	0.29
FGF-basic (pg/ml)	0.25 (0.14-0.44)	**<0.001**
G-CSF (pg/ml)	2.82 (1.81-4.41)	**<0.001**
IFN-γ (pg/ml)	0.40 (0.24-0.64)	**<0.001**
IP-10 (pg/ml)	140 (55.4-355)	**<0.001**
MCP-1 (pg/ml)	7.28 (4.45-11.9)	**<0.001**
MIP-1α (pg/ml)	0.80 (0.53-1.21)	0.29
MIP-1β (pg/ml)	2179 (555-8546)	**<0.001**
PDGF-BB (pg/ml)	0.30 (0.19-0.47)	**<0.001**
RANTES (pg/ml)	0.04 (0.02-0.09)	**<0.001**
VEGF (pg/ml)	2.58 (1.65-4.04)	**<0.001**
TNF-α (pg/ml)	5.60 (3.58-8.78)	**<0.001**
IL-6 (pg/ml)	3.53 (2.66-4.68)	**<0.001**
IL-10: TNF-α	40.4 (20.7-79.0)	**<0.001**

[a] OR, odds ratio, comparing severe malaria to community controls
[b] Models adjusted for age, sex and weight for age z-score. All cytokine levels were log transformed (log base 10)

Baseline characteristics of children with cerebral malaria and severe malarial anemia

Fifty-four of the 239 children with CM also had SMA. This group is presented here as CM/SMA and was separated from the CM group to clearly address the question of whether CM children have a unique immunologic profile as compared to SMA. Children with SMA or CM/SMA were younger than children with CM, had lower hemoglobin levels by definition, and a higher white blood cell count (Table 6.3). Children with CM or CM/SMA had a lower platelet count, higher *Pf*HRP-2 levels and sequestered parasite biomass, and also were more likely to have taken anti-malarials prior to hospitalization than children with SMA (Table 6.3). Children with CM/SMA had the highest sequestered

biomass of any group. The prevalence of co-infections (HIV, bacteremia, stool helminths) was low and similar between all three groups (Table 6.3).

Table 6.3. Baseline characteristics of children with cerebral malaria and severe malarial anemia

	CM (n=185)	CM/SMA (n=54)	SMA (n=174)	P[a]
Age (months), median (IQR)	43.8 (32.5-63.6)	35.7 (26.7-46.9)	31.6 (24.2-49.4)	<0.0001[b]
Sex (male), n (%)	112 (60.5)	31 (57.4)	110 (63.2)	0.72
Weight for age z-score, mean (SD)	-1.27 (1.22)	-1.40 (1.17)	-1.68 (1.45)	0.01[c]
Anti-malarial prior to hospitalization, n (%)	147 (79.5)	45 (83.3)	105 (60.3)	<0.0001[d]
HIV positive, n (%)	4 (2.38) n=168	1 (1.92) n=52	4 (2.34) n=171	0.98
Hookworm , n (%)	1 (0.54)	0 (0)	2 (1.15)	0.63
Stool positive for parasites, n (%)	8 (4.37) n=183	3 (5.66) n=53	7 (4.24) n=165	0.89
Positive blood culture, n (%)	16 (8.70) n=184	7 (13.0) n=54	17 (9.88) n=172	0.59
Hemoglobin (g/dL), mean (SD)	7.84 (1.96)	4.10 (0.77)	3.81 (0.86)	<0.0001[b]
Platelet number ($\times 10^3/\mu L$), median (IQR)	57.5 (34-101) n=182	79 (47-129) n=53	146 (88-218) n=172	<0.0001[d]
White blood cell count ($\times 10^3/\mu L$), median (IQR)	8.75 (6.50-12.3) n=182	12.4 (7.9-18.8) n=53	10.9 (8.25-14.6) n=172	<0.0001[b]
Parasite density (/µl), median (IQR)	55620 (15040-353305) n=180	23225 (9050-145700) n=52	46240 (12560-200620) n=173	0.09
Parasite load (PfHRP2, ng/ml), median (IQR)	2335 (845-5083)	3926 (1802-5887)	1072 (512-2790)	<0.0001[d]
Sequestered biomass (x10^8), median (IQR)	13300 (4614-33888) n=180	30026 (14446-49311) n=52	7853 (2475-17977) n=173	<0.0001[e]

[a] ANOVA followed by Tukey post-hoc adjustment for continuous variables. For continuous variables that did not have a normal distribution, values were log (log10) transformed. For categorical values, Chi2 test was used and $P<0.017$ was considered significant to account for multiple comparisons.
[b] CM differed from CM/SMA and SMA
[c] CM differed from SMA
[d] SMA differed from CM and CM/SMA
[e] All groups differed from each other

Immunologic profile in Ugandan children with cerebral malaria or severe malarial anemia

In order to determine which cytokines and chemokines best distinguish CM from SMA we compared the levels for each of these analytes between CM, SMA and CM/SMA. We hypothesized that the analytes differentially regulated in CM and CM/SMA, as compared to SMA, would be important in unique pathways that contribute to the pathogenesis of CM. Additionally, the immune markers differentially regulated in SMA and CM/SMA, as compared to CM, would contribute to the pathogenesis of SMA. Children with CM or CM/SMA had higher levels of IL-1ra, IL-6, IL-8, IL-10, and IP-10 and lower levels of RANTES than children with SMA (Figure 6.1), suggesting these cytokines and chemokines could be contributing specifically to the cerebral manifestations of the disease. The IL10:TNF-α ratio and MCP-1 were lower in SMA and CM/SMA as compared to CM, suggesting their importance in severe malarial anemia. G-CSF differed only between CM and SMA (Supplemental Figure 6.1) when adjusting for multiple comparisons. TNF-α (Figure 6.1), IFN-γ, MIP-1β and VEGF (Supplemental Figure 6.1) did not significantly differ between the three groups.

Since CM and SMA differed in age, weight for age z-score, number of platelets, white blood cell count, *Pf*HRP-2 levels, and the use of anti-malarials prior to enrollment, all of which could potentially affect cytokine levels we adjusted for these factors in a logistic regression model that assessed the levels of cytokines, chemokines and growth factors in children with CM compared to SMA. For this analysis, children with CM only were

compared to children with SMA only. In the regression model, increased levels of IFN-γ, IL-1ra, IL-8, IL-10, IL-10:TNF-α, MCP-1, IP-10, and G-CSF were associated with increased risk of CM ($P<0.05$ for all, Table 6.4). In a multivariate regression model including all the immune markers that had $P<0.10$ for the univariate regression models (Table 6.4), adjusting for age, weight for age z-score, anti-malarial treatment prior to admission, platelet and white blood cell count, and *Pf*HRP-2 levels, elevated IL-10:TNF-α (one log10 increase in IL10:TNF-α, odds ratio (OR) 4.80, 95% CI 1.94-11.9, $P=0.001$) was independently associated with increased risk of CM.

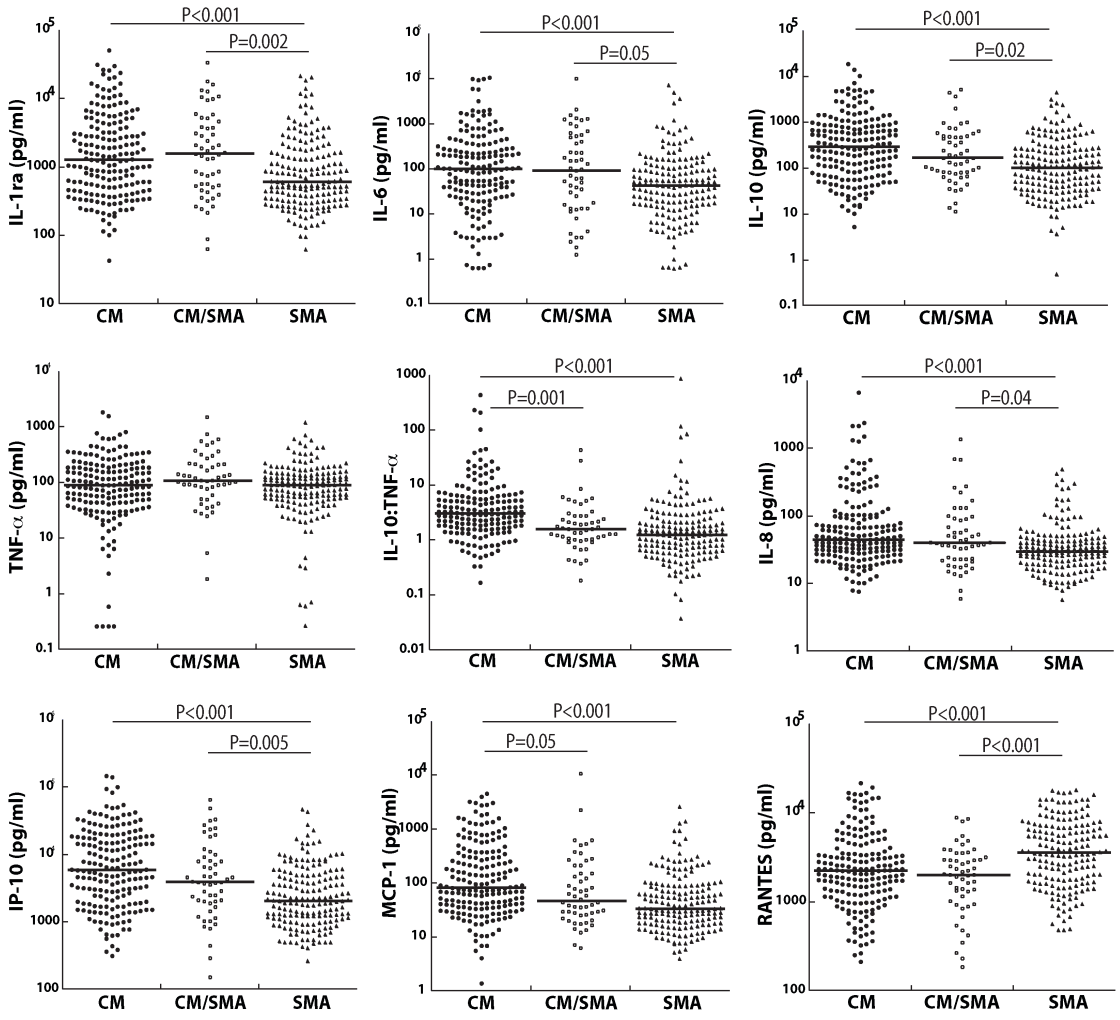


Figure 6.1. Differences in plasma cytokines and chemokines between children with cerebral malaria, severe malarial anemia and children that manifest with both forms of severe malaria

Cytokines and chemokines (on a logarithmic scale) at enrollment that differentiate between the three groups. The horizontal line represents median values. P-values represent ANOVA on log 10 transformed values followed by Tukey post-hoc adjustment for multiple comparisons. CM (cerebral malaria, hemoglobin >5g/dL), CM/SMA (cerebral malaria, hemoglobin ≤5g/dL), and SMA (severe malarial anemia).

Table 6.4. Association of plasma cytokines with cerebral malaria compared to severe malarial anemia

	OR[a] (95% CI)	P[b]
IL-1ra (pg/ml)	2.05 (1.25-3.36)	**0.004**
IL-8 (pg/ml)	4.47 (2.19-9.14)	**<0.001**
IL-10 (pg/ml)	1.85 (1.17-2.91)	**0.008**
G-CSF (pg/ml)	2.72 (1.53-4.86)	**0.001**
IFN-γ (pg/ml)	2.64 (1.43-4.87)	**0.002**
IP-10 (pg/ml)	2.75 (1.53-4.93)	**0.001**
MCP-1 (pg/ml)	2.08 (1.29-3.34)	**0.002**
MIP-1β (pg/ml)	0.81 (0.37-1.78)	0.60
RANTES (pg/ml)	0.59 (0.30-1.16)	0.13
VEGF (pg/ml)	1.21 (0.65-2.24)	0.55
TNF-α (pg/ml)	0.77 (0.49-1.22)	0.27
IL-6 (pg/ml)	1.37 (0.98-1.91)	0.07
IL-10: TNF-α	2.79 (1.60-4.88)	**<0.001**

[a] OR, odds ratio, comparing cerebral malaria to severe malarial anemia
[b] Models adjusted for age, weight for age z-score, anti-malarial treatment, platelet and white blood cell count, and *Pf*HRP-2 levels. All cytokine levels were log transformed (log base 10)

Elevated plasma IL-8 and IL-10 are associated with increased the risk of mortality in children with CM

The role of systemic inflammation in mortality in CM is still poorly defined. 31 children with CM died (13.0%); 11 of these children died the same day they were enrolled in the study, 14 children within 24 hours, 5 children within 48 hours, and 1child within 72 hours of enrollment. *Pf*HRP-2 levels were higher in children with CM who died compared to those who survived (died n=31, 4514ng/ml [2308, 7517] and survived n=208, 2389ng/ml [899, 5145], *P*=0.008). When adjusting for age, sex, weight for age z-score and *Pf*HRP-2 level, only elevated levels IL-8 and IL-10 were associated with increased risk of mortality

(Table 6.5). IL-8 and IL-10 did not differ significantly between children with CM who died within the first 24 hours (n=25) as compared to those who died later (n=6). However, the number of children with CM who died after 24 hours is low. In a multivariate regression model including all the immune markers that had $P<0.10$ for the univariate regression models (Table 6.5), adjusting for age, weight for age z-score, and *Pf*HRP-2 levels, neither IL-8 nor IL-10 were independently associated with mortality risk in CM. Receiver operating curve analysis showed that IL-10 (AUROC 0.62, 95% CI 0.51-0.73) was a slightly better predictor of CM mortality than IL-8 (AUROC 0.60, 95% CI 0.47-0.72), however none of these markers would qualify as a good predictor of mortality in CM for diagnostic purposes.

Table 6.5. Correlation of plasma cytokines and chemokines with mortality, and neurologic deficits at discharge or six-month follow-up in children with cerebral malaria

	Mortality		Neurologic deficits at discharge		Neurologic deficits 6-month follow-up	
	OR[a] (95% CI)	P[b]	OR[a] (95% CI)	P[b]	OR[a] (95% CI)	P[b]
IL-1ra (pg/ml)	1.50 (0.74-3.02)	0.26	0.78 (0.46-1.33)	0.36	0.82 (0.25-2.67)	0.75
IL-8 (pg/ml)	2.51 (1.18-5.33)	**0.02**	1.32 (0.65-2.65)	0.44	1.86 (0.38-8.97)	0.44
IL-10 (pg/ml)	1.96 (1.01-3.82)	**0.05**	0.68 (0.41-1.12)	0.13	0.87 (0.27-2.83)	0.81
GCSF (pg/ml)	1.29 (0.65-2.56)	0.47	1.19 (0.66-2.12)	0.57	1.70 (0.49-5.87)	0.40
IFN-γ (pg/ml)	1.24 (0.47-3.30)	0.67	1.08 (0.55-2.14)	0.82	0.33 (0.09-1.17)	0.09
IP-10 (pg/ml)	1.74 (0.81-3.73)	0.16	0.79 (0.46-1.37)	0.40	1.39 (0.38-5.11)	0.62
MCP-1 (pg/ml)	1.25 (0.68-2.32)	0.47	0.59 (0.35-0.99)	**0.05**	1.54 (0.48-4.90)	0.46
MIP-1β (pg/ml)	1.96 (0.63-6.11)	0.25	0.26 (0.10-0.66)	**0.005**	0.55 (0.06-4.98)	0.59
RANTES (pg/ml)	0.73 (0.27-1.96)	0.54	0.85 (0.42-1.74)	0.66	0.30 (0.05-1.72)	0.18
VEGF (pg/ml)	1.72 (0.61-4.88)	0.31	0.84 (0.43-1.68)	0.63	0.50 (0.13-1.90)	0.31
TNF-α (pg/ml)	1.22 (0.55-2.73)	0.63	0.61 (0.36-1.04)	0.07	1.28 (0.26-6.42)	0.76
IL-6 (pg/ml)	1.57 (0.95-2.61)	0.08	0.96 (0.68-1.36)	0.81	1.57 (0.60-4.10)	0.36
IL10:TNF-α ratio	1.93 (0.91-4.13)	0.09	1.13 (0.62-2.03)	0.69	0.59 (0.12-2.82)	0.51

[a] OR, odds ratio, comparing children with CM who died vs. survived; children with CM who were discharged with neurologic deficits vs. not , and children with CM who had neurologic deficits at 6-months follow-up vs. not.
[b] Models adjusted for age, weight for age z-score, and *Pf*HRP-2 levels. All cytokine levels were log transformed (log base 10)

MIP-1β and MCP-1 levels are associated with reduced risk of neurologic deficits at discharge in children with CM

Of the 206 children with CM who survived and had neurologic assessment done at discharge, 77 (37.4%) showed at least one sign of neurologic deficit. 200 of these children came back for the 6-month follow-up neurologic assessment and 11 (5.5%) children had neurologic deficits at this timepoint. Children that were discharged with neurologic deficits were younger, tended to be male more frequently and tended to have

lower weight for age z-score than those who were discharged without any neurologic deficits. When adjusting for age, sex, weight for age z-score, and *Pf*HRP-2 only MIP-1β and MCP-1 were associated with reduced risk of neurologic deficits at discharge (Table 6.5). However, in a multivariate regression model including all the immune markers that had $P<0.10$ in the univariate analyses (Table 6.5), adjusting for age, sex, weight for age z-score, and *Pf*HRP-2 neither MIP-1β nor MCP-1 were independently associated with risk of neurologic deficits at discharge. There was no association between any of the immune markers and neurologic deficits at the 6-month follow-up timepoint.

Increased IL-10, IFN-γ, RANTES and VEGF are associated with reduced coma duration in children with CM

Number of seizures and coma duration during admission are other markers of disease severity in CM. Elevated levels of IL-10, IFN-γ, RANTES and VEGF were associated with shorter coma duration at admission when adjusting for age, sex, weight for age z-score and *Pf*HRP-2 levels (Table 6.6). To make sure that the association of RANTES and VEGF with coma duration was independent of platelet number we also adjusted for platelet number in those models. Elevated levels of RANTES and VEGF remained associated with shorter coma during admission (β-coefficient -0.14, 95%CI -0.27- -0.02, $P=0.02$; β-coefficient -0.16, 95% CI -0.27- -0.05, $P=0.006$, respectively). In a model including all immune markers that had $P<0.10$ in linear regression models (Table 6.6), adjusted for age, sex, weight for age z-score, and *Pf*HRP-2 none of the immune markers

remained independently correlated with coma duration. There was no association between any of these markers and number of seizures during admission.

Table 6.6. Correlation of plasma cytokines and chemokines with coma duration and seizure numbers during hospitalization in CM children

	Coma duration (h)		Seizure numbers	
	β[a] coefficient (95% CI)	P[b]	β[a] coefficient (95% CI)	P[b]
IL-1ra (pg/ml)	-0.06 (-0.14- 0.03)	0.18	0.03 (-0.06-0.12)	0.53
IL-8 (pg/ml)	-0.03 (-0.15-0.08)	0.56	-0.02 (-0.13-0.09)	0.71
IL-10 (pg/ml)	-0.09 (-0.16- 0.007)	**0.03**	-0.04 (-0.12-0.05)	0.39
G-CSF (pg/ml)	-0.05 (-0.14-0.05)	0.33	-0.06 (-0.15-0.04)	0.24
IFN-γ (pg/ml)	-0.15 (-0.26- -0.05)	**0.005**	-0.08 (-0.20-0.04)	0.19
IP-10 (pg/ml)	-0.06 (-0.15-0.02)	0.15	-0.007 (-0.10-0.09)	0.89
MCP-1 (pg/ml)	-0.07 (-0.15-0.01)	0.09	-0.03 (-0.12-0.04)	0.33
MIP-1β (pg/ml)	-0.10 (-0.24-0.04)	0.18	-0.08 (-0.24-0.08)	0.30
RANTES (pg/ml)	-0.13 (-0.24- -0.01)	**0.03**	0.07 (-0.07-0.21)	0.31
VEGF (pg/ml)	-0.14 (-0.25- -0.03)	**0.01**	-0.04 (-0.17- 0.09)	0.58
TNF-α (pg/ml)	-0.06 (-0.14-0.03)	0.18	-0.04 (-0.14-0.05)	0.37
IL-6 (pg/ml)	-0.05 (-0.11- 0.004)	0.07	-0.02 (-0.08-0.05)	0.58
IL10:TNF-α	-0.04 (-0.13- 0.06)	0.46	-0.005 (-0.11-0.10)	0.93

[a] β, beta coefficient, comparing association of coma duration and number of seizures during admission with cytokine, chemokine levels and growth factor levels at enrollment.
[b] Models adjusted for age, weight for age z-score, and *Pf*HRP-2 levels. All cytokine levels were log transformed (log base 10)

6.5 Discussion

Identifying an immunologic profile that distinguishes between cerebral malaria (CM) and severe malarial anemia (SMA) is important in understanding the distinct pathological processes that contribute to these two forms of severe malaria and in informing the design of better diagnostics and therapies. In the current study, elevated IL-1ra, IL-8, IL-10, G-CSF, IFN-γ, IP-10, MCP-1, and IL-10:TNF-α increased the risk of CM over SMA. However, only IL-10:TNF-α remained independently associated with CM in a multivariate analysis. Elevated IL-8 and IL-10 were associated with increased risk of

mortality in CM, elevated MCP-1 and MIP-1β with neurologic protection at discharge, and elevated IL-10, IFN-γ, VEGF and RANTES with shorter coma during admission. However, none of these immune markers remained independently associated with mortality, neurologic deficits at discharge or coma duration in multivariate analysis.

IL-10 and IL-10:TNF-α appear to be particularly important in the pathogenesis and outcomes of CM. In the present study, IL-10 was uniquely upregulated in CM and CM/SMA as compared to SMA (Figure 6.1), distinguished well between CM and SMA (Table 6.4), and was also associated with mortality (Table 6.5). IL-10 has been previously associated with mortality in Vietnamese adults with SM[209] and in Ugandan children 5-12 years old with CM, the latter a prior study by our group[43]. IL-10 is also important in controlling TNF-α, which can limit the growth of erythroid precursors[172] and promote erythrophagocytosis and dyserythropoiesis[173]. Low IL-10:TNF-α values have been previously associated with SMA[201-203]. In our study, IL-10:TNF-α values were also lower in CM/SMA and SMA as compared to CM (Figure 6.1), emphasizing the importance of IL-10 controlling TNF-α in SMA. However, we did not determine reticulocyte counts in these children to establish a more direct association between IL-10:TNF-α and bone marrow function. Moreover, high IL-10:TNF-α was associated with increased risk of CM over SMA and remained independently associated with the risk of CM in a multivariate regression model. This suggests that IL-10 is important in controlling the anti-erythropoietic effects of TNF-α, however, very high levels of IL-10 could indicate an imbalanced anti-inflammatory response preventing parasite clearance in

CM. It is also possible that high levels of IL-10 reflect a strong anti-inflammatory response to a prior strong inflammatory reaction, and we are seeing a reflection of the consequences of earlier inflammation. However, the ability of IL-10, among multiple cytokines, to differentiate CM from SMA and, in children with CM, the survivors from those who died, does suggest a more specific role for this cytokine in the disease pathogenesis in CM.

IP-10, IL-8, and MCP-1 were associated with increased risk of CM as compared to SMA, and IL-8 was also associated with increased mortality in CM. IP-10, IL-8, and MCP-1 are chemokines important in recruiting monocytes, activated T cells, dendritic cells, neutrophils, and natural killer cells to sites of inflammation[210]. Elevated IP-10 and IL-8 have been previously reported in severe malaria [44,196,197,205,211,212], but they have been studied very little between CM and SMA[196,197], especially in the case of IL-8. Additionally, MCP-1 was seen elevated in CM vs. mild disease in a study of Indian adults[213] and trended higher in another study of adult CM[211]. The data in pediatric population is limited. Our group has previously shown elevated MCP-1 in children with CM compared to community controls and in children with CM who died[44]. Recruitment of neutrophils, other leukocytes and platelets, to the sites of endothelium activation in the brain has been reported in autopsy studies of human CM[46,47]. As a result, the elevation of IP-10, IL-8, and MCP-1 in CM and the association of IL-8 with mortality could be due to the ability of these chemokines to recruit immune cells to the brain microvasculature promoting mechanical sequestration and local inflammation, disease characteristics that

are not typical of SMA[47]. Lastly, IL-8 and MCP-1 can also be released by activated endothelial cells and platelets[208,214]. Since we have adjusted for platelet number in our models, elevated IL-8 and MCP-1 could be markers of endothelial activation, which is more predominant in CM than SMA[17]. Overall, more work is needed to characterize the expression of IL-8, MCP-1, and IP-10 in the brain microvasculature from fatal CM cases; characterize immune cells that express the receptors for these chemokines, and understand the role of IL-8, MCP-1, and IP-10 on chemotaxis in the context of a *P.falciparum* infection using *in vitro* assays.

MIP-1β and MCP-1 were the only immune markers associated with protection against neurologic deficits at discharge in our cohort. This association was surprising since one log 10 increase in their levels was associated with a 7- and 2000-fold increased risk of SM as compared to CC, respectively, and MCP-1 was associated with increased risk of CM over SMA. Higher levels of these beta chemokines in CM vs. SMA, and association of higher MCP-1 and MIP-1β levels with neurologic protection in CM, could suggest that elevated levels of these chemokines are an indicator of a proper immune response against the pathogen. Additionally, children with CM present at the hospital at different stages of the disease. As a result, increased levels of MIP-1β and MCP-1, could be an indicator of being enrolled and starting treatment earlier in the infection. Similarly, the association of elevated levels of RANTES, VEGF, IFN-γ and IL-10 with shorter coma could suggest presentation of children with CM closer to disease onset. However, in the case of RANTES and VEGF there are indicators of lower levels being pathogenic in SM. Low

RANTES was associated with mortality in 5-12 year old Ugandan children with CM [43] and with severity of anemia in malaria[64]. Low VEGF levels have been associated with increased severity in some studies of adult severe malaria[205,206] and are thought to contribute to disease partially *via* the effect of VEGF on the parasite itself and parasite biomass[215]. However, in a multivariate regression models none of these markers remained independently associated with neurologic protection or coma duration. There was no association of any of the immune markers studied here with neurologic deficits at 6-months follow-up or number of seizures during admission.

The finding that IFN-γ was not elevated in children with SM as compared to CC was surprising. Contrary to other studies[43,211,216], including an earlier study by our group in the same hospital[43], IFN-γ was significantly lower in SM as compared to CC. However, in other studies, IFN-γ was detectable in only 23% of mild malaria and 35% of cerebral malaria patients[197] or did not differ between severe malaria patients and controls[169,205]. Timing of sample collection (at time of coma for CM) was similar to timing in our earlier study[43]. However, timing could still affect levels, if sample collection is after peak of IFN-γ production, and IFN-γ tends to peak quite early in the infection and is highly transient[191,192]. Additionally, the presence of soluble IFN-γ receptors, normally elevated upon inflammation could be affecting the detection of IFN-γ in the context of a malaria infection. Within SM, high IFN-γ levels differentiated between CM over SMA. This emphasizes the need to better understand the factors that could affect the detection of IFN-γ in SM, and whether it could serve as a reliable marker of disease severity.

Similarly, we did not see elevated IL-1β and IL-12p70 levels in SM as compared to CC. This could be due to the highly elevated levels of IL-10 inhibiting the pro-inflammatory response, or due to the delay between disease onset and admission of children with SM; therefore we could be missing the initial peak of IL-1β, IFN-γ, and IL-12, and instead we are catching the downstream effectors, such as IL-6 and IL-10.

There are a number of limitations to studies of this nature. We measured levels of cytokines and chemokines at a single time point in the disease. Multiple time points during the child's admission and recovery might give a better idea of the factors that are specifically associated with coma and mortality, though we have shown previously that most cytokine and chemokine levels normalize by 72 hours[43]. Additionally, plasma does not reflect what could be happening locally in the secondary lymphoid organs, brain, and bone marrow of these patients. Obtaining and characterizing peripheral blood mononuclear cells might give a better idea about the source of these immune markers and their functionality. Moreover, we quantified levels rather than activity of these immune mediators. Quantification of their respective soluble receptors could give a better idea on the activity of these markers[217]. Genetic polymorphisms could also influence the variability in cytokine levels seen in this study. Lastly, while our study quantified 18 immune markers, this is not an exhaustive characterization. Recently IL-15, IL-5, eotaxin, IL-17 and IL-27 were associated with various aspects of severe malaria and would be of interest to investigate in our unique setting[211,213,218]. We are conducting

ongoing studies to assess how levels of these cytokines in the cerebrospinal fluid relate to mortality and neurologic morbidity in CM.

In conclusion, our study suggests that IL-10:TNF-α is an independent predictor of CM over SMA, that elevated levels of IL-10 and IL-8 differentiate well between CM and SMA and are also associated with mortality in CM, and that elevated IFN-γ, IL-10, RANTES and VEGF levels are associated with shorter coma duration in CM. The findings provide new insight into how pro- and anti-inflammatory cytokines and chemokines may contribute to disease phenotype and mortality in severe malaria, but also highlight the complexity of the host immune response and the need for additional study of this response to better define how interventions can successfully produce the optimal balance between too much and not enough of an inflammatory response to the parasite.

Chapter 7

High plasma erythropoietin levels are associated with prolonged coma duration and increased mortality in children with cerebral malaria

7.1 Objectives

- ✓ Determine EPO levels in plasma and CSF of children with CM
- ✓ Evaluate the association of systemic and local EPO levels with markers of disease severity in CM such as coma duration, neurologic deficits and mortality

7.2 Introduction

Erythropoietin (EPO) is a hematopoietic factor that promotes survival and proliferation of bone-marrow progenitor cells during erythropoiesis [219]. Expression of EPO under the control of hypoxia-inducible factor-1 alpha (HIF1-α) occurs primarily in the kidney, but EPO and EPO receptor (EPOR) expression has been identified in other organs, including the brain of rodents, monkeys, and humans [220]. *In vitro* studies demonstrating that EPO reduced glutamate-induced neuronal apoptosis [221] initiated intensive investigations on the use of EPO as a neuroprotective agent.

Exogenous recombinant human EPO (rHuEPO) has been shown to be neuroprotective in animal models of cerebral ischemic neuronal damage [222], autoimmune encephalomyelitis (EAE) [223] and cerebral malaria [224,225]. Consequently, rHuEPO was rapidly introduced into human clinical trials. A large phase I trial in acute ischemic stroke patients showed improved clinical outcome at the one-month primary endpoint [226]. However, longitudinal follow-up of these patients revealed increased mortality in the rHuEPO arm and provided evidence, along with other studies, that in critically ill patients, EPO can increase the risk of thrombosis, endothelial cell activation, and platelet aggregation [227-229]. rHuEPO has been shown to correlate with better cognitive outcomes in preterm infants [230], however the use of high doses of rHuEPO in children undergoing dialysis has been associated with an increased risk of hypertension [231], raising questions on the safety of rHuEPO as a neurotropic agent in children, particularly children with a pro-thrombotic disease process.

Severe malaria induces upregulation of endogenous EPO levels [232,233], which can be greater than the increase induced by similar anemia without malaria [68]. High plasma levels of endogenous EPO were associated with protection from acute neurologic deficits in Kenyan children with CM [204]. This study and murine cerebral malaria studies [224,225] prompted a small (n=35) phase I clinical trial in which EPO-beta adjunctive treatment for children with CM did not cause obvious adverse events [234]. In this study clinical assessment was limited and children were followed for only seven days. In light of recent studies showing increased mortality with rHuEPO in other neurologic conditions [227] and the lack of studies confirming the Kenyan study findings, we conducted a study to assess

the relationship of plasma and CSF EPO levels with mortality and acute and long-term neurologic deficits in children with CM. As EPO can increase endothelial activation [229] which can further promote sequestration [30,235], the relationships between plasma and CSF EPO levels and markers of endothelial activation and levels of *P. falciparum* histidine-rich protein-2 (*Pf*HRP2), a measure of sequestered and circulating parasite biomass [73], were also assessed.

7.3 Methods

Laboratory testing

Plasma and CSF EPO levels were tested via a high sensitivity radioimmunoassay (RIA), as previously described [236].

Plasma analyte testing

Plasma levels of soluble intracellular adhesion molecule-1 (sICAM-1), vascular cellular adhesion molecule-1 (sVCAM-1), and soluble P-Selectin and E-Selectin were measured by cytometric bead assay according to the manufacturer's instructions in plasma diluted 1:300 (R&D Systems, Minneapolis, MN) with a Bioplex-200 system (Bio-Rad, Hercules, CA).

Statistical Analysis

Clinical and laboratory factors in children with vs. without neurologic deficits and in survivors vs. non-survivors were compared by χ^2 testing if categorical and if continuous, by Student's t-test or by the Wilcoxon rank-sum test for measures with skewed distributions. Variables with a $P<0.2$ in these analyses were adjusted for in the respective regression models. Plasma and CSF EPO levels, coma duration, and number of seizures had skewed distributions, so for these variables, Spearman's rank correlation (rho) was used for unadjusted analyses and log-transformed (natural log) values were used for regression analyses. Associations between log-transformed EPO levels and neurologic outcomes and mortality were tested by logistic regression for categorical variables and linear regression for continuous variables, with adjustment for potential confounding factors after initial testing for interactions of pairs of potential confounding factors and of log EPO with each potential confounder.

7.4 Results

Study cohort follow-up, sample collection and baseline characteristics

Figure 1 shows the number of children tested for plasma and CSF EPO levels, the number of survivors and those who died, and the number of surviving children with neurologic deficits at discharge. Of the 204 children who had plasma tested, 147 had CSF tested. CSF was not tested on 57 of the 204 children for reasons specified in Figure 7.1. Children with HIV (n=5) and sickle cell disease (n=1) were not excluded from the study.

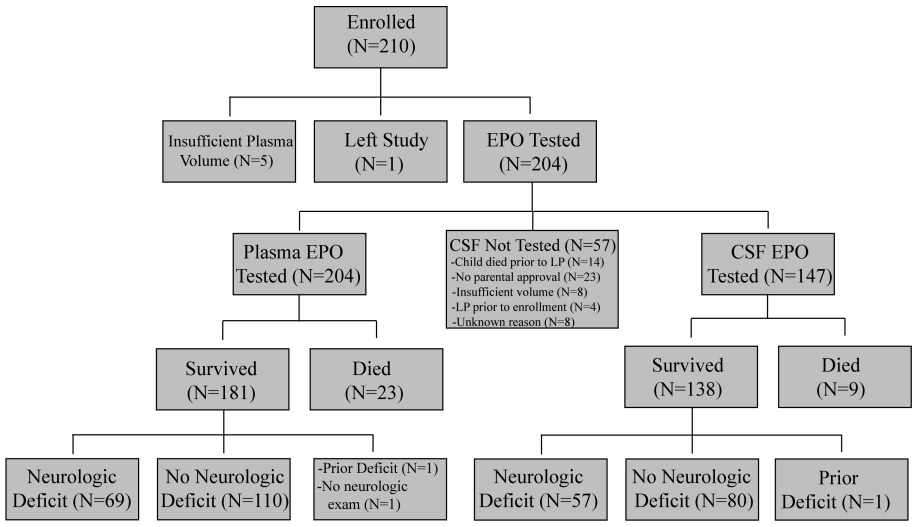


Figure 7.1. Study profile

Clinical and laboratory predictors of neurologic deficits and mortality

At discharge, 69 of 179 children who survived CM and had a neurologic exam (38.5%) had neurologic deficits, including motor deficits (n=37), ataxia (n=35), or disorders of movement (n=5), behavior (n=10), hearing (n=5), or speech or vision (n=34). At six months follow-up, 10 of 173 children who survived and completed follow-up (5.8%) had neurologic deficits, including motor deficits (n=4), ataxia (n=4), movement disorder (n=2), behavior (n=1), and speech or visual disorders (n=6).

Clinical and laboratory variables that were biologically plausible as predictors of neurologic deficits or mortality in children with CM were compared in children with vs. without neurologic deficits at discharge and six months, and in children who survived vs.

children who died (Tables 7.1 and 7.2). Among these factors, children with neurologic deficits at discharge had a lower age, higher sP-Selectin levels and longer coma duration during admission than children without deficits, and children with neurologic deficits at six-month follow-up had a lower weight for age z-score, lower Blantyre coma score, longer coma and received a blood transfusion more frequently than children without deficits (Table 7.1). Lower sP-Selectin levels and a trend toward lower hemoglobin levels were seen in children who survived vs. children who died (Table 7.2).

Table 7.1. Clinical and laboratory findings in children with cerebral malaria with vs. without neurologic deficits, at discharge and 6-month follow-up

	Discharge			6 month follow-up		
	Deficits (n=69)	No deficits (n=110)	P[a]	Deficits (n=10)	No deficits (n=163)	P[a]
Demographic and clinical findings						
Age (months) median (IQR)	38.1 (26.7-47.8)	43.7 (32.5-61.6)	0.02	37.1 (24.8-42.3)	41.5 (31.1-52.9)	0.09
Sex, male n (%)	45 (65.2)	60 (54.6)	0.16	3 (30.0)	97 (59.5)	0.07
Weight for age z-score, mean (SD)	-1.46 (1.29)	-1.11 (1.10)	0.06	-1.96 (1.11)	-1.20 (1.15)	0.04
Seizures before admission, n (%)	65 (94.2)	105 (95.5)	0.71	10 (100.00)	157 (96.3)	0.54
Deep acidotic breathing, n (%)	4 (5.80)	9 (8.18)	0.55	0 (0)	12 (7.36)	0.37
Abnormal posturing, n (%)	4 (5.80)	8 (7.27)	0.70	1 (10.00)	11 (6.75)	0.69
Blantyre coma score, median (IQR)	2 (1-2)	2 (2-2)	0.18	1 (1-1)	2 (2-2)	<0.0001
Coma duration (hours), median (IQR)	73.0 (40.0-119)	47.0 (27.5-70.0)	0.0004	155 (86.5-227)	49.0 (32.0-78.7)	0.0001
Seizures after admission, n (%)	33 (47.8)	66 (60.0)	0.11	6 (60.00)	90 (55.2)	0.77
No. of seizures after admission, median (IQR)	0 (0-1)	1 (0-2)	0.29	3 (0-8)	1 (0-2)	0.11
Transfused, n (%)	47 (68.1)	65 (59.1)	0.23	10 (100)	97 (59.5)	0.01
Clinical laboratory tests						
Hemoglobin (g/dL), mean (SD)	6.84 (2.33)	6.60 (2.21)	0.49	6.39 (1.47)	6.68 (2.23)	0.68
White blood cell count, median (IQR)	10.2 (6.70-16.1)	8.90 (6.50-12.3)	0.11	8.30 (5.30-15.70)	9.10 (6.70-12.9)	0.74
Platelet count, median (IQR)	63.0 (33.5-130)	61.0 (37.0-105)	0.96	58.0 (31.0-75.0)	61.0 (37.0-115)	0.62
Hypoglycemia[c], n (%)	6 (8.70)	6 (5.45)	0.40	1 (10.0)	11 (6.75)	0.69
P. falciparum peripheral blood density, median (IQR)	37580 (8160-249560)	59180 (14180-407940)	0.20	72370 (6420-801060)	43990 (12680-309060)	0.62
PfHRP2 level (ng/ml), median (IQR)	2678 (1042-5369)	2274 (943-4949)	0.54	1001 (470-3036)	2561 (996-5148)	0.14
Endothelial and platelet activation markers						
Soluble VCAM-1 (ng/ml), median (IQR)	4034 (2551-6815)	3944 (2869-6601)	0.78	5836 (3781-11977)	3845 (2715-6364)	0.09
Soluble ICAM-1 (ng/ml), median (IQR)	729 (225-1433)	505 (238-1340)	0.60	657 (220-1170)	521 (225-1433)	0.86

Soluble E-Selectin (ng/ml), median (IQR)	172 (121-238)	187 (142-254)	0.22	211 (158-246)	179 (134-250)	0.78
Soluble P-Selectin (ng/ml), median (IQR)	61.0 (40.6-83.1)	48.6 (35.8-72.8)	0.05	58.8 (51.7-83.2)	50.0 (36.8-76.1)	0.40
Erythropoietin (EPO) levels						
Plasma EPO (mU/ml), median (IQR)	741 (330-3234)	933 (288-2560)	0.76	680 (549-1763)	783 (259-2835)	0.76
CSF EPO (mU/ml) d, median (IQR)	8.34 (4.21-14.8)	8.50 (4.17-18.4)	0.99	8.34 (4.21-12.0)	8.76 (4.20-17.9)	0.71

Abbreviations: no., number; CSF, cerebrospinal fluid; IQR, inter-quartile range. See text for laboratory variable abbreviations.

[a] Variables with medians reported compared by Wilcoxon rank-sum score; means compared by t-test; proportions compared by X^2 test

[b] Blantyre coma score assessed in children <5 years of age; at discharge, deficits, n=56, no deficits=82; at 6 months, deficits= 9, no deficits=125

[c] Hypoglycemia defined as blood glucose <2 mmol/L

[d] CSF EPO tested on admission in 57 and 80 children with and without deficits, respectively, and at 6 months, in 9 and 122 children with and without deficits, respectively

Table 7.2 Clinical and laboratory findings in children with cerebral malaria who survived compared to those who died

	Survived (n=181)	Died (n=23)	P[a]
Demographic and clinical findings			
Age (months) median (IQR)	41.46 (31.05-52.17)	35.63 (25.79-46.98)	0.13
Sex, male n (%)	106 (58.56)	14 (60.87)	0.83
Weight for age z-score, mean (SD)	-1.25 (1.18)	-1.66 (1.29)	0.13
Seizures before admission, n (%)	171 (94.48)	22 (95.65)	0.81
Deep acidotic breathing, n (%)	13 (7.18)	3 (13.04)	0.33
Abnormal posturing, n (%)	12 (6.63)	0 (0)	0.20
Blantyre coma score[b], median (IQR)	2 (1-2)	2 (1-2)	0.41
Coma duration (hours), median (IQR)	54.0 (32.0-83.0)	NA	
Seizures after admission, n (%)	100 (55.25)	13 (56.52)	0.91
No. of seizures after admission, median (IQR)	1 (0-2)	1 (0-2)	0.73
Transfused, n (%)	112 (61.88)	12 (52.17)	0.37
Clinical laboratory tests			
Hemoglobin (g/dL), mean (SD)	6.69 (2.24)	7.63 (2.35)	0.06
White blood cell count, median (IQR)	9.30 (6.70-13.90)	10.70 (7.40-13.20)	0.38
Platelets count, median (IQR)	61.00 (35.00-113)	55.00 (35.00-83.00)	0.34
Hypoglycemia[c], n (%)	12 (6.63)	3 (13.04)	0.27
P. falciparum peripheral blood density, median (IQR)	45600 (11780-302060)	49040 (6480-121100)	0.53
PfHRP2 level (ng/ml), median (IQR)	2486 (996-5112)	3532 (1598-5822)	0.15
Endothelial and platelet activation markers			
Soluble VCAM-1 (ng/ml), median (IQR)	3945 (2770-6601)	3225 (2532-6018)	0.23
Soluble ICAM-1 (ng/ml), median (IQR)	621 (238-1400)	976 (321-1864)	0.16
Soluble E-Selectin (ng/ml), median (IQR)	180 (137-246)	189 (158-283)	0.25
Soluble P-Selectin (ng/ml), median (IQR)	53.07 (37.45-76.78)	67.09 (48.53-82.99)	0.05
Erythropoietin (EPO) levels			
Plasma EPO (mU/ml), median (IQR)	783 (288-2759)	1566 (473-2852)	0.39
CSF EPO (mU/ml)[d], median (IQR)	8.42 (4.20-17.64)	9.70 (6.66-89.00)	0.21

Abbreviations: no., number; CSF, cerebrospinal fluid; IQR, inter-quartile range. See text for laboratory variable abbreviations.

[a] Variables with medians reported compared by Wilcoxon rank-sum score; means compared by t-test; proportions compared by X^2 test

[b] Blantyre coma score assessed in children <5 years of age; survived n=139, died =17

[c] Hypoglycemia defined as blood glucose <2 mmol/L; [d] CSF EPO testing performed in 138 children who survived and 9 children who died

Plasma and CSF EPO levels and neurologic outcomes, adjusted for hemoglobin level and age

Plasma and CSF EPO levels were strongly correlated (rho= 0.68, $P<0.0001$), and both plasma and CSF EPO correlated inversely with age (rho = -0.31, $P<0.0001$ and rho = -0.29, $P=0.0002$, respectively) and hemoglobin level (rho = -0.78, $P<0.0001$ and rho= -0.54, $P<0.0001$, respectively). Age and hemoglobin level were therefore included as adjusters when assessing the relationship of plasma and CSF EPO levels to primary outcomes. After adjusting for age and hemoglobin level, endogenous plasma and CSF EPO levels were not associated with neurologic deficits (at discharge or six-month follow-up) or number of seizures post-admission, but plasma and CSF EPO levels positively correlated with increased coma duration during hospitalization (Table 7.3). Plasma EPO levels in a cohort of asymptomatic and otherwise healthy children from this area were significantly lower than in children with CM (n=136, median [25th percentile, 75th percentile], 20.02 mU/ml [15.62, 35.25], $P<0.0001$ compared to children with CM).

Table 7.3. Association of plasma and CSF EPO levels with neurologic deficits, number of seizures and coma duration

	Neurologic deficit (discharge)		Neurologic deficit (6 mo)		Number of seizures after admission		Coma duration (hours)	
	OR[a] (95% CI)	P	OR[a] (95% CI)	P	β[a] coefficient (95% CI)	P	β[a] coefficient (95% CI)	P
Plasma EPO (mU/ml)	1.16 (0.83-1.62)	0.39	0.98 (0.49-1.98)	0.96	-0.01 (-0.14-0.12)	0.85	0.15 (0.03-0.27)	0.02
CSF EPO (mU/ml)	1.15 (0.75-1.75)	0.53	0.74 (0.31-1.76)	0.49	0.09 (-0.08-0.27)	0.30	0.20 (0.04-0.35)	0.01

[a] Adjusted for age and hemoglobin level; EPO levels, seizures after admission and coma duration were log-transformed (natural log).
Odds ratios (OR) denote the increase in odds of the clinical outcome (neurologic deficit or log of number of seizures or coma duration) for each log increase in EPO level. β-coefficients denote the increase in clinical outcome (neurologic outcome) or log of clinical outcome (number of seizures, coma duration) for each log increase in EPO level.

Plasma and CSF EPO levels and mortality

Unadjusted plasma and CSF EPO levels did not differ in CM survivors as compared to those who died (Table 7.2), but after adjustment for age and hemoglobin level, log-transformed plasma EPO levels were associated with mortality (odds ratio (OR) 1.74, 95% CI 1.09-2.77, *P*=0.02, Table 7.4). In this analysis, hemoglobin level (OR 1.70, 95% CI 1.27-2.26, *P*<0.001) but not age (OR 0.78, 95% CI 0.58-1.06, *P*= 0.11) was also independently associated with mortality. For any given hemoglobin level, children who died typically had a higher EPO level than children who survived (Figure 7.2). Using an 8 g/dL cutoff for moderate anemia, plasma EPO was associated with mortality and prolonged coma duration in children with hemoglobin levels <8g/dL (adjusted OR 3.11, 95% CI 1.30-7.41, *P*=0.01 and β=0.23, 95% CI 0.02-0.44, *P*=0.03, respectively) but not in children with hemoglobin levels ≥8g/dL (adjusted OR 1.37, 95% CI 0.76-2.47, *P*=0.29 and β=0.11, 95% CI -0.12-0.34, *P*=0.34, respectively). Plasma EPO levels remained

associated with mortality and prolonged coma after adjustment for receipt of a blood transfusion or number of transfusions (data not shown).

Table 7.4. Association of plasma and CSF EPO levels with mortality

	OR[a] (95% C.I)	P	OR[b] (95% C.I)	P
Plasma EPO (mU/ml)	1.74 (1.09-2.77)	0.02	1.69 (1.03-2.77)	0.04
CSF EPO (mU/ml)	1.70 (0.81-3.56)	0.16	1.73 (0.80-3.74)	0.17

[a] Adjusted for age and hemoglobin level; EPO levels were log-transformed (natural log)
[b] Adjusted for age and levels of hemoglobin, sP-Selectin, sICAM-1 and PfHRP2; EPO levels were log-transformed (natural log)

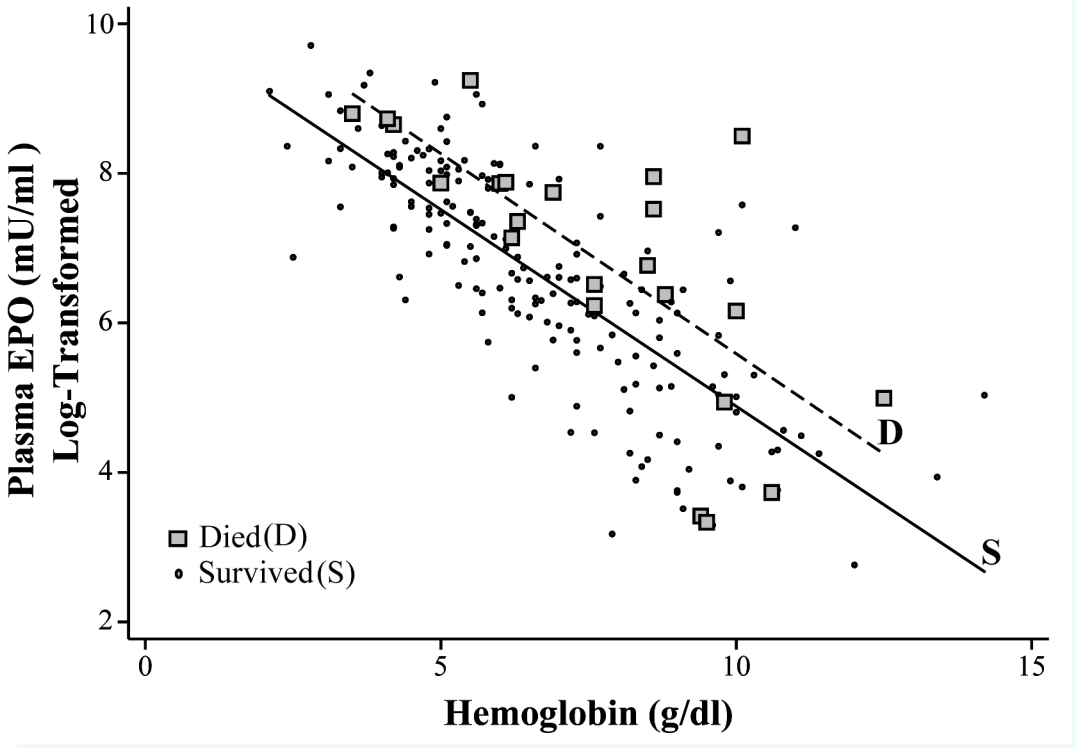


Figure 7.2. Hemoglobin and EPO levels in children with cerebral malaria who survived vs. died

EPO levels (log-transformed) and hemoglobin level are depicted for children who died and survived. Solid and dashed lines show average EPO level for each hemoglobin level for children who survived (S) vs. children who died (D), respectively. In 19 of the 23 children who died, EPO values were higher than the mean EPO value of survivors for the same hemoglobin level.

CSF EPO levels showed a very similar trend toward association with mortality as plasma EPO levels (one natural log increase in CSF EPO level, odds ratio (OR) 1.70, 95% CI 0.81-3.56, *P*=0.16, Table 7.4), but the trend did not achieve significance, likely because of smaller number of children with CSF samples for testing (n=147).

Plasma EPO levels, markers of endothelial activation and *Pf*HRP-2

Log-transformed plasma EPO levels, adjusted for age and hemoglobin level, were positively associated with levels of sP-Selectin, sE-Selectin, sICAM-1, and sVCAM-1 (Table 7.5). Plasma and CSF EPO levels also correlated strongly with plasma *Pf*HRP-2 levels after adjustment for age and hemoglobin level (β=0.44, 95% CI 0.27-0.61, *P*<0.001 and β=0.50, 95% CI 0.27-0.73, *P*<0.001, respectively), but not with peripheral parasite density (p>0.71 for both). Levels of PfHRP-2, sP-Selectin, and sICAM-1 met the pre-determined adjuster cutoff for differences between children who survived vs. died (Table 7.2), so they were adjusted for in a final model assessing plasma EPO levels and mortality. Log-transformed plasma EPO levels remained independently associated with mortality after this further adjustment (OR 1.69, 95% CI, 1.03, 2.77, *P*=0.04, Table 7.4).

Table 7.5. Association of plasma EPO with markers of platelet and endothelium activation

	β[a] coefficient (95% CI)	P
sP-Selectin	0.11 (0.04-0.18)	0.002
sE-Selectin	0.11 (0.06-0.17)	<0.001
sICAM-1	0.19 (0.007-0.38)	0.04
sVCAM-1	0.11 (0.02-0.19)	0.01

[a] Adjusted for age and hemoglobin level; EPO levels were log-transformed (natural log)

7.5 Discussion

The present study showed that in children with CM, both high plasma and CSF levels of endogenous EPO are associated with prolonged coma duration, while only plasma EPO

levels are associated with increased mortality. In contrast to an earlier study in Kenyan children with CM [204], we found no association between plasma and CSF EPO levels and protection from acute neurologic deficits; we also found no association with protection from long-term neurologic deficits. Further studies are needed to confirm these findings, but in conjunction with studies showing no neuroprotective benefit of endogenous EPO in adult CM patients [237] and increased risks of exogenous rHuEPO in other diseases [227,231], the current findings argue for caution in using systemic rHuEPO as adjunctive therapy for children with CM.

Association of endogenous and exogenous EPO with adverse outcomes has precedent in several severe diseases such as chronic kidney disease, dialysis and stroke [227,228,231]. These studies, along with others that found an increase of exogenous EPO-mediated platelet and endothelial activation [229,238], suggest that a balance between neuroprotective and erythropoietic events is needed for erythropoietin to be both useful and safe. We observed a positive correlation of endogenous plasma EPO levels with markers of platelet and endothelial activation, factors important in CM pathogenesis [235]. EPO could lead to increased disease severity in CM by endothelial and platelet activation, as this increased activation could lead to sequestration [30,235]. In support of an association with sequestration, plasma EPO was not associated with parasite density at enrollment but was associated with plasma PfHRP-2 levels, a marker of total parasite biomass, including sequestered parasite biomass [73].

In vitro and *in vivo* studies have shown that EPO, as a neurotropic agent, can either be neuroprotective or harmful [239,240]. The continued presence of high levels of EPO, when combined with moderate hypoxia leads to increased neuronal apoptosis in cultured rat neurons [239]. Impaired local perfusion due to sequestration, rosette formation and reduced nitric oxide (NO) bioavailability leads to hypoxia in CM, which could tip the balance of EPO from neuroprotective to damaging. In addition, EPO levels at 10nM or higher increased apoptosis in cultures of rat hippocampal neurons [240]. The levels of endogenous plasma EPO seen in this study are lower than the levels of EPO reached in adults after rHuEPO treatment [226], and since the affinity of EPO for its receptor in the brain is lower than for its homodimeric receptor on erythroid progenitors, it is possible that EPO levels above those produced endogenously are neuroprotective [241]. Moreover, the timing of plasma EPO elevation could have detrimental effects. Untimely onset of EPO-induced reticulocytosis augmented parasitemia and was fatal in a mouse malaria model [242]. In our study, endogenous EPO was not associated with parasitemia at enrollment, but we do not have consecutive EPO and parasitemia measurements to compare our findings with those of the mouse model.

Our study findings contrast with those of a previous study done in Kenyan children with CM, in which high EPO levels were associated with protection from acute neurologic deficits and mortality [204]. Children in the present study had a lower mortality rate and slightly more frequent neurologic deficits at discharge than children in the prior study, but these differences should not strongly alter associations between EPO and neurologic

deficits. Differences in age might partially explain the different study findings. The median age was higher in the present study, and increased age has been associated with greater upregulation of endogenous EPO levels in response to a similar decrease in hemoglobin levels [243], and with decreased clearance of both endogenous and exogenous EPO [243,244]. Slower clearance of high EPO levels could increase the risk of thrombotic events. Our regression models controlled for age but we did not enroll children as young as the youngest children in the Kenyan study. The other primary differences seen between the current study population and the Kenyan study population were that children in the current study had lower mean hemoglobin levels and platelet counts and had higher median EPO levels. Among children in the present study, the elevated EPO levels were likely due to the lower hemoglobin levels, and the lower platelet counts could reflect increased platelet sequestration. Elevated EPO levels via their pro-thrombotic effect could have aggravated already increased platelet and infected erythrocyte sequestration in the children in our study. The association of EPO with mortality in children with a hemoglobin level <8 g/dL, but not in children with a hemoglobin level ≥8 g/dL is consistent with low hemoglobin levels being a driver of mortality in our study cohort, though transfusion did not alter outcomes. In the Kenyan study, high levels of EPO were associated with decreased mortality, after adjustment for deep breathing, number of seizures, coma duration, hyperparasitemia and papilledema. In our cohort, the association of EPO with mortality was unaltered in a model that included these predictors (adjusted OR for log-transformed plasma EPO 1.68, 95% CI 1.03-2.76, p=0.04). In summary, age, severity of anemia and degree of sequestration could explain some of the differences

between the Kenyan study and the present study, but studies in additional cohorts are needed to resolve the study differences.

As a longitudinal, observational study, the present study cannot determine causality. Given the multiple factors that can cause EPO levels to increase, such as hypoxia, inflammation and suppression of erythropoiesis it will be important to determine whether the association of high endogenous EPO levels with coma duration and mortality is causative. Several factors lead us to believe that endogenous EPO levels are most likely causally related to prolonged coma and mortality. First, in randomized clinical trials of rHuEPO in stroke, which has some similarities in pathogenesis to CM, individuals in the rHuEPO arm had increased mortality [227]. Second, in our study the association between EPO and mortality remained after adjustment for important confounding factors, including age, hemoglobin level and PfHRP2 level [73]. Third, although exogenous EPO can be neuroprotective, evidence of EPO-related adverse events has been demonstrated in animal models and human studies of other diseases [227,231,239,240]. However hypoxia, a major driver of EPO levels, can be caused by multiple factors and it remains possible that elevated endogenous EPO is a marker for another as yet undefined process that leads to mortality in CM. New analogues of EPO have been formulated that lack erythropoietic effects but retain the neuroprotective characteristics of EPO, such as carbamylated EPO [238,245]. These derivatives have been tested as neuroprotective agents in animal models of stroke and EAE [245], and may hold promise in CM treatment.

In summary, the present study showed that high plasma levels of endogenous EPO are associated with prolonged coma duration and increased mortality in CM children above 18 months of age, and not with protection from neurologic deficits. In conjunction with other studies showing adverse effects from systemic rHuEPO therapy in adults and children with pro-thrombotic disease states, the present study findings suggest caution in considering recombinant systemic rHuEPO as adjunctive therapy for children above 18 months of age with cerebral malaria.

Chapter 8

Summary

Cerebral malaria (CM) and severe malarial anemia (SMA) remain drivers of morbidity and mortality due to *P. falciparum* infection in children in Sub-Saharan Africa. Adjunctive therapies to date have not shown success in controlled clinical trials[28], suggesting that we need a better understanding of both host and pathogen factors that contribute to severe malaria (SM). Moreover, it remains unclear how *P.falciparum* infection leads to CM vs. SMA. This dissertation attempted to identify both host and parasite factors that contribute to disease severity in malaria. The sample size and study design also allowed us to investigate host and parasite factors that differentiate between CM and SMA, and factors associated with mortality and neurocognitive outcomes in CM.

Morbidity and mortality due to *P.falciparum* infection has exerted strong selective pressure on the human genome[246]. As an example, heterozygous individuals for the sickle cell gene (HbS) have ~10 fold decreased risk of SM[247]. In our cohort, the sickle cell trait was prevalent in 19% of community children (CC) as compared to 0.8% and 0.9% in CM and SMA, respectively. CC had no history of SM, and only one CC returned to the clinic with SM in the two years of follow-up. Therefore, we hypothesized that other protective genes against SM would be present in our cohort. We investigated the prevalence of a functional polymorphism in the endothelial protein C receptor (EPCR) gene. EPCR binds to infected erythrocytes (IEs) in SM[52] and rs867186-G variant is associated with less

bound and more soluble EPCR [94,95]. Consequently, we hypothesized a higher prevalence of the rs867186-G variant in CC. We observed a significantly higher prevalence of the rs867186-GG genotype in our CC group (4.1%) as compared to SM (0.6%, *P*=0.002), suggesting an association of this genotype with protection against SM. As expected, we saw an association between the prevalence of rs867186-G and increased levels of soluble EPCR (sEPCR) in the plasma for each disease group, suggesting that the protection against SM could be due to less available bound EPCR and higher sEPCR. Nevertheless, our CC group was relatively small to make strong conclusions about protection, and larger multi-center studies are needed. Interestingly, despite inflammation, SM had lower levels of sEPCR than CC, unlike other diseases characterized by inflammation such as SLE [108,109], Wegener's granulomatosis [110], Behcet's disease [111] and sepsis[108,112,113]. These findings suggest that EPCR biology is quite different in SM and needs further studying. We hypothesized that IEs could be acting as a sink for sEPCR and *in vitro* studies with parasite strains that bind specifically to EPCR and sEPCR would help address this hypothesis. Also, how the shedding of EPCR is affected by IE-EPCR binding is unclear. *In vitro* studies with EPCR binding parasite strains in a simple endothelial monolayer model could start asking the questions of whether IE-EPCR binding prevents shedding of EPCR in the context of inflammation. We did not see a correlation of sEPCR with mortality, neurocognitive deficits or coma in CM, suggesting that sEPCR would not be a good biomarker of disease severity in CM. Moreover, we did not see a difference in the prevalence of rs867186-G or in the levels of sEPCR in CM vs. SMA, suggesting that IE-EPCR binding is a common phenomenon in both these forms of SM, and that potentially

the *P.falciparum* erythrocyte membrane 1 (PfEMP1) variant types and their expression levels could be determining whether the child gets CM vs. SMA.

Cytoadhesion is a characteristic of all *P.falciparum* IEs mediated by parasite variant surface antigens such as PfEMP1, suggesting that it is not simply the presence of cytoadhesion that leads to SM, but potentially a unique combination of PfEMP1 variants and host receptors. Expression of group A[131-135] *var* genes and *var* genes encoding DC8 (*var* B/A) and DC13 (*var* A) PfEMP1[85] have been associated with SM. In addition, PfEMP1 variants that bind to EPCR, including DC8 and DC13 reduce the production and cytoprotective effects of aPC[53,55,56]. However, it has been unclear whether EPCR-binding PfEMP1 variants are equally transcribed and prevalent in CM and SMA. In the current study we show that EPCR-binding PfEMP1 domains (DBLα2/1.1/2/4/7/9, CIDRα1.1, and overall group A EPCR) were higher in parasites from CM children compared to SMA and elevated DBLα2/1.1/2/4/7/9 and group A EPCR PfEMP1 transcripts increased the risk of CM independently of parasite biomass. More work is needed to understand how PfEMP1 binding to EPCR could contribute to SMA. EPCR is expressed on long-term hematopoietic stem cells (HSCs), and EPCR signaling is important in hematopoiesis[148]. Therefore, bone marrow smears staining for both EPCR and IEs could start elucidating whether co-localization of IEs with EPCR is also associated with abnormal bone marrow morphology, however these studies would be challenging to perform. Instead, *in vitro* models of erythropoiesis using CD34+ HSCs could be used to study whether parasites expressing certain PfEMP1 variants affect development and

survival of erythroid progenitors. As mentioned above, *in vitro* models are also needed to address how exactly PfEMP1-EPCR binding affects the blood brain barrier (BBB). Future directions should also include quantification of group B and C *var* genes, and quantification of transcript levels for the second head structure of PfEMP1, which could be providing binding to a second host receptor. Epigenetic mechanisms regulate *var* transcription[149], however more work is needed to understand how host environmental signals can affect these epigenetic mechanisms and lead to expression of a specific PfEMP1.

Host immune mediators can also promote IE binding in CM. TNF-α, is one of these mediators due to its role in promoting endothelial activation, which can further increase binding of IEs to host endothelium[150,151]. The association of TNF-α with severity markers remains controversial and the use of antibodies against TNF-α had adverse effects in children with CM[152]. In our study, plasma and cerebrospinal CSF TNF-α levels were elevated at enrollment in children with CM compared to controls. In addition, elevated CSF, but not plasma TNF-α levels were associated with longer duration of coma, higher risk of neurologic deficits at discharge and 6-months follow-up, suggesting a pathogenic role of CSF TNF-α in CM. Our results emphasize the importance of studying both systemic and local immune responses since they do not always tell the same story. We hypothesize that adjunctive treatment with anti-TNF-α monoclonal antibodies was not successful in CM due to the inability of these antibodies to cross the BBB and inhibit local functions of TNF-α. However, in our study, we were not able to determine the

source of TNF-α in periphery or in the CNS, and we were not able to associate CSF TNF-α levels with CNS or BBB damage due to the lack of tissue samples. These studies would be difficult to perform logistically and would limit our understanding only to the fatal cases of CM. Future directions should include estimating the prevalence of functional TNF-α polymorphisms in this population. Moreover, *in vitro* co-culture BBB models are needed to understand how physiologically relevant TNF-α levels affect primary glial cells in addition to BBB endothelium in the context of *P.falciparum* infection.

An imbalanced immune response is typical of SM episodes. Whether a certain systemic immunologic profile can differentiate between CM and SMA and between different outcomes in CM remains understudied due to the mixed inclusion of severe malaria cases and small sample size in many studies. In studies like ours, where patients present with coma or severe anemia it is difficult to determine the pathways that led to severe malaria. However, identifying immunologic markers that can differentiate between CM and SMA or between severe outcomes in CM at enrollment could have important diagnostic and therapeutic functions. In the current study, we quantified the levels of 18 cytokines, chemokines, and growth factors. IL-1ra, IL-8, IL-10, G-CSF, IFN-γ, IP-10, MCP-1, and IL-10:TNF-α increased the risk of CM over SMA. However, only IL-10:TNF-α remained independently associated with CM in a multivariate analysis. Elevated IL-8 and IL-10 were associated with increased risk of mortality in CM, elevated MCP-1 and MIP-1β with neurologic protection at discharge, and elevated IL-10, IFN-γ, VEGF and RANTES with shorter coma during admission. However, none of these immune markers remained

independently associated with mortality, neurologic deficits at discharge or coma duration in multivariate analysis. Our study warrants further investigation of chemokines in CM. Quantifying expression of IL-8, MCP-1 and IP-10 in the brain microvasculature and tissues from fatal CM cases, as well as quantifying and characterizing the immune cells that express their receptors could increase our understanding of these chemokines in CM. Moreover, functional *in vitro* chemotaxis assays with patient cells would further elucidate the role of IL-8, MCP-1 and IP-10 in CM. Another important immune mediator in our study was IL-10, which differentiated well between CM and SMA and was also associated with mortality in CM. Together with higher IL-10:TNF-α being associated with CM, our findings suggest that IL-10 is important in controlling the anti-erythropoietic effects of TNF-α, however very high levels of IL-10 could be an indicator of an imbalanced anti-inflammatory response preventing parasite clearance in CM and promoting parasite replication and sequestration.

Lastly, we also investigated the role of endogenous erythropoietin (EPO) in CM patients to elucidate the factors that need to be considered when thinking of targeting any marker identified from clinical studies with adjunctive therapies. The present study showed that in children with CM, both high plasma and CSF levels of endogenous EPO were associated with prolonged coma duration, and plasma EPO levels were associated with increased mortality in CM children above 18 months of age, and not with protection from neurologic deficits. These findings, in conjunction with studies showing no neuroprotective benefit of endogenous EPO in adult CM patients [237] and increased risks

of deleterious outcomes following therapy with exogenous recombinant human EPO (rHuEPO) in other diseases [227,231] argue for caution in using systemic rHuEPO as adjunctive therapy for children with CM. New analogues of EPO have been formulated that lack erythropoietic effects but retain the neuroprotective characteristics of EPO, such as carbamylated EPO [238,245], which may hold promise in CM. Our study overall emphasizes that a single target might not be an ideal approach for adjunctive therapies in CM, especially when that target has multiple functions physiologically. An important limitation of our study is the lack of causality for the association of EPO with prolonged coma and mortality. However, we hypothesize that the pathogenic role of EPO in CM is due to its role in promoting platelet and endothelial activation [229,238], which could be tested in an *in vitro* BBB model. This would allow the investigation of physiologically relevant EPO levels, either rHuEPO or EPO that lacks erythropoietic effects on endothelial and platelet activation, as well as on IE sequestration.

Overall, the work presented in this dissertation identifies both IE cytoadhesion and host immune factors as important contributors to SM pathogenesis. We have shown that polymorphisms associated with less bound and more soluble EPCR are associated with reduced risk of SM; that EPCR-binding PfEMP1 are important in SM and that their expression is higher in CM than SMA; that the immune profile, while quite similar in CM and SMA, is differentiated especially by elevated levels of chemokines and IL-10 in CM. Our studies on the association of TNF-α and EPO with disease severity in CM highlight the importance of understanding both systemic and local effects of host mediators when

considering targets for adjunctive therapies. In addition, successful adjunctive therapies must recognize and address the various physiological roles of a potential target and selectively inhibit only pathogenic effects without compromising essential and beneficial roles.

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Appendix

Appendix for Chapter 3

Supplemental Table 3.1. Prevalence of rs9574-C variant in malaria disease groups and community control

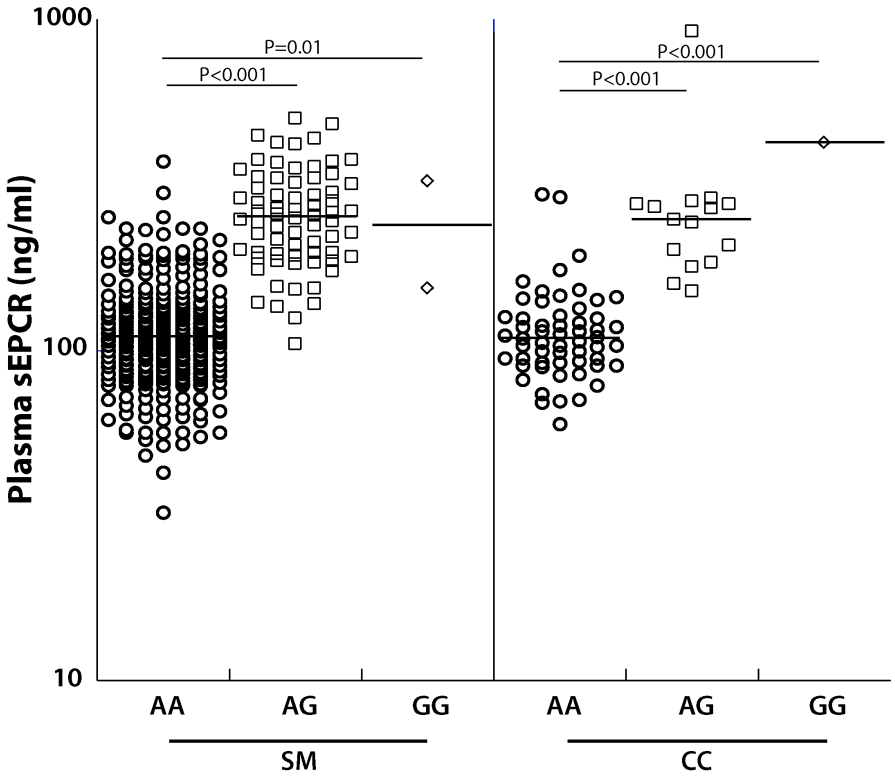
	rs9574 (G4678C)			P[a] Additive model	P[a] Recessive model	P[a] Dominant model
	GG, N (%)	**GC, N (%)**	**CC, N (%)**		**CC vs. GC+GG**	**GG vs. GC+CC**
SM (N=550)	382 (69.5)	155 (28.2)	13 (2.3)	0.14[b]	0.28[b]	0.29[b]
UM (N=71)	59 (83.1)	11 (15.5)	1 (1.4)	0.32[c]	0.44[c]	0.18[c]
CC (N=170)	126 (74.1)	37 (21.8)	7 (4.1)	Reference	Reference	Reference

SM, severe malaria (cerebral malaria or severe malarial anemia); UM, uncomplicated malaria ; CC, community children
[a]Fisher's exact test used. *P*<0.008 considered significant to control for multiple comparisons
[b]SM vs. CC
[c]UM vs. CC

Supplemental Table 3.2. Prevalence of rs9574-C and rs867186-G variants in malaria disease groups and community control

	H1/H3, N (%)	H1/Hx or H3/Hx, N (%)	Hx/Hx, N (%)	P[a] (H1/H3)
SM (N=550)	21 (3.8)	231 (42.0)	298 (54.2)	0.99[b]
UM (N=71)	2 (2.8)	22 (31.0)	47 (66.2)	0.99[c]
CC (N=170)	6 (3.5)	69 (40.6)	95 (55.9)	Reference

SM, severe malaria (cerebral malaria or severe malarial anemia); UM, uncomplicated malaria; CC, community children
[a] Fisher's exact test used. P<0.025 considered significant to control for multiple comparisons; H1/H3 having at least one copy of each variant (heterozygous or homozygous for H1 and heterozygous or homozygous for H3)
[b]SM vs. CC
[c]UM vs. CC



Supplemental Figure 3.1. rs867186-G is associated with increased levels of systemic sEPCR at 6-month follow-up

sEPCR levels are represented on a logarithmic scale and each disease group is separated by rs867186 genotype: AA, AG or GG. The horizontal line represents median values. Severe malaria (SM), community controls (CC).

Appendix for Chapter 4

Supplemental Table 4.1. Primers used in the study and their targets

Primer name	Represented in the paper	Primers	Target
CIDR1δ	CIDR1δ	F: TAAATGTAACTTAGATGTATGTGAAC F: TAAATGTAACTTACATGTATGTGAAC F: TAAATGTAACTTAGACGTATGTGAAC F: TAAATGTTACTTAGATGTATGTGAAC F: TAAATGTAACTTAGATATATGTGAAC F: TAAATGTAAGTTAGATGTATGTGAAC R: AATACTTTAACCAACGTTTAATCAATAC R: AATACTTTAACCAACGCTTAATCAATAC R: AATACTGCAACCAACGTTTAATCAATAC R: AATGCTCTAACCAACGTTTAATGAATAC R: AATACATCAACCAACGCTTAATCAATAC	Rosetting PfEMP1
DBLα1ALL	Group A	F: TTGGGAAATGTRTTRGTTACAGCAAA F: TTGGGAAATGTGTTAGTTATGGCAAA F: TTGGGGAATTTGTTAGTTATGGCAAG F: TTGGGGAACCTATTAGTTATGGCAA F: TTAGGAAATATATTGGTAGCAGCAA F: TTAGGAAATATCTTGGTCACAGCAA R: CCTATATCNGCAAAACTKCKWGC	All group A
DBLα1.5/6/8 types	DBLα1.5/6/8 types	F: TGGTWYRANGAATGGGCAGAAGA F: TGGTTYGAGGAATGGAGTGAAGA R: GATTTGTTTTWTTACAATCGTAACCCTC R: ACAATCCTCACCATCACCACTACAAT R: CGTGATATATCTGTTTKAGTACAATC R: GATCTGTTCGTTTACAATCGTAACCCTC	Group A mostly non EPCR-binders, associated with rosetting
DBLα2/1.1/2/4/7/9 types	DBLα2/1.1/2/4/7/9 types	F: TGGTWYRANGAATGGGCAGAAGA F: TGGTTYGAGGAATGGAGTGAAGA R: TACAATCATATCCATTAWGACTACAA R: TCACAATCGCATCCATTATGACTACAA	Group A EPCR-binders

CIDRα1.4	DC13	F: AACTATCAAAAATGGGAATGCTATTA F: AACTATGAACAATGGAAATGCTATTA F: AACTATCAAAAATGGAATTGCTATTA F: AACTATGAAAACTGGCAATGCTATTA F: AACAATCAAATATGGAAATGCTATTA R: TTTCCCACTTTATAGTGTCTATTA R: TTTCCCATTTTATAGTGTCTATTA R: TTTCCCACTTTATACTGTCTATTA R: TTTCCCAGTTTATAGTGTCTATTA R: TTTCCCACTCTATAGAGTCTATTA	Group A DC13
CIDRα1.5a		F: GATTTATGGATTAAGAATTTATTAAG F: GATTTGTGGGTTACGAATTTATTAAG F: GATTTGTGGGTTACATATTTATTAAG R: TAATTCATCCGTAAATTTCTTCCA R: CAAATCTTCCTTAAGTTTTTTCCA R: TAATTCATCCGTAAATTGATTCCA R: CAAATCTTCTTTAAGTTTTTTCCA	Group A EPCR binders
CIDRα1.5b		F: ACGATACTATAGACTGGAAATACG F: ATTGGGAAWATAAACTTAAGACCTG F: TGGATACTACAGATTGGGATCGTA R: AACCCATTGTTCAAAACATTTACA R: AACCCATTTATCAAAACACGTACA R: AACCCATTTATCAAAACACATACA	Group A EPCR binders
CIDRα1.6b		F: ATAATACTAATGTSACGGATTGT R: CAGTTTCTTTATACTATCCCATTC R: ACATCCTTTATACTACCCCATTCC R: AATTCCTTTATACTCTTCCATTCTG	Group A EPCR binders
CIDRα1.7		F: CGGAAACTATAACGTGGAACGATAA F: CGGAAACTATAAGGTGGAACGATAA F: CGGAAACTATAACGTGGAAAGATAA F: GGATACTATAATGTGGAATGATAAA R: TAGTTTCTTTATACTATTCCATTC R: TAGTTCCTTTATACTATTCCATTC R: TAGTTTCTTTATATTATTCCATTC R: TAGTTTCTTTATACTACTCCATTC R: TAATTCCTTTATATTATTCCATTC	Group A EPCR binders
Group A-EPCR binders	Median of CIDRα1.4, CIDRα1.5a, CIDRα1.5b, CIDRα1.6b and CIDRα1.7		
CIDRα1.1	DC8	F: TGGGAACATCAACTTAAGGATTGCATA F: TGGGAACATCAACTTAAGAATTGCATA F: TGGGAACATGAACTTAAGGATTGCATA R: TAAATCTTYCNTAAATTGATHCCAT	Group B DC8
CIDRα1.8a		F: ATAATTGTGAAATGAAAGGTTCA R: TATGCAMTTCTTAAGTTTGGTTTCC	Group B EPCR

			binders
CIDRα1.8b		F: AATAGACAGTATAATGTGGGAA F: AAAGGATACTATAAAGTGGGAA R: CAAAACATWTACAATTTTCGTTACA	Group B EPCR binders
Group B – EPCR binders	Median of CIDRα1.1, CIDRα1.8a and CIDRα1.8b		
CIDRα1-EPCR	Median of CIDRα1.1-CIDRα1.8b		

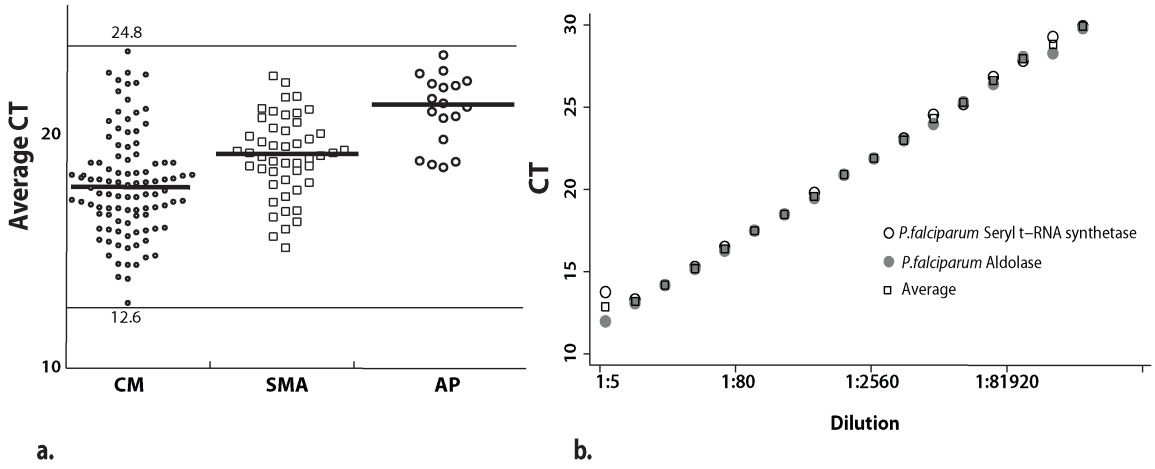
Supplemental Table 4.2. Transcript abundance of var domains in children with cerebral malaria vs. those that have both CM and SMA

	CM/SMA (n=21)	**CM (n=77)**	**P**
CIDR1δ	2.73 (1-6.05)	1 (1-3.44)	0.12
DBLa1ALL	73.7 (25.0-140)	40.5 (9.88-77.6)	0.09
DBLa1.5/6/8 types	13.7 (8.23-31.1) n=18	10.4 (3.45-22.6) n=73	0.11
DBLa2/1.1/2/4/7/9 types	48.6 (27.3-98.1) n=18	43.5 (24.5-60.5) n=73	0.27
CIDRα1.4	11.1 (2.85-20.3)	4.16 (1-11.7)	0.02
Group A-EPCR binders	1.35 (1-3.12)	1 (1-2.38)	0.26
CIDRα1.1	14.3 (1-55.6)	10.4 (1-43.4)	0.72
Group B-EPCR binders	1 (1-5.04)	1.37 (1-4.93)	0.87
CIDRa1-EPCR binders	1.76 (1-6.06)	1.22 (1-3.62)	0.29

Supplemental Table 4.3. Transcript abundance of var domains in CM children with higher and lower than 1700ng/ml PfHRP-2 levels

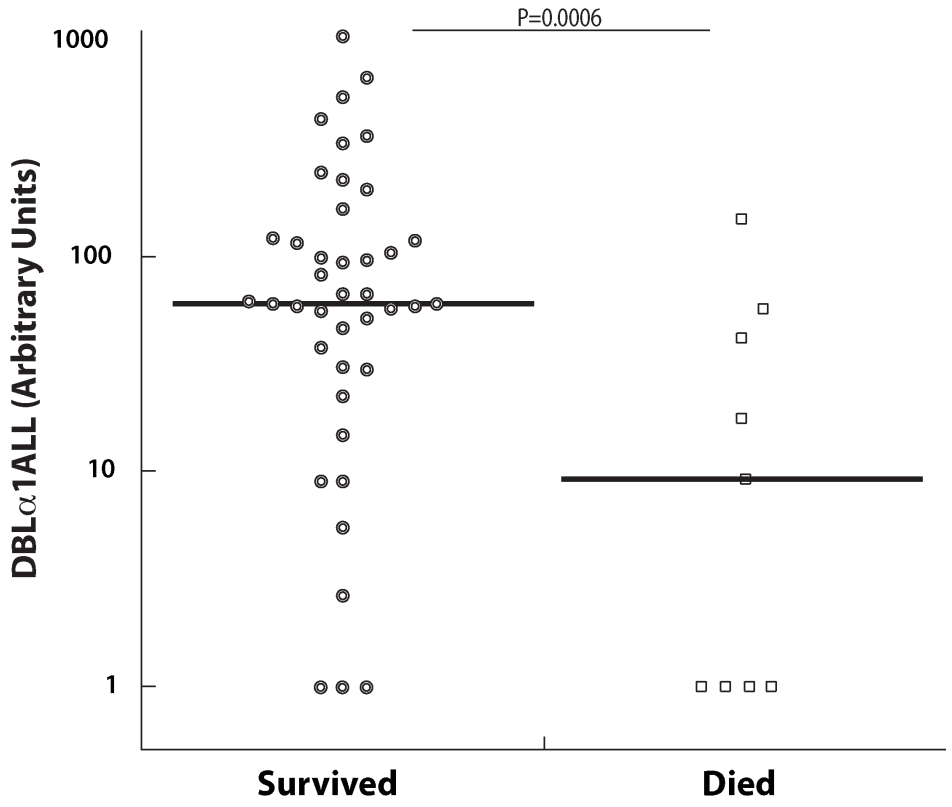
	***Pf*HRP-2-high (n=62)**	***Pf*HRP-2-low (n=35)**	**SMA (n=47)**	**P[a]**
CIDR1δ	1.34 (1-4.02)	1 (1-5.57)	1 (1-2.21)	0.58
DBLa1ALL	53.8 (20.8-78.0)	36.9 (1-107)	25.4 (1-59.1) n=46	0.06[b]
DBLa1.5/6/8 types	10.8 (5.21-26.6) n=55	11.5 (2.75-22.9)	5.95 (1.39-17.0) n=44	0.09
DBLa2/1.1/2/4/7/9 types	43.5 (23.6-69.8) n=55	49.1 (28.4-70.2)	27.3 (10.1-45.7) n=43	0.009[c]
CIDRα1.4	4.76 (1.35-16.6)	5.08 (1-12.7)	1.27 (1-13.0)	0.26
Group A-EPCR binders	1 (1-2.15)	1 (1-2.92)	1(1-1)	0.03[d]
CIDRα1.1	13.0 (1-43.3)	10.9 (1-48.3)	3.11 (1-21.4)	0.11
Group B-EPCR binders	1 (1-4.09)	1.91 (1-5.88)	1 (1-2.25)	0.17
CIDRα1-EPCR binding	1.23 (1-3.37)	1.82 (1-4.07)	1 (1-1.57)	0.06

[a] ANOVA, Tukey post-hoc test adjustment for multiple comparisons with log10 transformed values.
[b] In post-hoc testing, SMA differed from RP
[c] In post-hoc testing, SMA differed from RP and RN
[d] In post-hoc testing, SMA differed from RN



Supplemental Figure 4.1. Detection of housekeeping genes in parasites infecting asymptomatic controls

(a) Median comparison of average Ct values for the two housekeeping genes (seryl t RNA synthetase and fructose-bisphosphate aldolase) between disease groups. Average Ct values are shown on a logarithmic scale. The horizontal line represents median values. b) Standard curves for both housekeeping genes and the average based on dilutions of 3D7 gDNA. Cerebral malaria (CM), severe malarial anemia (SMA) and asymptomatic control (AC).



Supplemental Figure 4.2. DBL*α*1 transcripts, targeting all group A *var* genes are lower in parasites from retinopathy positive patients that died

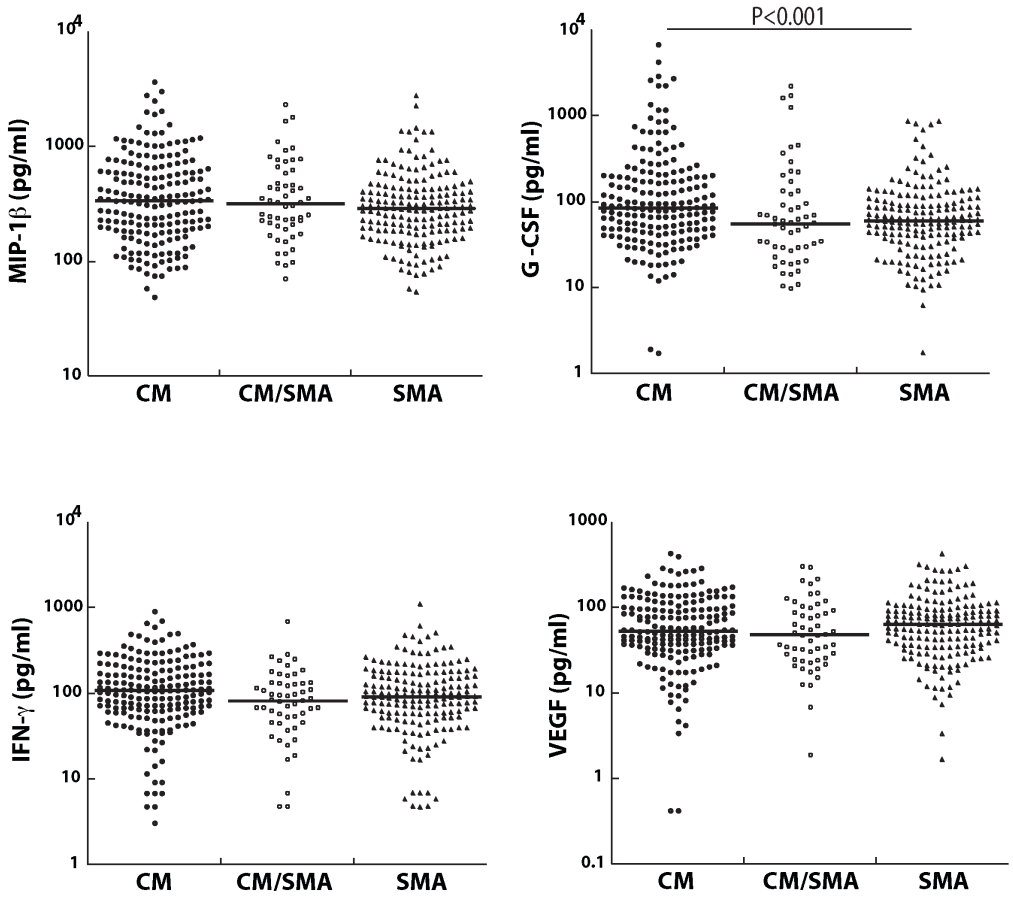
Arbitrary unit values for DBLα1ALL (all group A var genes). Medians are compared by Mann-Whitney test. Only retinopathy positive cerebral malaria cases are presented in this figure.

Appendix for Chapter 6

Supplemental Table 6.1. Differences in plasma cytokines between severe malaria and healthy Ugandan controls without P.falciparum parasitemia by PCR

	SM (n=413)	CC (n=123)	P[a]
IL-1ra (pg/ml), median (IQR)	900 (398-2840)	214 (134-318)	<0.0001
IL-8 (pg/ml), median (IQR)	36.8 (23.5-66.8)	21.1 (12.9-33.1)	<0.0001
IL-10 (pg/ml), median (IQR)	163 (55.4-560)	8.27 (4.13-16.5)	<0.0001
IL-12p70 (pg/ml), median (IQR)	20.3 (11.6-36.8)	23.5 (13.7-42.5)	0.09
FGF-basic (pg/ml), median (IQR)	32.7 (12.8-48.6)	46.6 (30.7-68.1)	<0.0001
G-CSF (pg/ml), median (IQR)	67.2 (39.5-137)	53.4 (35.5-85.3)	0.003
IFN-γ (pg/ml), median (IQR)	96.9 (58.4-170)	158 (92.5-286)	<0.0001
IP-10 (pg/ml), median (IQR)	3566 (1421-9540)	557 (407-816)	<0.0001
MCP-1 (pg/ml), median (IQR)	56.4 (25.6-180)	23.8 (15.2-33.3)	<0.0001
MIP-1α (pg/ml), median (IQR)	6.73 (3.93-11.4)	6.95 (4.37-10.9)	0.61
MIP-1β (pg/ml), median (IQR)	306 (185-533)	76.8 (58.5-105)	<0.0001
PDGF-b (pg/ml), median (IQR)	730 (301-1424)	1197 (679-2049)	<0.0001
RANTES (pg/ml), median (IQR)	2563 (1412-5351)	8248 (4976-12830)	<0.0001
VEGF (pg/ml), median (IQR)	55.0 (33.0-98.1)	40.5 (22.6-67.9)	<0.0001
TNF-a (pg/ml), median (IQR)**	93.0 (49.3-175)	25.5 (18.1-40.6)	<0.0001
IL-6 (pg/ml), median (IQR)**	53.7 (18.8-216)	13.2 (1.71-38.4)	<0.0001
IL10: TNF-a ratio, median (IQR)**	1.88 (0.97-4.11)	0.23 (0.14-0.58)	<0.0001

[a] Wilcoxon rank-sum (Mann-Whitney) test.
*SM (n=391), CC (n=119)



Supplemental Figure 6.1. Cytokines and chemokines that did not differentiate well between CM and SMA

The horizontal line represents median values. P-values represent ANOVA on log 10 transformed values followed by Tukey post-hoc adjustment for multiple comparisons. CM (cerebral malaria, hemoglobin >5g/dL), CM/SMA (cerebral malaria, hemoglobin ≤5g/dL), and SMA (severe malarial anemia).