THE ROLE OF CIRCULATING ENDOTHELIAL PROGENITOR CELLS (CEPCs) AND OTHER BIOMARKERS IN THE PATHOGENESIS OF CEREBRAL MALARIA

BY

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DECLARATION

I do declare that this thesis except for the references to other persons' investigations which I have duly acknowledged, is the result of my own original research and that it has neither in whole nor in part been submitted for another degree anywhere. This work was done under the supervision of the persons mentioned below.

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DEDICATION

To my wife, Mrs. Salomey Oduro and my kids; Maame Afua, Nana Akua and Daniel (Jnr). Also to my parents, siblings and entire staff of Immunology Department, NMIMR especially Prof. Ben Gyan.

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ABBREVIATIONS

Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APC	Activated Protein C
APC	Allophycocyanin
BBB	Blood Brain Barrier
BCS	Blantyre Coma Score
BD	Becton Dickinson
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
C-C BCT	Cyto-Chex Blood Collection Tube
CECs	Circulating Endothelial Cells
CEPCs	Circulating Endothelial Progenitor cells
CI	Confidence Interval
CNS	Central Nervous System
CM	Cerebral Malaria
CSF	Cerebrospinal Fluid
ECs	Endothelial Cells
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPCs	Endothelial Progenitor Cells
EPCR	Endothelial Protein C Receptor
FACS	Fluorescence –Activated Cell Sorting

FITC	Fluorescein Isothiocyanate
GDP	Gross Domestic Product
Hb	Haemoglobin
HC	Healthy Control
HCT	Haematocrit
HIV	Human Immunodeficiency-Virus
HRP2	Histidine Rich Protein 2
HSCs	Hematopoietic Stem Cells
ICAM-1	Intercellular Adhesion Molecule-1
IFN-ɣ	Interferon-ɣ
Ig	Immunoglobulin
IL	Interleukin
IRB	Institutional Review Board
iRBC	Infected Red Blood Cell
ITNs	Insecticide-Treated Nets
KDR	Kinase-insert Domain Receptor
LEKMA	Ledzokuku Krowoh Municipal Authority
MAb	Monoclonal Antibody
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Content
MCV	Mean Corpuscular Volume
MMP-9	Matrix Metalloproteinase-9
NGS	Normal Goat Serum

NMIMR	Noguchi Memorial Institute for Medical Research
NO	Nitric Oxide
OPD	Out Patient Department
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PC	Protein C
PE	Phycoerythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule
PerCP	Peridinin-Chlorophyll-Protein Complex
*Pf*EMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
*Pf*GPI	*Plasmodium falciparum* Glycosylphosphatidylinositol
RBM	Roll Back Malaria
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
SAH	Sub-Arachnoid Haemorrhage
SDF-1	Stromal Cell-Derived Growth Factor-1
sEPCR	Soluble Endothelial Protein C Receptor
SMA	Severe Malaria Anaemia
sTM	soluble Thrombomodulin
TGF	Transforming Growth factor
TM	Thrombomodulin
TSP	Thrombospondin
TNF- α	Tumor Necrosis Factor-α

UCB	Umbilical Cord Blood
UM	Uncomplicated Malaria
UNICEF	United Nations International Children's Emergency Fund
VCAM-1	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor Receptor 1
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
VWF	Von Willebrand Factor
VWFpp	Von Willebrand Factor propeptides
WBC	White Blood Cell
WHO	World Health Organization
WPB	Weibel-Palade Bodies

ABSTRACT

Cerebral malaria (CM) is known to be the most severe complication of *Plasmodium falciparum* infection. Despite effective anti-parasitic treatment, it remains a major cause of morbidity and mortality in infected children. Sequestration of infected red blood cells in the brain microvasculature, occlusion of blood flow, activation and subsequent damage of the endothelium are hallmarks of the pathogenesis of CM. This study sought to quantify and compare time course levels of cEPC and other biomarkers of endothelial injury and repair in Ghanaian children between the ages of 2 and 12 years with cerebral malaria, uncomplicated malaria as well as uninfected healthy controls. Participants with malaria were recruited from five main referral hospitals in Accra, Ghana and healthy uninfected controls from community schools around the hospitals. Matrix metalloproteinase-9 (MMP9), stromal cell-derived growth factor 1 (SDF-1), Angiopoietin 1 and 2, Endothelial protein C receptor (EPCR), thrombomodulin (TM) and Histidine Rich Protein 2 (HRP2) levels were also determined by enzyme-linked immunosorbent assay (ELISA) at the same time points in all study participants. Stability of cEPCs and CECs were assessed in whole blood stored in the cell preservative Cyto Chex BCT (C-C BCT) at 4^{o}C and compared with that stored in ethylenediamine tetra acetic acid (EDTA) antocoagulant at baseline. CM patients showed a baseline mean percentage cEPC of 0.042% which increases to 0.117% at recovery from coma. cEPC levels in uncomplicated malaria (UM) and uninfected healthy controls (HC) were 0.147% and 0.83% respectively at baseline with no significant difference in time course. CEC levels in CM was higher (0.003%) at initial presentation compared with uncomplicated (0.001%) and uninfected healthy controls (0.0009%). However, CEC levels in UM patients spiked (0.009%) at day 7. HC maintained a low CEC levels in time course. SDF-1 levels remained unchanged in all study groups in time course whiles MMP9 levels were higher in UM patient compared with CM

and HC at baseline. Angiopoietin 1 (Ang-1) levels were higher (5960pg/ml) in CM compared to UM (4041pg/ml) and HC (4909pg/ml) though not significant. TM, EPCR and HRP2 level was highest in CM compared with other groups at baseline. The study has shown that cEPCs and the mediators associated with their release and migration are very critical in the resolution of coma in children and has placed CM within the context of current paradigms of microvascular repair, offering strategies that could predict who is at risk of developing CM. Therapies that mobilize and improve cEPC function will therefore be of immense utility in the prevention and treatment of CM.

CHAPTER ONE

1 INTRODUCTION

1.1 Background

Malaria is a major global health problem that poses enormous burden on mankind, both socially and economically (Rénia *et al.*, 2012). A report from World Health Organization (WHO) in 2014 shows that there were an estimated 198 million episodes of malaria worldwide in 2013 from which 584,000 resulted in death. Ninety percent (90%) of these deaths were recorded in Africa. It is also estimated that nearly a quarter of all childhood deaths are caused by malaria (Miller *et al.*, 2013). Out of about 500 million clinical malaria cases that are reported each year, one percent of these cases may become complicated and develop into severe malaria (Idro *et al.*, 2010). In some cases, however, the disease becomes so severe and may lead to death. Malaria is known to be caused by five different species of the *Plasmodium* parasite of which *Plasmodium falciparum* is responsible for nearly all of the severe morbidity and mortality in malaria endemic areas (*WHO*, 2000). About 91% of malaria cases worldwide are caused by *P. falciparum*, with the majority (86%) occurring in the African region (*WHO*, 2008).

Cerebral malaria is known to be the deadliest form of severe malaria and probably amongst the most common non-traumatic encephalopathies in the world due to the fact that its case fatality rate is about 20% and about 7% of children who survive are left with permanent neurological disability, epilepsy or behavioural problems (Birbeck *et al.*, 2010). CM collectively involves the clinical manifestations of *P. falciparum* malaria that induces changes in mental status and coma (Ozen *et al.*, 2006). Malaria can occur in less than two weeks in non-immune individuals after a mosquito bite and CM may develop if not treated after 24 hours of onset of symptoms (*WHO*, 2000; *WHO*,

2014a). Sequestration of infected red blood cells (iRBCs) in the brain microvasculature has been shown to be the hallmark of CM and the resultant damage to the vascular endothelium has been postulated as a major initiator of CM (Cooke *et al.*, 2000; Silamut *et al.*, 1999a; Taylor *et al.*, 2004b; Weatherall *et al.*, 2002). The ability to balance microvascular damage and repair may therefore be critical in the pathogenesis of cerebral malaria.

Studies have shown that recovery from endothelial damage as seen in CM, is defined by a balance between the magnitude of microvascular damage and the capacity for repair (Hill *et al.*, 2003). Repair of damaged endothelium can occur by migration and proliferation of surrounding mature endothelial cells (ECs). However, mature ECs are terminally differentiated cells with a low proliferative potential, and their capacity to substitute damaged endothelium is limited (Hristov *et al.*, 2003a). Therefore, endothelial repair may need the support of other cell types such as bone marrow–derived endothelial progenitor cells (EPCs) which migrate to sites of damage and incorporate into the microvasculature (Lin *et al.*, 2000; Rafii, 2000) to augment the local response which may be insufficient to repair extensive or chronic injury (Gyan *et al.*, 2009). Some studies have shown EPCs to be capable of facilitating vascular repair in different ischaemic tissues (Medina *et al.*, 2010). A cross-sectional evaluation of circulating EPC (cEPC) levels in cerebral malaria by Gyan *et al.* (2009) associated low levels of cEPCs with CM in African children, indicating the importance of cEPCs in microvascular repair in *P. falciparum* infection.

The presence of circulating ECs (CECs) has been recognized as a useful marker of microvascular damage (Goon *et al.*, 2006b). Acute vascular injury has been correlated with an increase in the number of CECs and bone marrow-derived cEPCs in the peripheral blood (Wu *et al.*, 2007).

Although rare in healthy individuals, increased CECs in peripheral blood reflects significant vascular damage and dysfunction (Goon *et al.*, 2006b). Elevated levels of CECs in peripheral blood in CM patients could therefore be predictive of the endothelial damage.

Microvascular damage induces the expression or activation of a series of molecules such as stromal cell-derived growth factor 1 (SDF-1) and the matrix metalloproteinase-9 (MMP-9) which mediate the mobilization and release of EPCs from the bone marrow (Adams *et al.*, 2004; Carmeliet and Collen, 2000; Heissig *et al.*, 2002; Hristov *et al.*, 2003b; Ruhrberg, 2003; Szmitko *et al.*, 2003; Urbich and Dimmeler, 2004). Elevated levels of SDF-1 in acute malaria infection could determine to a larger extent, the mobilization and release of EPCs from the bone marrow and subsequent repair of damaged endothelium (Gyan *et al.*, 2009). These chemokine/proteases could therefore play a major role in the pathogenesis of CM.

Other endothelial mediators such as the Angiopoietins (Ang-1 and Ang-2) are known to be important regulators of vascular structure and function, and are hallmark indicators of vascular injury (Chittiboina *et al.*, 2013). These proteins have shown promise as targets in the treatment of diseases such as traumatic brain injury, sub-arachnoid haemorrhage (SAH) and sepsis (Chittiboina *et al.*, 2013; Parikh, 2013). Angiopoietins have also been shown to discriminate cerebral malaria and severe, non-cerebral, malaria from uncomplicated malaria (Conroy *et al.*, 2009; Lovegrove *et al.*, 2009) indicating the importance of these mediators in the pathogenesis of severe malaria.

Thrombomodulin (TM) and Endothelial Protein C Receptor (EPCR) are receptors expressed on endothelial cells and are essential components of the anticoagulant protein C pathway, an

endothelial homeostatic signal critical in regulating coagulation, inflammation, endothelial barrier function, and neuro-protection (Esmon, 2000). These receptors are shed into peripheral blood when the endothelium is inflamed and increased levels of soluble forms of these receptors in peripheral blood have been shown in cerebral malaria (Boehme *et al.*, 1996; Moxon *et al.*, 2013). Soluble TM and EPCR therefore show promise as predictors of cerebral malaria.

Sequestration of asexual parasites in the brain microvasculature and other organs in the severe malaria host makes identification of peripheral parasitaemia difficult. Histidine Rich Protein 2 (HRP2), a protein produced by *P. falciparum* has been used as a diagnostic marker and to estimate parasite burden in severe malaria (Storm and Craig, 2014). Elevated levels of this protein has also been used to distinguish coma caused by CM and other infections (Kariuki and Newton, 2014).

This study therefore aimed at characterizing the hosts' response to *P. falciparum* induced microvascular damage and determine its relationship to the development of, and recovery from, cerebral malaria. This was done by longitudinally determining the levels of CECs, EPCs, chemokines/proteases (SDF-1 and MMP-9) associated with the release of EPCs and endothelial mediators (Angiopoietins), endothelial receptors (soluble TM and soluble EPCR) as well as *P. falciparum* parasite protein, HRP2, in children with cerebral malaria, uncomplicated malaria and uninfected healthy controls.

EPCs by nature are very rare in peripheral blood (Duda *et al.*, 2007) and their determination by flow cytometric analysis must be done immediately after collection and staining of fresh whole blood samples (NCCLS, 1998, as cited in (Schumacher and Burkhead, 2000)). The study also

aimed at developing methods that would allow preservation of samples for delayed flow cytometric analysis and for the extension of this technique to biological samples from remote settings where flow cytometers are not readily available.

1.2 Justification and Objectives

1.2.1 Justification

Cerebral malaria is the most severe neurological complication of infection with *Plasmodium falciparum* and is a major cause of child death in sub-Saharan Africa. Several theories have evolved to define the pathogenesis of cerebral malaria. However, the sequestration of infected red blood cells in the brain microvasculature, activation and subsequent damage of the endothelium are hallmarks (Cooke *et al.*, 2000; Silamut *et al.*, 1999a; Taylor *et al.*, 2004b; Weatherall *et al.*, 2002).

Damaged microvasculature was believed to be repaired solely by the replication or migration of local preexisting vascular wall endothelial cells to sites of injury (Asahara *et al.*, 1997; Lin *et al.*, 2000; Rafii, 2000). It is now known that circulating bone marrow derived EPCs are involved in the repair of microvascular damage (Asahara *et al.*, 1999). They augment the local response, which may be insufficient to repair extensive or chronic injury by replication or migration. Studies suggests that therapies and tests based on microvascular homeostasis are currently being aggressively developed by industry to treat and determine who is at risk for cardiovascular events, stroke, asthma and ischemic disease, and might be of utility in cerebral malaria. The capability of cEPCs as biomarkers and their use as therapeutic agents for microvascular repair have shown promise in cardiovascular disease (Timmermans *et al.*, 2009).

Gyan *et al* (2009) associated cerebral malaria with low levels of cEPCs, thereby placing cerebral malaria pathogenesis within the context of the current paradigms of microvascular homeostasis. Their study postulated that an increase in cEPC levels could correlate with recovery from cerebral malaria. The body's ability to elevate peripheral levels of cEPCs could therefore be predictive of recovery from CM.

Several molecules have been implicated in the loss of endothelial integrity and by extension also implicated in the pathogenesis of cerebral malaria. These molecules may include stromal cell derived growth factor 1 (SDF-1) and the matrix metalloproteinase-9 (MMP-9) which mediate the mobilization and release of cEPCs (Adams *et al.*, 2004; Carmeliet and Collen, 2000; Heissig *et al.*, 2002; Hristov *et al.*, 2003b; Ruhrberg, 2003; Szmitko *et al.*, 2003; Urbich and Dimmeler, 2004), endothelial mediators such as Angiopoietin 1 and 2 (Lovegrove *et al.*, 2009) and endothelial receptors such as EPCR and TM (Moxon *et al.*, 2014).

The repair role of EPCs and the other endothelial mediators could help address some questions that have remained unanswered in the pathogenesis of CM: why is coma so rapidly reversible with treatment despite the large number of parasites in the brain of most patients? Why do some children recover quickly from coma while others die?

This study therefore aimed at determining factors that affect the levels and function of cEPCs and CECs and their relation with the progression of uncomplicated malaria to cerebral malaria. This knowledge would be important in addressing some unanswered questions as stated above, predict

who will develops cerebral malaria and explore the possibility of cEPCs as therapeutic agents for cerebral malaria and in the development of vaccines.

1.2.2 Aims and Objectives

Generally the aim of the study was to firstly determine and compare the time course host response to microvascular damage in Ghanaian children with CM, UM and HC. An additional objective was to investigate the ability of Cyto-Chex BCT to maintain the viability of receptors expressed by circulating endothelial cells and their progenitors

1.2.2.1 Specific objectives

1. To determine and compare levels of cEPC/CEC in whole blood stored with EDTA and C-C BCT at 4^{o}C over seven days.
2. To determine levels of cEPC/CEC in CM patients at initial clinical presentation and at recovery from coma as well as at days seven and fourteen post recovery.
3. To determine levels of cEPC/CEC in UM patients and uninfected healthy controls at initial presentation and at days seven and fourteen after initial presentation.
4. To compare cEPC/CEC levels within the patient groups and across the three study groups.
5. To measure and compare levels of chemokines/proteases (SDF-1, and MMP-9), endothelial mediators (Angiopoietin 1 and 2), endothelial receptors (EPCR and TM), and HRP 2 at all sampling time points in the three study groups.

1.2.2.2 Study hypothesis

The study hypothesized that:

1. Increasing levels of cEPCs and decreasing levels of CECs and chemokine/protease correlate with recovery from cerebral malaria as shown in Figure 1.1 below:

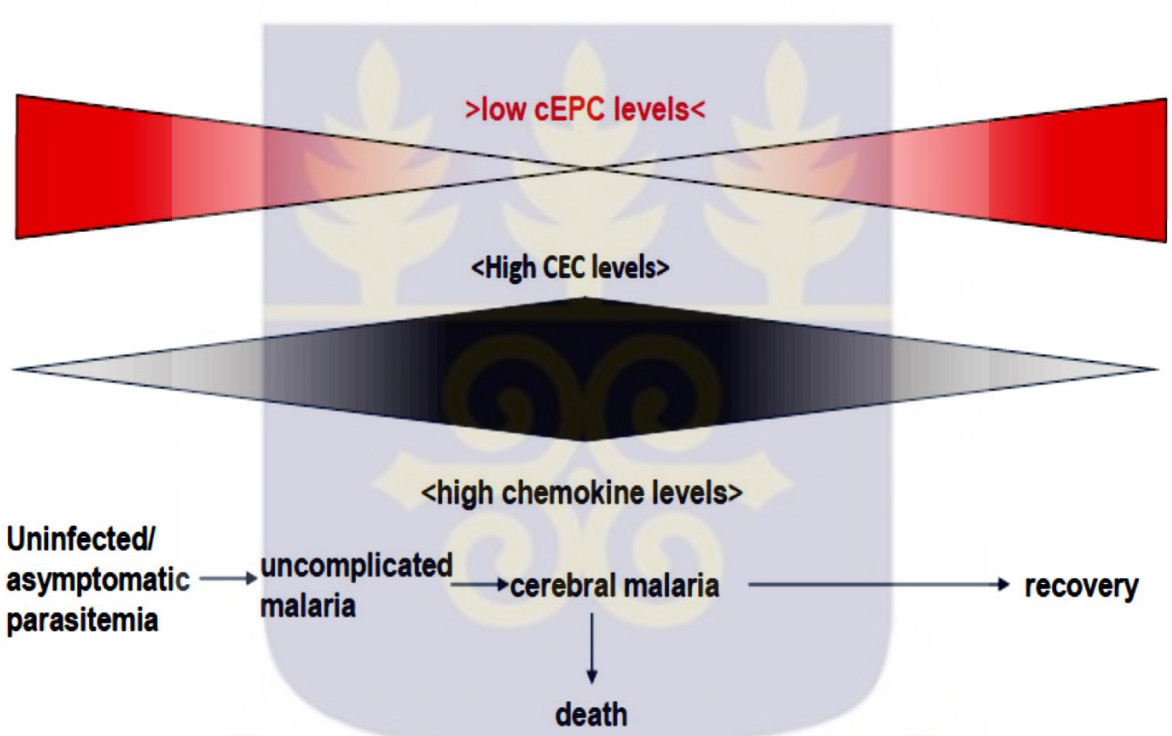


Figure 1. 1 Model of the development and resolution of cerebral malaria

2. Lower Ang-2: Ang-1 ratio correlates with recovery from cerebral malaria.
3. Increased levels of sEPCR and sTM are associated with cerebral malaria.
4. Higher HRP2 levels is higher in true CM than uncomplicated malaria

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Socio-economic Burden of Malaria

2.1.1 Global malaria burden

The World Malaria Report of 2014 shows that approximately 3.3 billion people were at risk of malaria around the world and 219 million cases are estimated to have occurred. Africa alone accounts for 89% of malaria cases and 91% of malaria deaths (WHO, 2014b; WHO, 2014c). In 2013, malaria alone caused an estimated 453 000 under-five deaths globally and an estimated 437 000 African children died before their fifth birthday due to malaria in the same year (WHO, 2014c). In high-risk areas, more than one malaria case occurs per 1000 population and with the estimated number of deaths in 2013, it indicates 1300 children deaths every day (WHO, 2014c). More than 90 countries are known to have ongoing malaria transmission and hence malaria is considered a public health problem (WHO, 2014b). Despite the considerable public health efforts directed at treatment and control worldwide, malaria remains the most important parasitic disease globally with almost one-quarter of the world's population at risk (Hay *et al.*, 2009). In Ghana, despite several gains in malaria control initiatives worldwide, malaria situation remains high (between 25-65 death rate per 100,00 population) [Figure 2.1], with a prevalence of 67% of households reporting an episode of malaria every two weeks (Musah, 2013).

Malaria inflicts serious negative impact on health and lays a heavy economic and social burden on families, communities and societies in the poorest countries of the world and has thus been tagged as a disease of poverty (Karunamoorthi, 2012). It is the major cause of repeated work absenteeism in endemic regions and this results in short and long term losses in productivity as the main

transmission periods coincide with the peak agricultural and harvesting seasons (Karunamoorthi and Bekele, 2009). In regions where malaria thrives, jobs and school days are lost, productivity plummets and entire communities remain locked in an unbreakable cycle of disease and poverty (RBM, 2013). When it does not kill, the disease can lead to permanent neurological and cognitive damage in children, thus impeding education, reducing career opportunities and lowering productivity in adult age. The direct and indirect costs of malaria have been shown to be a major constraint to economic development with the direct costs being a combination of personal and public expenditures on both prevention and treatment of the disease. At the micro level the personal expenditures include individual or family spending on insecticide-treated nets (ITNs), doctors' fees, anti-malarials, transport to health facilities, and support for the patient and an accompanying family member during hospital stays (RBM, 2003). At the macro level the economic burden of malaria is estimated at an annual reduction in economic growth of 1.3% for those African countries with the highest burden (WHO, 2009). An estimated 12 billion USD loss to the African continent's Gross Domestic Product (GDP) annually as a result of malaria (RMB, 2013).

2.1.2 Malaria burden in Ghana

In Ghana, malaria has been reported as the major cause of poverty and low productivity, accounting for about 32.5 % of all OPD attendances and 48.8 % of children under five years admissions in the country (Aregawi *et al.*, 2009). It is generally believed that malaria is the cause of the highest loss of number of days of healthy life in Ghana although reliable information on the impact of malaria on labour productivity and the economy is absent (Asenso-Okyere and Dzator, 1997).

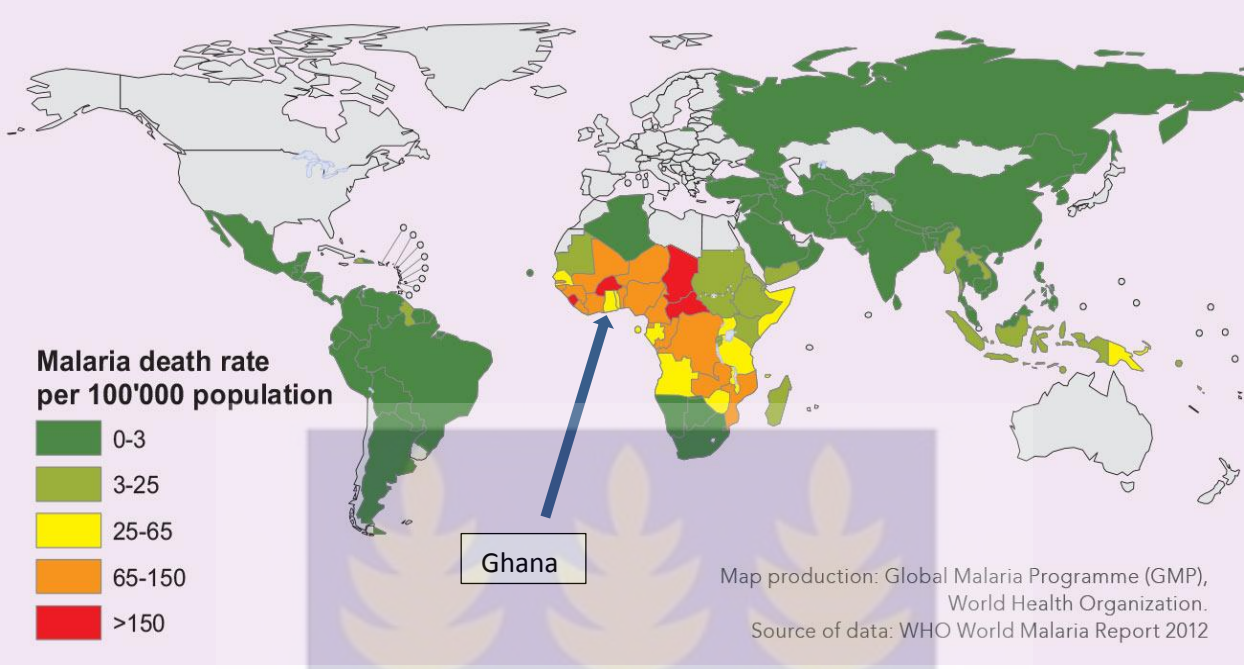


Figure 2. 1 Worldwide Malaria death rate. Adapted from Factsheet on WHO Report, 2013**.**

2.3 Malaria Infection

2.3.1 Malaria parasite

Malaria is caused by infection of red blood cells by the protozoan parasite *Plasmodium*. There are five species of *Plasmodium* that infect humans (*falciparum*, *vivax*, *ovale*, *malariae* and *knowlesi*). These are transmitted by over 30 species of anopheline mosquitoes. *Plasmodium falciparum* is known to be the most deadly among the five species and results in a number of different pathologies associated with specific organ systems. It is responsible for almost all the mortality from malaria and is the only species that appears to directly affect the central nervous system (CNS) causing neurologic deficits and cognitive sequelae. *P. falciparum* is also known to be the predominant species in sub-Saharan Africa where over 90% of all malaria deaths occur (WHO, 2012).

A key feature of the biology of *P. falciparum* is its ability to cause infected red blood cells (iRBCs) to adhere to the linings of small blood vessels. Such sequestered parasites cause considerable obstruction to tissue perfusion. In addition, in severe malaria there may be marked reductions in the deformability of uninfected RBCs (Dondorp *et al.*, 2008).

The majority of malarial deaths in Africa occur in children under 5 years of age, as non-sterile immunity develops with increasing age and recurrent exposure to malaria (Marsh, 1992). It remains unclear why some children develop the severe manifestations of disease while others suffer only mild symptoms or remain asymptomatic (Rowe *et al.*, 1995).

2.3.2 Malarial life cycle

Even-though the pathophysiology and management of malaria are well understood there is still high mortality in children (Miller *et al.*, 2002). Understanding the malaria disease process begins with an understanding of the complex life cycle of *Plasmodium* species. Figure 2.2 shows a simplified malaria life cycle in the mammalian host.

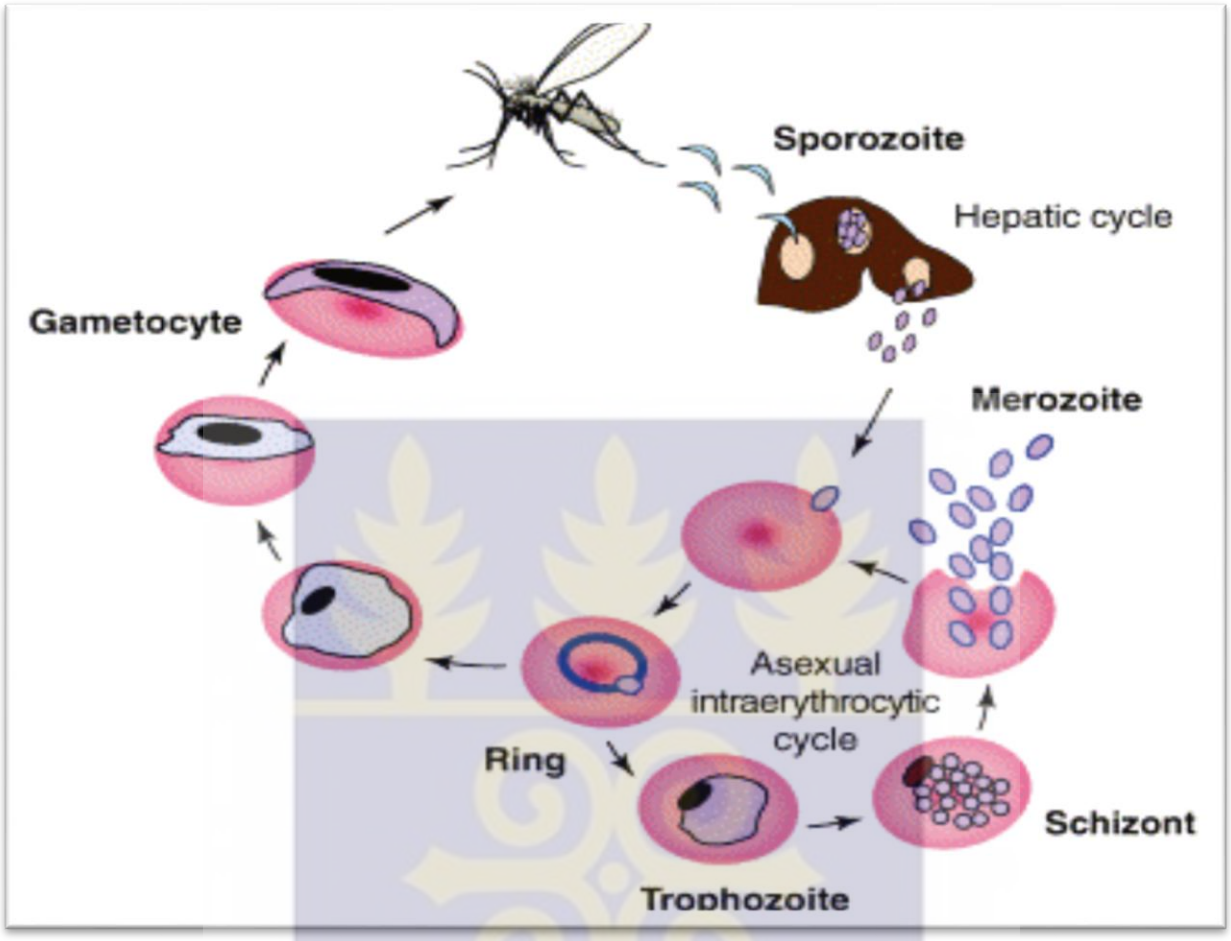


Figure 2. 2 Simplified malaria lifecycle within the mammalian host. Adapted from Serghides *et al.* (2003). *Trends Parasitol*. 19(10): 461-9.

When a mosquito injects motile sporozoites into the human blood stream during a blood meal, they invade the liver cells where they multiply and generate thousands of merozoites. These merozoites are then released into the blood stream and start to invade healthy red blood cells (RBCs). The iRBCs go through a sequence of three main developmental stages – ring, trophozoite and schizont, also known as the asexual multiplication cycle. After 48 hours this sequence ends in the bursting and releasing of 16–32 new merozoites from the iRBCs, and the cycle of invasion and infection starts again.

Some intraerythrocytic parasites take a different developmental path and produce male and female gametocytes to begin the sexual stage of the malaria life cycle within the mosquito midgut. Disease in *P. falciparum* is related to the latter half of the erythrocytic cycle, where: i) schizonts express parasite proteins that mediate the cytoadherence of iRBCs in the microvasculature; and ii) schizonts rupture releasing infective merozoites and other parasite-derived bioactive products.

2.4 Severe Malaria

Despite several breakthroughs in understanding *Plasmodium* biology, including the sequencing of the *Plasmodium falciparum* genome (Gardner *et al*., 2002) and efforts to eradicate the mosquito vector through widespread insecticide campaigns, people (mostly children) are still dying as a result of severe complications of malaria.

Severe malaria is most commonly caused by infection with *P. falciparum*, although *P. vivax* and *P. knowlesi* can also cause severe disease. However, nearly all deaths from severe malaria result from infections with *P. falciparum*. The risk is increased if treatment of an uncomplicated attack of malaria caused by these parasites is delayed. Sometimes, however, especially in children, severe *P. falciparum* malaria may develop so rapidly that early treatment of uncomplicated malaria is not feasible (Michael and World Health Organization, 2000). In high-transmission areas, the risk for severe *falciparum* malaria is greatest among young children and visitors of any age from non-endemic areas. It is defined by clinical or laboratory evidence of vital organ dysfunction. Severe malaria can mimic many other diseases that are also common in malaria-endemic countries. The

most important of these are central nervous system infections, septicaemia, severe pneumonia and typhoid fever.

Severe malaria develops due to the fact that parasites sequester themselves in various organs including heart, lung, brain, liver, kidney, subcutaneous tissues and placenta (Miller *et al.*, 2002). It has been shown that one or more of these organs can be affected with different levels of severity and this can be classified as neurologic and renal dysfunction, haematologic, cardiovascular, and respiratory dysfunction, as well as hepatic and metabolic dysfunction depending on the organ affected (Mohapatra and Das, 2009).

These may be due to fact that, during the malaria cycle, iRBCs circulating in the blood stream begin to lose their deformability (MacPherson *et al.*, 1985), thus becoming potential targets for filtering and destruction by the spleen. To avoid this, the parasite expresses and exports adhesive proteins to the surface of the host iRBC, causing the cell to stick to microvascular endothelial cells in different organs, thereby preventing its clearance by the spleen. Available evidence suggests that organ dysfunction and severe pathology follow the accumulation of iRBCs at high density in particular organs (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991).

Adhesion is therefore believed to be one of the main causes of lethal complications resulting in cerebral malaria and placental malaria. Rapid expansion of iRBC mass, destruction of both infected and uninfected RBCs, microvascular obstruction as a result of parasite sequestration, and inflammatory processes are basic processes that combine to lead to reduced tissue perfusion in severe malaria (Miller *et al.*, 2002). These, in turn, may lead to downstream events at the cellular level that further exacerbate the situation.

The frequent presentations of severe *falciparum* malaria include cerebral malaria, metabolic malaria (hyperlactaemia, acidosis or respiratory distress) and severe anaemia (Dzeing-Ella *et al.*, 2005; Miller *et al.*, 2013). Most often than not, seizures, impaired consciousness, or metabolic acidosis presenting as respiratory distress or severe anaemia are usual manifestations of severe *falciparum* malaria in African children growing up in areas where malaria is endemic. African children rarely develop renal failure or pulmonary oedema (MacPherson *et al.*, 1985).

2.5 Cerebral Malaria

Most malaria-related deaths are associated with cerebral malaria (CM) and is arguably one of the most common non-traumatic encephalopathies in the world and remains a major cause of morbidity (Mishra and Newton, 2009). Cerebral malaria is considered the most severe form of malaria and is caused by infection with *P. falciparum* parasites (Miller *et al.*, 2002). *Plasmodium falciparum* parasite is responsible for almost all the neurological complications associated with malaria, although *P. vivax* causes seizures in children, and is also associated with coma in both children and adults (Mishra and Newton, 2009). It is also considered one of the most dangerous diseases due to the fact that up to 30% of patients who develop cerebral malaria can die (Adams *et al.*, 2002)..

Children in sub-Saharan Africa account for 90% of CM-associated deaths (Dorovini-Zis *et al.*, 2011) and it is estimated that over 785000 children younger than 9 years are affected every year in sub-Saharan Africa, (Newton and Krishna, 1998). Peak incidence is recorded in preschool

children where approximately 575000 children are affected with cerebral malaria annually (Breman, 2001).

The clinical hallmark of cerebral malaria is coma and this collectively involves the clinical manifestations of *P. falciparum* malaria that induces changes in mental status (Idro *et al.*, 2005). The commonly accepted clinical definition of CM is the neurological syndrome with patients in unarousable coma (Newton *et al.*, 1990). The World Health Organization also defines cerebral malaria as a clinical syndrome characterized by coma at least 1 hour after termination of a seizure or correction of hypoglycemia, asexual forms of *Plasmodium falciparum* parasites on peripheral blood smears, and no other cause to explain the coma (WHO, 2000) .

In African children, cerebral malaria can occur in less than two weeks after a mosquito bite and coma develops suddenly with seizure onset often after 1–3 days of fever (Rénia *et al.*, 2012). A few children develop coma after progressive weakness and prostration (Idro *et al.*, 2010).

Without treatment, cerebral malaria is invariably fatal. In children, parenteral anti-malarials (cinchonoids or artemisinin derivatives) are indicated, but even with this treatment, 15– 20% die (Idro *et al.*, 2010; Kain *et al.*, 1998). Although highly effective anti-malarial drugs are widely available, CM case fatality remains 15-20% globally. If a person is not treated, CM is fatal in 24 - 72 hours (Babikir, 2010).

Earlier studies suggested that surviving patients fully recover (Muntendam *et al.*, 1996) but over the past 20 years, it became clear that many children sustain significant brain injury; 11% are discharged with gross neurological deficits and these may include weakness, spasticity, blindness,

speech problems and epilepsy (Birbeck *et al.*, 2010; Brewster *et al.*, 1990; Newton and Krishna, 1998). There is also evidence that suggests that some children who appear to have made a complete neurological recovery from cerebral malaria may develop significant cognitive problems (attention deficits, difficulty with planning and initiating tasks and language problems), which can adversely affect school performance and persist for years after the attack (Brewster *et al.*, 1990; Idro *et al.*, 2007; Njuguna and Newton, 2004).

2. 5.1 Pathogenesis of cerebral malaria

There has been continuous effort to understand the pathogenesis of coma in paediatric CM because it is not clearly known what mechanisms determine the outcome of the illness. One major issue in the pathogenesis of paediatric CM is the nature of tissue injury that leads to severe central nervous system (CNS) damage and death in some of the infected children (Dorovini-Zis *et al.*, 2011).

Histopathological analysis of brain tissue from CM patients at autopsy has identified large numbers of *P. falciparum* iRBCs sequestered in the cerebral vessels with cerebral edema and localized haemorrhages (Aikawa *et al.*, 1990; MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991; Porta *et al.*, 1992; Taylor *et al.*, 2004a). In this regard two main hypothesis have been proposed for human cerebral malaria. The first being the mechanical hypothesis which involve impaired tissue perfusion because of sequestration of parasitized erythrocytes and immune-mediated injury secondary to host responses to parasite products. The second is an immune-pathological hypothesis which proposes that hyper-inflammatory responses responsible for eliminating *P. falciparum* parasite causes cerebral edema resulting from increased permeability and dysfunction of the blood-

brain barrier (BBB) and organ failure (Brown *et al.*, 1999; MacPherson *et al.*, 1985). Figure 2.3 below attempts to summarize the mechanical hypothesis of the pathogenesis of human CM.

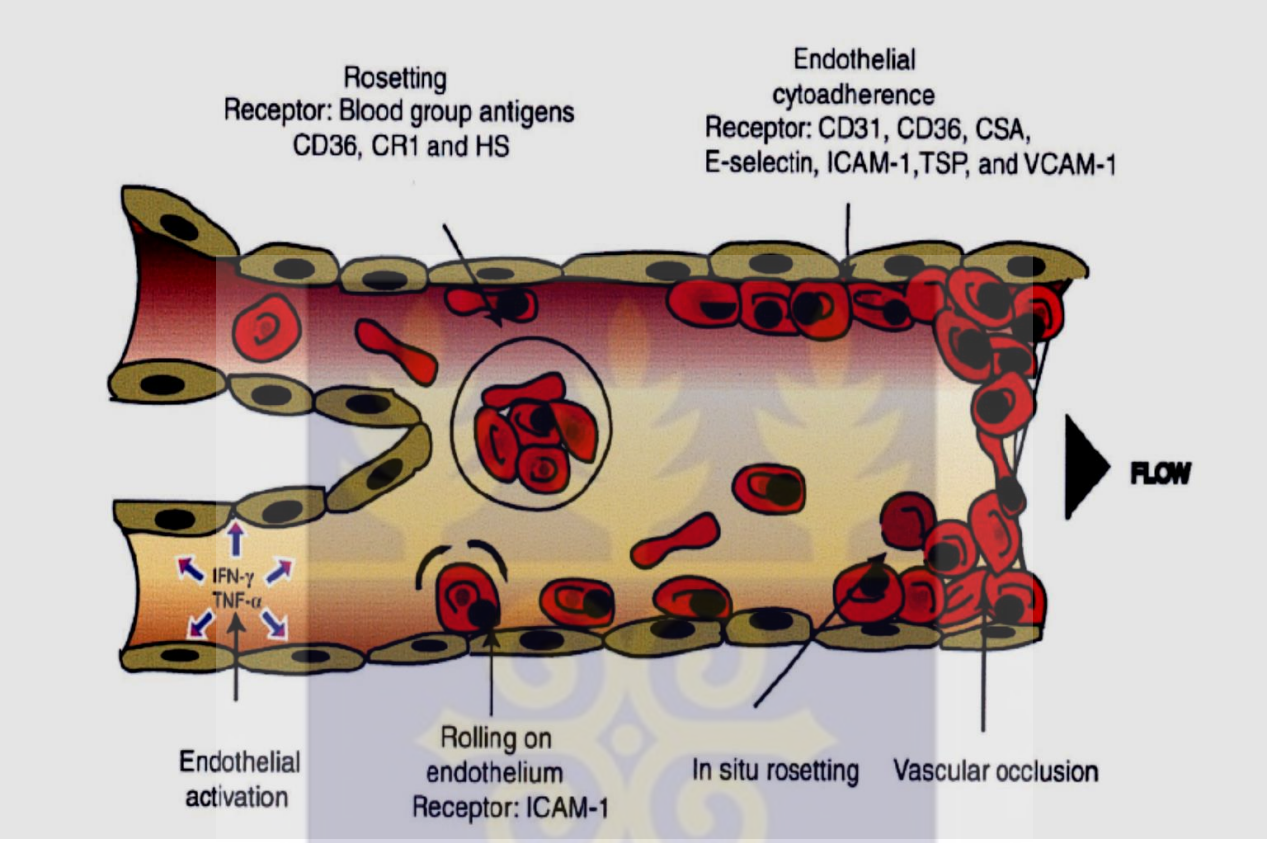


Figure 2. 3 Diagram showing proposed mechanical hypothesis of the pathogenesis of cerebral malaria. Adapted from Chen *et al.* (2000)

2.5.2 Sequestration in CM

It is now widely accepted that the histopathologic hallmark of CM is sequestration of infected red blood cells in the microcirculation of the brain and retina (Gyan *et al.*, 2009). Approximately 16 hours after invasion of the red blood cells (RBCs), structural changes occur on the surface of these iRBCs and this results in the increase in their rigidity and adhesiveness to endothelial cells (Gardner *et al.*, 1996). Owing to the increased adhesiveness, the red cells infected with late stages of *P. falciparum* (during the second half of the 48 hour life cycle) adhere to the capillary and

postcapillary venular endothelium in the deep microvasculature. This is a process that allows the parasite to avoid splenic clearance mechanisms, but comes at a cost to the host. An important difference between *P. falciparum* and other human malarias is the way in which *P. falciparum* modifies the surface of the RBCs so that asexual parasites and gametocytes can adhere to the endothelium (Miller *et al.*, 2002). This is facilitated by excessive inflammatory conditions that contribute to the adhesion of erythrocytes infected with *P. falciparum* to endothelial cells in brain capillaries (Schofield and Grau, 2005).

Adherence to the microvasculature is promoted by the expression of parasite protein including *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of the infected red blood cell (Baruch *et al.*, 1995; Rowe *et al.*, 2009; Su *et al.*, 1995). This protein is expressed by the parasite and exported to the surface of the infected erythrocyte, where it mediates adhesion to various host cell adhesion receptors (Baruch *et al.*, 1995; Turner *et al.*, 1994). The most well-described endothelium receptors for PfEMP1 are ICAM-1 in the brain, although binding to other receptors, including thrombospondin (TSP), PECAM-1, P-selectin, E-selectin, CD 36, CSA and VCAM has been reported (Gardner *et al.*, 1996; Ockenhouse *et al.*, 1992; Roberts *et al.*, 1985; Rock *et al.*, 1988). Sequestration of the growing *P. falciparum* parasites in these deeper tissues provides them the microaerophilic venous environment that is better suited for their maturation and the adhesion to endothelium allows them to escape clearance by the spleen and to hide from the immune system. The structural changes in parasitized RBCs results in the adherence of these parasitized RBCs to uninfected red cells leading to the formation of rosettes (Newbold *et al.*, 1999) as shown in Figure 2.3 above.

A detailed postmortem evaluation of cerebral microvessel sequestration in fatal pediatric CM has shown the presence of parasitized RBC sequestration in all patients with CM and an association of sequestration with microvascular pathology in 75% of patients with cerebral malaria (Taylor *et al.*, 2004a).

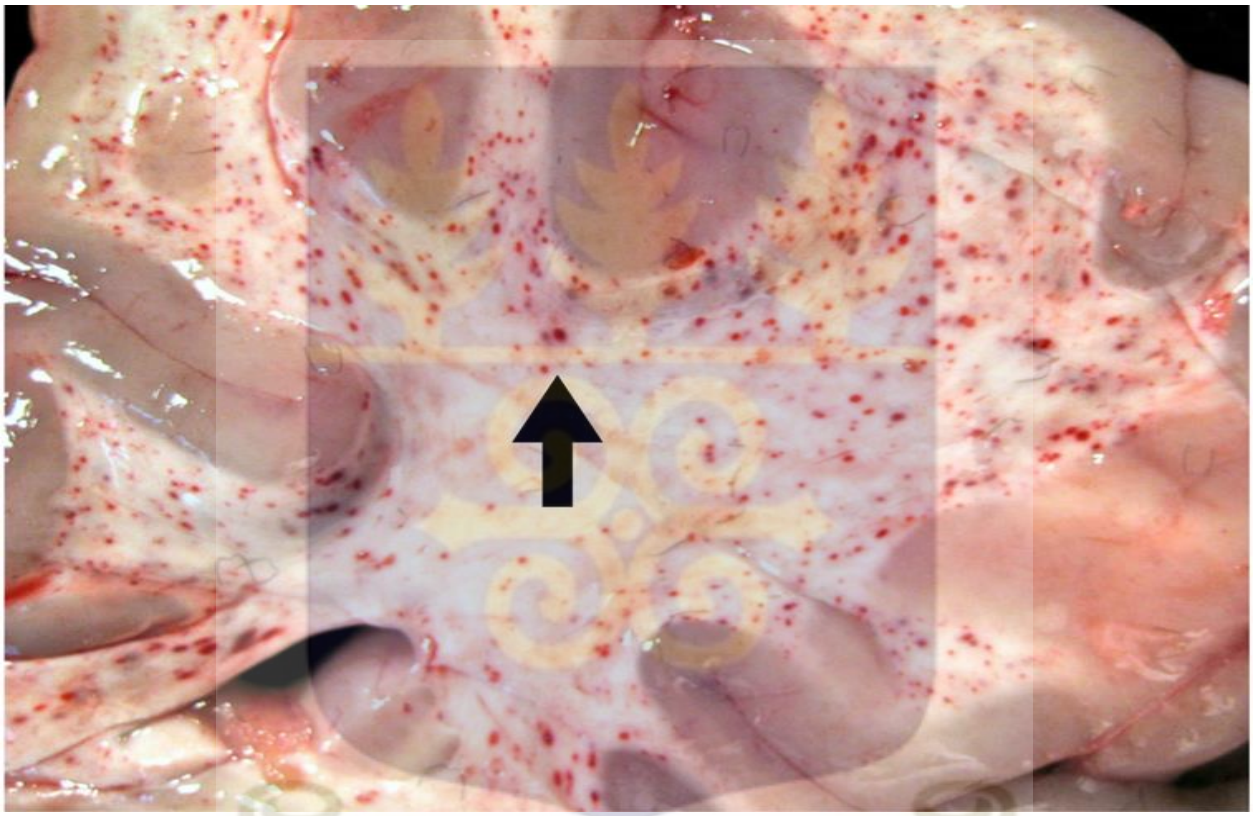


Figure 2. 4 Coronal section of the brain showing iRBC sequestration and microvascular thrombosis in fatal cerebral malaria. Adapted from Moxon *et al.* (2013)

2.5.3 Role of cytokines and immune mediators in CM

The body's response to the presence of infected RBC and subsequent disruption of microvascular blood flow as a result of sequestration may cause localized endothelial dysfunction. In addition

induction of proinflammatory and pro-adhesive molecules may compromise the integrity of the endothelial barrier (Francischetti *et al.*, 2008; Miller *et al.*, 2013; Moxon *et al.*, 2009).

There is now overwhelming evidence that within cerebral vessels, adherent iRBCs induce endothelial activation, with several consequences (Dorovini-Zis *et al.*, 2011). These consequences may be results of the host response to parasite products. Cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1, IL-6 and neuro-active mediators such as nitric oxide (NO) are produced as a result of sequestration of parasitized RBCs in the microvasculature (Figure 2.3). These cytokines are produced to inhibit parasite growth and promote parasite killing (Haidaris *et al.*, 1983). These mediators can however, be toxic to the central nervous system (CNS) when overproduced. These mediators may also cause inflammations of the endothelium as morphological alterations of brain endothelium at the site of iRBC sequestration have been described (Pongponratn *et al.*, 2003). Excessive levels of these cytokines have been associated with severe disease (Day *et al.*, 1999). Children with CM have recorded significantly higher plasma levels of TNF-α than those with mild disease (Babikir, 2010).

Malaria antigens have been shown to induce cytokine production. One major antigen is *P. falciparum* glycosylphosphatidylinositol (PfGPI), which are released from the iRBC when it ruptures at the schizont stage (Krishnegowda *et al.*, 2005). PfGPI has also been shown to be involved in the production of purified TNF- α release from macrophages (Schofield and Hackett, 1993). TNF- α levels in peripheral blood and brain tissues have been shown to be elevated in patients with CM compared to those with uncomplicated malaria (Brown *et al.*, 1999) and high

levels in *P. falciparum* infection have been associated with increased soluble ICAM-1, a marker of endothelial activation (McGuire *et al.*, 1996).

Other cytokines such as IFN- gamma- produced as part of the early response to *P. falciparum* infection (Hensmann and Kwiatkowski, 2001) is also known to sensitize monocytes to produce increased levels of pro-inflammatory cytokines (such as TNF- α) when exposed to parasite inflammatory mediators (Grau *et al.*, 1989).

Decreased levels of IL-10 and transforming growth factor beta (TGF-beta), which are major anti-inflammatory cytokines, has been associated with poor outcome in malaria (Day *et al.*, 1999). Other studies have also shown high ratios of pro:anti-inflammatory cytokines (Perkins *et al.*, 2000). The body's inability to regulate the pro-inflammatory cascade, has been suggested to play a role in severe malaria disease including cerebral malaria.

2. 5.4 Endothelial activation and blood-brain barrier integrity in CM

The brain contains a network of blood vessels which are necessary for providing nutrients and oxygen, and for removing carbon dioxide and waste (i.e. urea, creatinine, etc.). This network of capillaries together with the glia form a protective barrier called the blood-brain barrier (BBB). This barrier prevents large molecules and pathogens in the blood from entering the brain tissues and from altering the brain's functions (Cardoso *et al.*, 2010; Neuwelt *et al.*, 2011). The brain is very sensitive to blood chemistry variations and its homeostasis is tightly regulated (Levin *et al.*, 2011).

Maintenance of homeostasis is principally due to the brain endothelial cells, which are on the luminal side of the blood-brain barrier and correspond to the actual barrier site. Brain endothelial cells differ from those found in other tissues in many ways. They are attached by tight junctions of high electrical resistance preventing intercellular passage of molecules, and do not contain small openings called slit pores that allow the diffusion of molecules (Rénia *et al.*, 2012). Brain endothelial cells also have important functions in mediating and regulating the immune response in the nervous system (Miller, 1999).

Endothelial cells, line the inner surface of blood cells and form a structural barrier between the blood and the rest of the body. During vasculogenesis (the formation of new blood vessels) and angiogenesis (the growth and remodelling of existing blood vessels), proteins produced by endothelial cells and their underlying mural cells are critical for the migration and apposition of endothelial cells to each other and their supporting cells. While endothelial cell activation directs key immune responses, both activation and apoptosis may lead to a loss of the endothelial barrier integrity resulting in vascular leak and dysfunction in target organs preferably the brain. Many studies have been performed to uncover the extent of BBB alterations and their relationship to CM pathogenic processes (Adams *et al.*, 2002; Medana and Turner, 2006).

Two families that have been extensively studied for their role in normal and pathologic angiogenesis are: vascular endothelial growth factor (VEGF) and its receptors Flt-1 (fms-like tyrosine kinase-1, (VEGFR-1) and Flk-1 (VEGFR-2); and angiopoietin-1 and angiopoietin-2 and their cognate receptor Tie-2 (Risau, 1997). Changes in these two proteins have considerable effect in endothelial activation and dysfunction.

VEGF is a pro-vasculogenic, pro-angiogenic and pro-inflammatory protein that signals through two main receptors expressed on the endothelium, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). VEGFR-2 appears to be the dominant signaling receptor necessary for inducing vascular permeability and vessel formation, whereas VEGFR-1 may serve to modulate VEGF signaling (Yancopoulos *et al.*, 2000). VEGF is critical for vasculogenesis and angiogenesis and acts as a key destabilizing force during vessel remodelling. It has also been shown to influence other biomarkers such as angiopoietins that have been implicated in pathogenesis of cerebral malaria (Lobov *et al.*, 2002).

2.5.5 Role of Angiopoietin 1 and 2 in CM

The angiopoietins are among the most widely studied biomarkers involved in endothelial activation and dysfunction in diseases. They are important angiogenic proteins that are antagonistic ligands of the Tie-2 receptor, which belongs to a family of vascular tyrosine kinase receptors expressed primarily in endothelial cells (Page and Liles, 2013). These biomarkers have been identified as indicators of malarial disease severity (Fiedler and Augustin, 2006; Larkin *et al.*, 2009).

In healthy conditions, serum concentration of angiopoietin 1 (Ang-1) is higher than that of angiopoietin 2 (Ang-2). This favours the binding of Ang-1 to the Tie-2 receptor. Ang-1 is constitutively produced by pericytes and vascular smooth muscle cells that surround the endothelial cell and promote vascular stability and quiescence (Fiedler and Augustin, 2006). It is also expressed widely in normal adult tissues and is usually constitutively expressed in brain endothelium (Chittiboina *et al.*, 2013). Ang-2 on the other hand is contained within Weibel-Palade

bodies (WPB) in the endothelium and can be rapidly mobilized and released upon endothelial activation or exposure to inflammatory stimuli. The preferential binding of Ang-1 to the Tie-2 receptor in normal conditions therefore initiates pro-survival pathways and inhibit pro-inflammatory pathways. The net result is endothelial cell quiescence (Page and Liles, 2013). In contrast, inflammation stimulates Weibel–Palade body exocytosis and the release of Ang-2, allowing it to preferentially bind the Tie-2 receptor and promote proinflammatory and pro-thrombotic pathways, as well as microvascular leak or damage as shown in Figure 2.5 below. Ang 2 is also released alongside other WPB products such as von Willebrand factor (VWF) and its propeptide (Fiedler *et al.*, 2004).

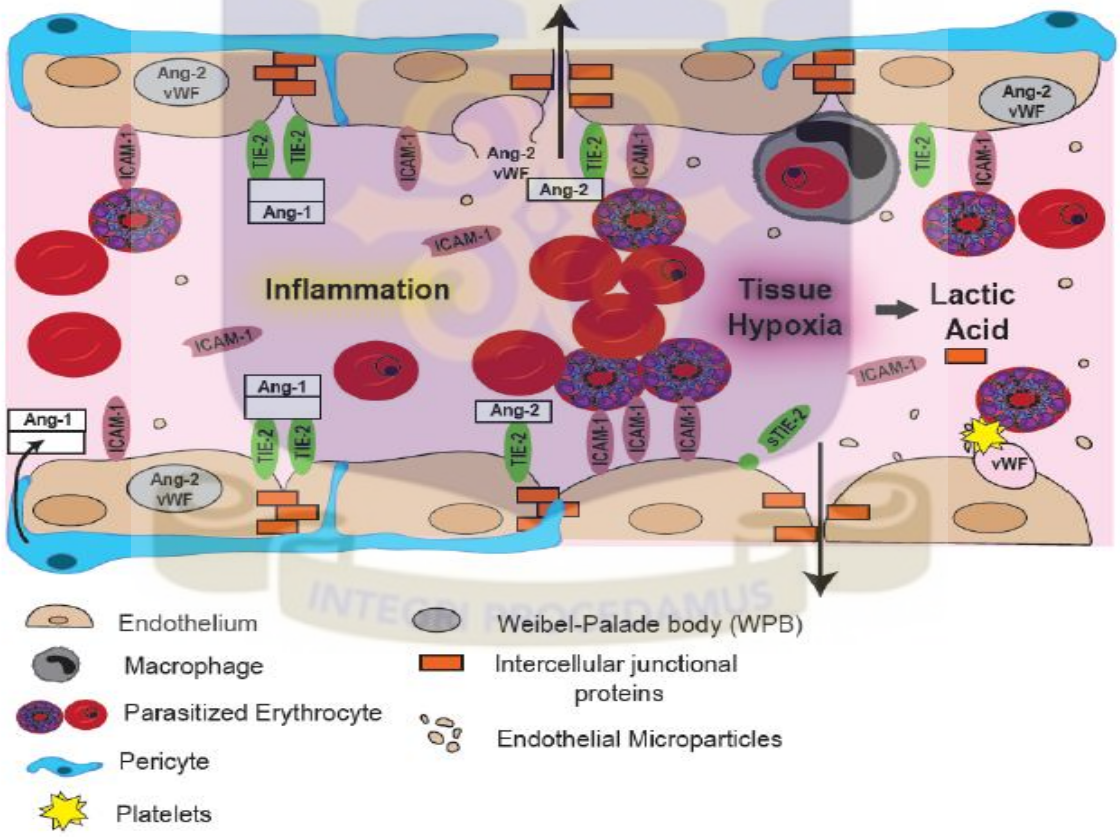


Figure 2. 5 Proposed mechanism of the role of Angiopoietins in immunopathogenesis of CM. Adapted from Conroy (2012)

In quiescence, clusters of Ang-1 aggregate and activate the transmembrane receptor tyrosine kinase, Tie-2, which is highly specifically expressed on endothelial cells. Tie-2 signals into the cell to favor phenotypes such as fortification of barrier function. In CM, Ang-2 is up regulated as a result of its release from the WBP and is believed to antagonize Ang-1. The tonic homeostatic signaling through Tie-2 is attenuated, contributing to the vascular leak and inflammation observed in CM.

Ang-2 has also been shown to induce the expression of ICAM-1 and VCAM-1 at sub-saturating concentrations of TNF (Fiedler *et al.*, 2006). The overexpression of Ang-2 destabilizes quiescent endothelial cells (EC) through an internal autocrine loop and leads to EC detachment and a vessel regression (Hu and Cheng, 2009).

Elevated levels of Ang-2 have been observed to increase mortality in diseases such as sepsis (Kümpers *et al.*, 2009; van der Heijden *et al.*, 2009) and have been reported to be predictive of mortality in certain subsets of critically ill patients, including those requiring renal replacement therapy and in children with septic (Giuliano Jr *et al.*, 2007)

Ang-2 has also been shown to promote several activities in the microvasculature including the migration of endothelial cells in the presence of VEGF whiles in absence of VEGF, Ang-2 promotes endothelial cell death and vessel regression (Lobov *et al.*, 2002).

A change in the normally low Ang-2:Ang-1 ratio may give an indication of endothelial dysfunction and could be as a result of either a decrease in Ang-1 or an increase Ang-2, or both (Lukasz *et al.*, 2008). The ratio of Ang-2/Ang-1 in serum therefore serves as a good predictor of disease severity

thereby implicating them in various disease pathogenesis (Page and Liles, 2013). Decreased levels of Ang-1 and an up regulated Ang-2 has been associated with early phase injury in the endothelium of rats (Chittiboina *et al.*, 2013).

In *Plasmodium falciparum* malaria, plasma Ang-2 levels have been found to be higher in patients with severe than non-severe disease. However, Ang-1 has been identified as a more consistent diagnostic biomarker in malaria, discriminating between cerebral malaria and uncomplicated malaria. (Lovegrove *et al.*, 2009).

Furthermore, in a study in malaria-exposed pregnant women, *Plasmodium falciparum* infection was associated with a decrease in maternal plasma Ang-1 levels and an increase in the Ang-2: Ang-1 ratio. Ang-1 levels were recovered after treatment of peripheral parasitemia (Silver *et al.*, 2010). Low birth weight have also been associated with increased Ang-2:Ang-1 ratios (Silver *et al.*, 2010).

2.5.6 Role of thrombomodulin in CM

Thrombomodulin (TM) is an integral membrane glycoprotein that has a major role in the regulation of intravascular coagulation (Sadler, 1997). TM is present in large quantities on the surface of the endothelium, particularly in the microcirculation, where it acts as an anticoagulant (Page and Liles, 2013)

TM is expressed at a lower constitutive levels in the brain compared with other organs. This glycoprotein forms a complex with thrombin and this complex alters the function of thrombin, the main pro-coagulant enzyme, to an anticoagulant through activation of the protein C pathway

(Salomaa *et al.*, 1999) . The TM-thrombin complex also prevents thrombin form converting fibrinogen to fibrin and thereby preventing coagulation (Levi and Van Der Poll, 2013). TM is known to exhibit an anti-inflammatory effect, regulate cell adhesion and proliferation through its lectin-binding domain. (Kodama *et al.*, 1990). Reduced expression of TM on the cell surface have been observed in activated endothelium and this indicates the shedding of the molecule into soluble forms, soluble TM (sTM) (Page and Liles, 2013). Studies in the early 90's had stated that the plasma soluble TM is derived from the degradation of that on endothelial cell membrane by various proteases during the process of endothelial cell injury and is then released into circulation without any evidence of active secretion (Ishii *et al.*, 1991).

Using a mouse model, (Esmon, 2000) demonstrated that, increased serum TM level is associated with a decreased expression of TM on the endothelial surface in vivo. Nevertheless, elevated serum TM levels are found in diseases associated with locally increased levels of inflammatory cytokines such as in malaria, dengue fever, sepsis and other diseases and syndromes such as cardiovascular diseases, acute coronary syndrome, pulmonary thromboembolism, and severe hemorrhage (Salomaa *et al.*, 1999).

In malaria, soluble TM levels have been observed to be higher in severe conditions compared to uncomplicated conditions (Faust *et al.*, 2001). In a study conducted by Mita-Mendoza *et al.* (2013) using Malian children with uncomplicated and non-cerebral severe malaria, they showed that sTM levels was elevated during infection and declined with convalescence. They also showed that the levels of sTM correlated with both parasitemia and disease severity, and were higher in children with severe malaria than in those with uncomplicated malaria. In another malaria study by Maya

et al. (2008), sTM levels was higher in uncomplicated *P. falciparum* malaria than in uninfected healthy individuals, and the sTM levels positively correlated with levels of pro-inflammatory cytokines and anaemia which is a marker of disease severity. The elevated serum levels of TM in both studies suggests that endothelial cell dysfunction occurs in the *P. falciparum* malaria. sTM has been proposed as both a diagnostic and prognostic tool of endothelial activation or dysfunction (Faust *et al.*, 2001).

2.5.7 Role of endothelial protein C receptor (EPCR) in CM

EPCR is a type 1 transmembrane protein that is expressed primarily by endothelial cells of the large blood vessels (Laszik *et al.*, 1997). It is homologous to major histocompatibility complex class I/CD1 family proteins, that is expressed mainly on the luminal surface of aortic endothelial cell, surface of monocytes, natural killer cells, neutrophils, eosinophils, immature hematopoietic stem cells, brain capillary endothelial cells and embryonic giant trophoblast (Stephenson *et al.*, 2006). However it is expressed at lower constitutive levels in the brain compared with other organs (Moxon *et al.*, 2013). EPCR is one of the most important components of the protein C (PC) pathway, classically known as the anticoagulant system. Studies have shown that mechanisms of the protein C anticoagulant pathway is triggered when thrombin binds to the endothelial cell receptor, TM (Castellino, 1995; Esmon *et al.*, 1999) as has been stated earlier. This complex activates protein C to generate the anticoagulant enzyme activated protein C (APC), which, in complex with protein S, inhibits coagulation by inactivating two critical regulatory proteins, factors Va and VIIIa. This pathway plays a critical role in the negative regulation of blood coagulation, as evidenced by the fact that total deficiencies of protein C or protein S are associated with severe and life-threatening thrombotic complications (Esmon and Schwarz, 1995).

EPCR also exists in plasma soluble form (sEPCR) that binds PC and APC with similar affinity (Fukudome *et al.*, 1996; Regan *et al.*, 1996). sEPCR may result from inflammation or activation of the endothelium and subsequent shedding of these receptor (Moxon *et al.*, 2013). Loss of EC bound EPCR and increasing levels of soluble forms of EPCR have been demonstrated in a study of Malawian children with CM (Moxon *et al.*, 2013). A recent study on children in Benin has also associated high plasma levels of sEPCR with increased mortality in children with CM (Moussiliou *et al.*, 2014). This studies have suggest strongly that increased levels of this receptor in plasma results from a pathophysiological mechanism of CM and measurement of soluble EPCR at initial clinical evaluation could predict severe malaria.

In a recent study by Turner *et al*, (2013), EPCR was observed to act as the endothelial receptor for PfEMP1 domain cassettes 8 and 13. The study as shown in Figure 2.6 demonstrated that parasites causing severe malaria exhibited stronger EPCR binding than parasites from children with uncomplicated malaria. Of considerable interest and importance, PfEMP1 was shown to bind EPCR near or at the same region of APC, implying that EPCR-mediated cytoadhesion likely inhibits APC-mediated EPCR-dependent cytoprotective effects on endothelial cells (Turner *et al.*, 2013).

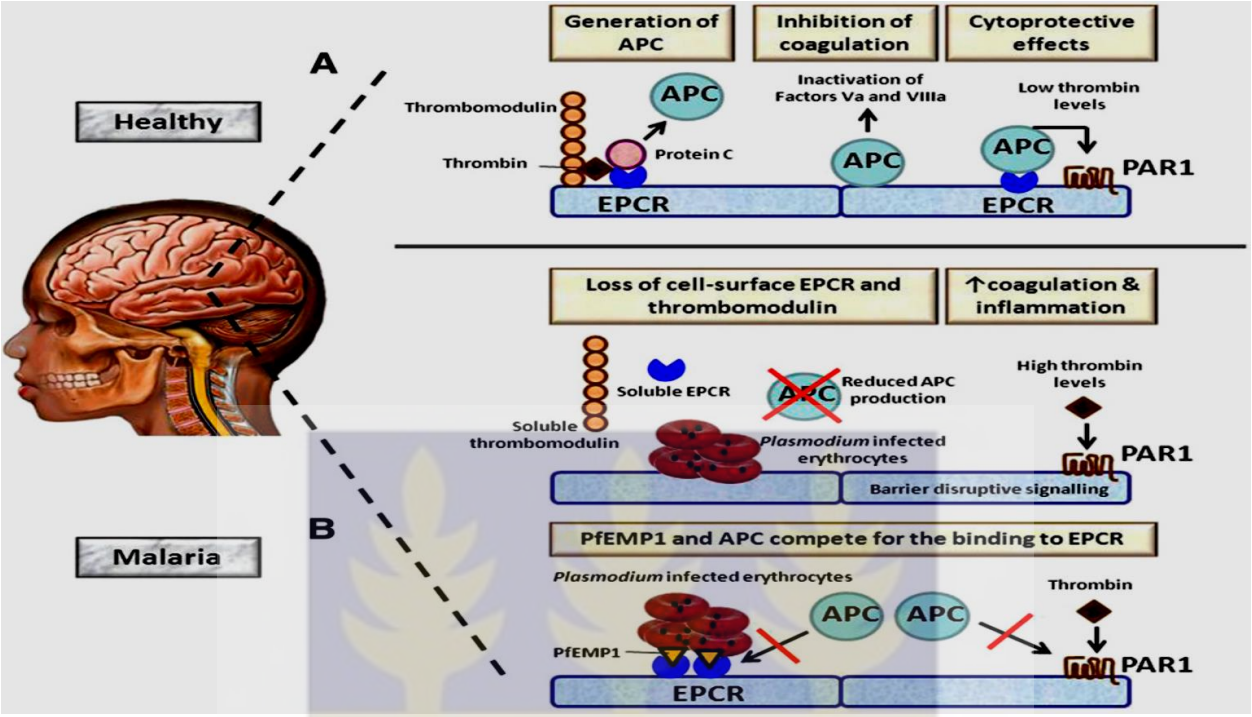


Figure 2. 6 Role of EPCR in CM. Adapted from Turner *et al.* (2013)

2.6 HRP 2 and Cerebral Malaria

Detection of malaria parasites on blood smears from peripheral blood and the presence of fever are the commonly used case definitions of malaria. However, in malaria-endemic settings asymptomatic parasitaemia complicates the diagnosis of malaria. The presence of parasites in peripheral blood, lacks specificity as symptoms of severe forms of malaria are nonspecific and can have different etiologies (English *et al.*, 1996). Peripheral blood parasitaemia does not represent the sequestered parasite burden, which is pivotal to the pathophysiology of severe *falciparum* malaria (Hendriksen *et al.*, 2013). Sequestration of asexual parasites occur in the second half of the erythrocytic stage of the life cycle and this prevents detection of these parasites in peripheral blood films (Silamut *et al.*, 1999b). Autopsies of cerebral malaria have demonstrated 26–40 times the burden of *Plasmodium falciparum* parasites in the deep tissue circulation of the brain compared to peripheral blood (Pongponratn *et al.*, 2003; Silamut *et al.*, 1999b). This phenomenon may

possibly explain the poor association between malaria severity and parasitemia measured by peripheral blood microscopy.

Plasmodium falciparum Histidine-Rich Protein 2 (PfHRP2) is a parasite-derived water-soluble protein which is released in discrete amounts into the plasma, predominantly during schizont rupture (Desakorn *et al.*, 2005). Released PfHRP2 is distributed over the plasma volume and, therefore, its concentration in plasma reflects the total body parasite burden, including the sequestered parasites. Studies involving Asian adults (Dondorp *et al.*, 2005) and African children (Rubach *et al.*, 2012) show that, in contrast with the peripheral blood parasite density, the plasma PfHRP2 concentration correlates strongly with disease severity and outcome.

Other studies have shown that elevated plasma pfHRP2 concentrations can identify children with histologically confirmed cerebral malaria (Seydel *et al.*, 2012) and can distinguish severe malaria from coincidental or uncomplicated malaria (Hendriksen *et al.*, 2013). Higher plasma concentrations of PfHRP2 (>1700ng/mL) has been shown to be a more field-friendly approach to confirming the diagnosis of CM compared to other techniques such as postmortem sampling or ophthalmoscopy (Seydel *et al.*, 2012).

2.7 Endothelial Cells (ECs)

The endothelium plays a pivotal role in the regulation of several biological processes relevant to clinical investigators such as inflammation, homeostasis and angiogenesis (Cines *et al.*, 1998). The endothelium is subjected to several pathophysiological stimuli including that from pro-inflammatory cytokines, growth factors, infectious agents, lipoproteins, and oxidative stress

(Dignat-George and Sampol, 2000). Prolonged subjection of the endothelium by these environmental pressures subsequently leads to dysfunction and damage (Dignat-George and Sampol, 2000; Goon *et al.*, 2006b).

2.7.1 Endothelial progenitor cell (EPC) and endothelial repair

Endothelial repair may occur by migration and proliferation of surrounding mature endothelial cells. Replication of local endothelial cells has been shown to be insufficient in the repair of extensive endothelial damage (Asahara *et al.*, 1999). It has been shown that, these mature endothelial cells are terminally differentiated cells with a low proliferative potential and their capacity to substitute damaged endothelial cells (Hristov *et al.*, 2003a) . Evidence suggest the presence of endothelial precursors in peripheral blood and these precursors originate from the bone marrow (Asahara *et al.*, 1997). These precursor cells have the potential to differentiate into mature endothelial cells and therefore they have been termed endothelial progenitor cells (Hristov and Weber, 2004). The presence of these bone marrow derived EPCs in the peripheral and umbilical cord blood had been demonstrated by (Asahara *et al.*, 1999). Bone marrow derived endothelial progenitor cells are thus known to migrate to sites of endothelial damage to augment the local response by incorporation into the microvasculature (Lin *et al.*, 2000). Interest in EPCs arises because of their potential as stem cells and thus providers of therapeutic neovascularization and the repair of existing, damaged endothelium (Blann and Pretorius, 2006).

EPCs account for approximately 0.1% of peripheral blood, have proliferative potential, and can differentiate into mature circulating endothelial cells (Thomas *et al.*, 2009). When required for vascular repair/angiogenesis or in cases of vascular stress, EPCs enter the peripheral blood and

migrate to areas of endothelial damage, differentiate and begin the reparative process as shown in Figure 2.7 below (Real *et al.*, 2008).

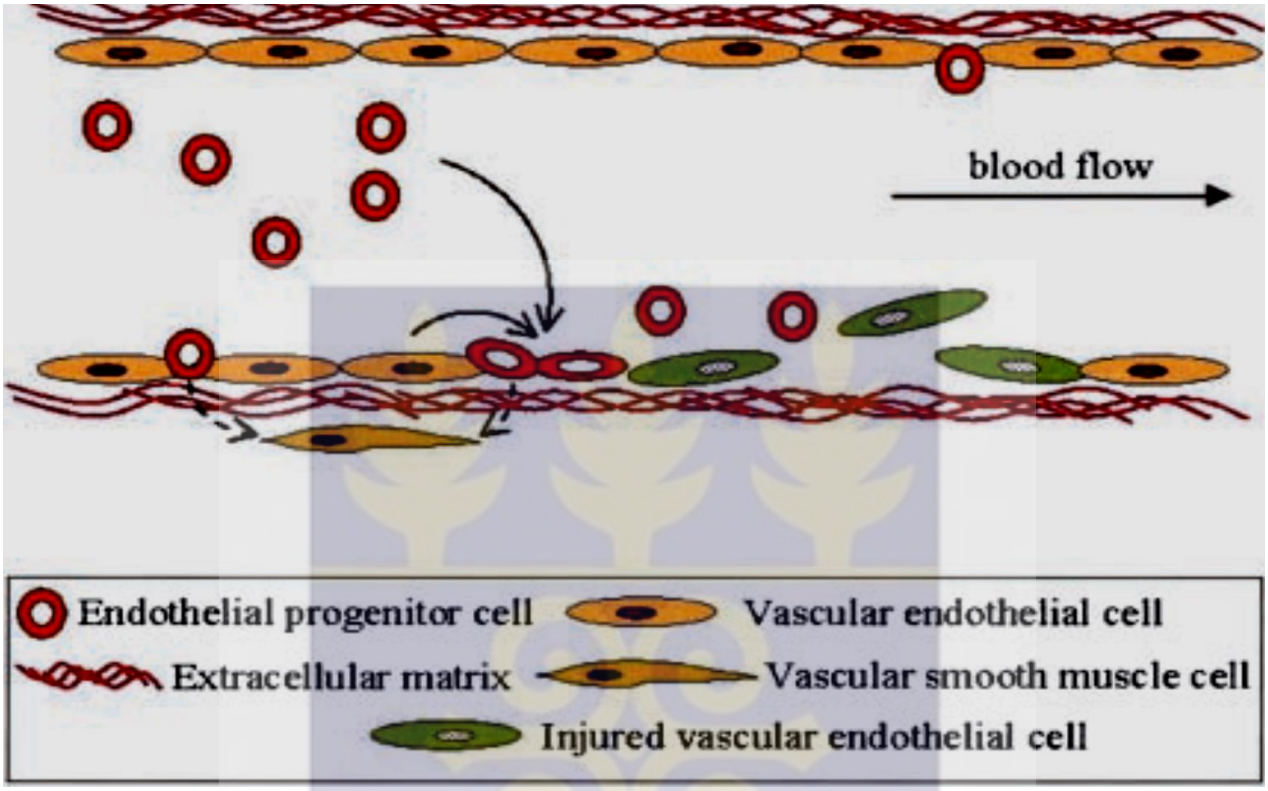


Figure 2. 7 Circulating EPC contribute to endothelial repair (Hristov *et al.*, 2003a)

Circulating EPC levels can also provide clinical information on the atherosclerotic burden and even on the future cardiovascular risk (Fadini *et al.*, 2012). Low EPC level has been demonstrated in patients with unstable angina and cerebrovascular (George *et al.*, 2004). Transfusion of EPCs into animal models have been shown to reduce neo-intima formation after vascular injury (Werner *et al.*, 2003)

These considerable interests in exploiting the function of bone marrow derived EPC are thus being extended to severe malaria. A study by Gyan *et al.*, (2009) determined EPC levels in Ghanaian children with cerebral and uncomplicated malaria as well as healthy children. The study associated

decreased levels of EPC with cerebral malaria, thus placing cerebral malaria within the context of current paradigms of micro vascular repair.

2.7.2 Circulating endothelial cells (CEC)

Endothelial cell damage or detachment usually results from continuous or exaggerated endothelial activation by environmental pressures (Figure 2.8).

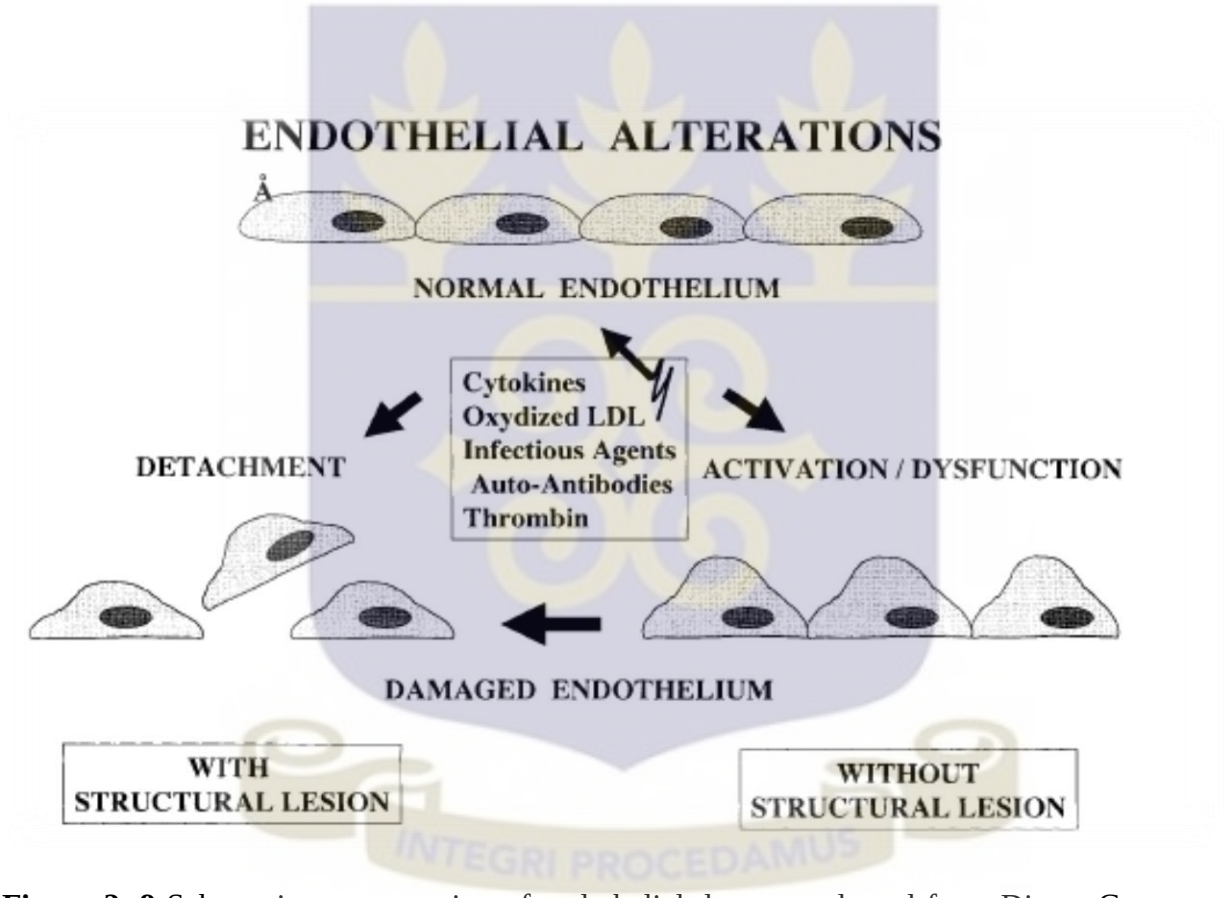


Figure 2. 8 Schematic representation of endothelial damage: adapted from Dignat-George and Sampol (2000).

Detached endothelial cells circulate in peripheral blood and are often termed circulating endothelial cells (CECs). Some CECs have phenotypes compatible with terminally differentiated endothelial cells in some cases being apoptotic or necrotic and thus most likely derived from the

turnover of vessel walls. The presence of CECs has been recognized as a useful marker of vascular damage (Goon *et al.*, 2006b). Usually absent in the blood of healthy individuals, CEC counts are elevated in diseases hallmarked by the presence of vascular insult, such as sickle cell anemia, acute myocardial infarction (Goon *et al.*, 2006b).

Other studies have also demonstrated increased CECs in other disorders encompassing vascular, autoimmune, infectious and ischemic diseases (Bertolini *et al.*, 2006). Some cancer patients have also demonstrated increased CEC counts (Farace *et al.*, 2007). CECs are considered 'rare' cells with a consensus around their level ranging from 0 to 1500 cells per millilitre of blood (Dignat-George and Sampol, 2000). In healthy subjects, a low basal level of endothelial turnover, respectively very low amounts of circulating and vessel wall–derived ECs (1 to 3/mL blood), has been described (Dignat-George and Sampol, 2000). Increased numbers, often up to 10-fold or more, are found in a broad tranche of diseases and conditions associated with vascular perturbation or damage and broadly speaking, correlate with plasma and physiological markers of endothelial damage/dysfunction such as flow mediated dilatation, von Williebrand factor (vWf), and soluble E selectin (Makin *et al.*, 2004b).

Subpopulations of CECs have been shown to express E-selectin and other markers of activation (Bull *et al.*, 2003), and also potentially bio-active tissue factor and thrombomodulin (Woywodt *et al.*, 2003). In various disease condition, the longitudinal quantitation of CECs showed that their levels vary according to the clinical evolution. Levels in patients who are acutely ill are higher than those in patients in clinical remission or in recovery phases of the disease (Dignat-George and Sampol, 2000).

Plasmodium falciparum infection represents a major endothelial infection as a result of sequestration of infected RBC on the endothelium. However, very little is known of circulating endothelial cells in *P. falciparum* infection. Studies with balb/c mice infected with *P. berghei* endothelial cells were correlated with cerebral symptoms and death (Neill and Hunt, 1992). CECs therefore show promise as a prognostic marker for cerebral malaria.

2.7.3 CEC and EPC identification and quantification

Immunomagnetic bead capture method and flow cytometry (FC) are the most common and widely accepted techniques of CEC and EPC enumeration. Immunomagnetic bead capture method which is a well validated technique is notably labour extensive and requires a high degree of operator skills (Woywodt *et al.*, 2006). Flow cytometry on the hand offers a multi-marker approach to EPC and CEC estimation, involving the concurrent use of endothelial-associated cellular markers (Goon *et al.*, 2006a). These cellular markers are expressed as surface receptors on the cells and are used to distinguish between CECs, EPCs and hematopoietic stem cells (HSCs) among others. Monoclonal antibodies against these surface receptors have made detection and quantification of these cells more practical.

Circulating EPC have been identified and enumerated by flow cytometry by the expression of progenitor receptors, CD34 and CD133 and endothelial vascular endothelial growth factor receptor 2 (VEGFR2) also known as CD309 (Asahara *et al.*, 1997). Earlier studies in the field have reported that CD34 positive (CD34+) and VEGFR2+ cells purified from various sources such as umbilical cord blood (UCB), peripheral blood (PB) and bone marrow are able to generate ECs in vitro, suggesting that CD34+ cells contains cEPCs (Asahara *et al.*, 1997; Shi *et al.*, 1998). Many

investigators therefore define EPCs by means of the co-expression of CD34 and VEGFR2 (Bertolini *et al.*, 2006; Shi *et al.*, 1998). Others have used a combination of CD34 and a more immature marker, CD133 to select for putative EPCs (Gehling *et al.*, 2000; Gyan *et al.*, 2009). However, it has been reported that $CD34^{+}CD133^{+}VEGFR2^{+}$ cells do not only give rise to endothelial cells but rather are a distinct subpopulation of HSCs (Timmermans *et al.*, 2009; Yoder, 2009). Studies have indicated that about 90% of CD34+ progenitor cells express CD45 at low intensity ($CD45^{dim}$), whereas less than 10% are CD45-negative (Fadini *et al.*, 2012). Hence a true population of EPCs may be defined as $CD45^{dim}/CD34^{+}/VEGFR\text{-}2^{+}/CD133^{+}$ (Jain *et al.*, 2012; Kondo *et al.*, 2004).

CEC estimation like EPCs involve the concurrent use of endothelial-associated cellular markers such as CD31, CD34, CD144, CD62E, CD105, CD106, CD146 and KDR, enabling better distinguish mature CECs from other cells such as lymphocytes and haematopoietic stem cells (Mancuso and Bertolini, 2010). CECs have been defined as negative for the leukocyte and HSC markers (CD45 and CD11b), positive for endothelial marker CD31 and CD34, negative for activation markers CD105 and CD106, and negative for the progenitor cell marker CD133 (Erdbruegger *et al.*, 2006; Goon *et al.*, 2006b). However, examination of transcripts of cell surface markers among endothelial cells have revealed the expression of CD133 by brain endothelial cells and partially by endothelial cells in the eye, testis and skin (Nolan *et al.*, 2013). CECs are known to express CD31 at high or bright intensity and hence CD45-/CD34+/CD31bright/CD133- (Mund *et al.*, 2012) or CD11b-/CD34+/CD31bright/CD133- may define putative CECs. For the purpose of CECs originating from brain endothelium, CECs may be defined as CD11b-/CD34+/CD31bright/CD133+.

Table 2. 1 Definition of EPCs and CECs

Markers	Cells	EPC	CECs	References
CD 45	Pan-leukocyte marker, human hematopoietic stem cells (HSC)	dim	-	**(Duda *et al.*, 2007)**
CD 11b	Granulocytes,monocytes/macrophages , dendritic cells, NK cells, and subsets of T and B cells	-	-	**(Nolan *et al.*, 2007)**
CD 31	Monocytes, platelets, granulocytes, endothelial cells and lymphocyte subsets	dim	Bright	**(Duda *et al.*, 2007)**
CD 34	HSC and progenitors bone marrow stromal cells, capillary endothelial cells, embryonic fibroblasts,	+	+	**(Asahara *et al.*, 1999; Lin *et al.*, 2000)**
CD 133	human hematopoietic stem cells, stem cells, endothelial progenitor cells, glioblastomas, neuronal,	+	(-/+)	**(Nolan *et al.*, 2007; Peichev *et al.*, 2000)**
CD309 (VEGFR2)	endothelial cells, embryonic tissues, and megakaryocytes	+	+	**(Duda *et al.*, 2007; Nolan *et al.*, 2007)**

(-): no expression on cells surface
(+): expression on cell surface
(-/+): expressed on cells from certain organs and absent in others
dim: reduced intensity of expression
bright: strong intensity of expression

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Chemicals, Reagents and Equipment

The sources and manufacturers of reagents, buffers, solutions and equipments used in the study are shown in Appendix 1.

3.2 Study Sites

The study was done in collaboration with five hospitals of the Ghana Health Service; The Princess Marie Louise Children's Hospital (PML), La General Hospital, Tema General Hospital, Ledzokuku Krowoh Municipal Assembly Hospital (LEKMA) and the Ridge Hospital. These hospitals are the main referral hospitals located within the Greater Accra Region of Ghana where all cases of cerebral malaria cases are managed (Figure 3.1). Healthy controls for the study were recruited from the Hayward Nursery School and Osu Home School in La community and LEKMA cluster of schools in Teshie. The hospitals and the schools are located within two hours' drive from Noguchi Memorial Institute for Medical Research (NMIMR), where the analysis of samples was done.

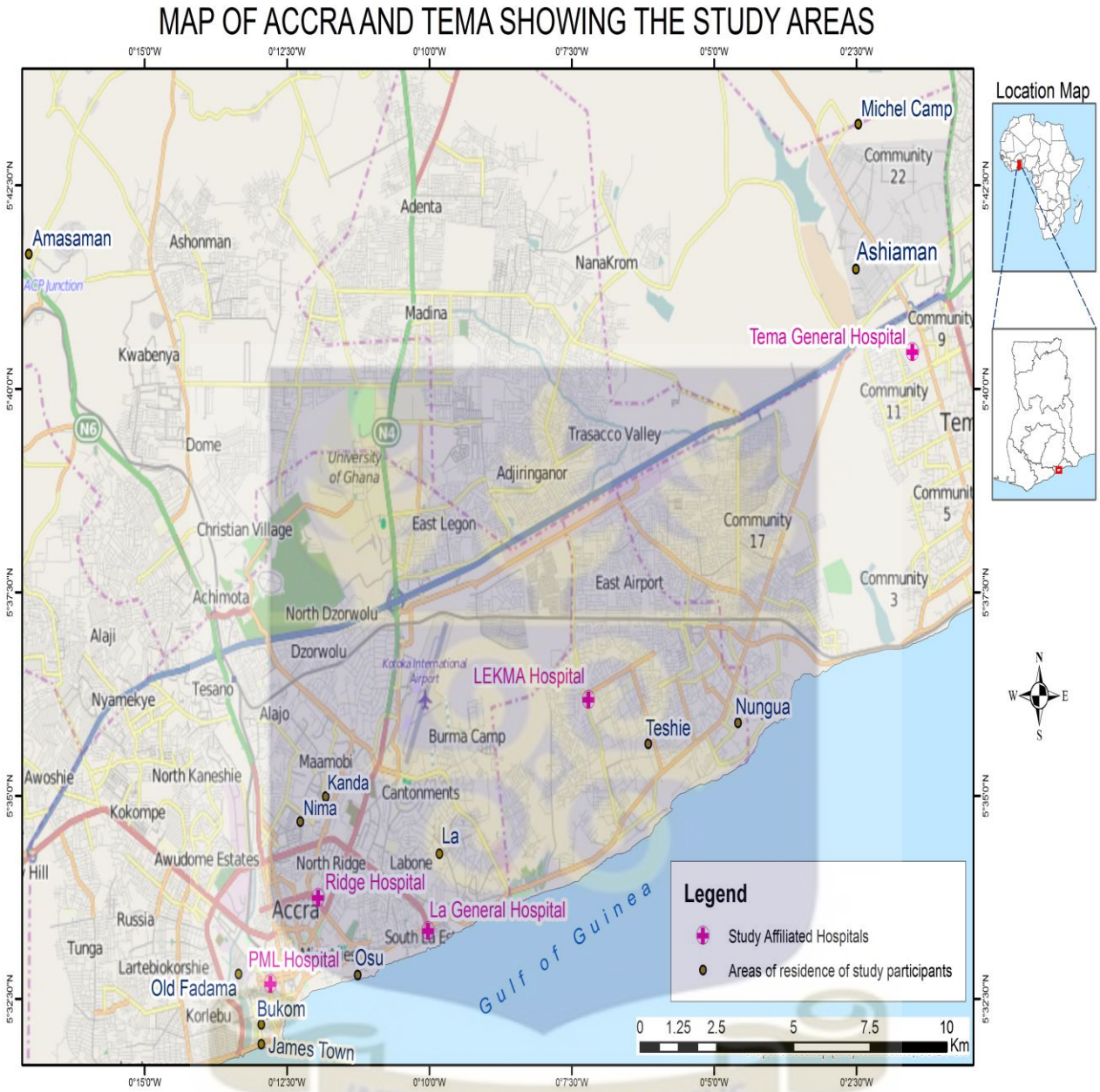


Figure 3. 1 Map of Greater Accra region showing the location of the study sites (red)

3.3 Study Design

In a longitudinal cohort study, children (2-12 years) who presented with coma to the emergency of any of the affiliated hospitals were screened by paediatricians for cerebral malaria whiles those presenting to the Outpatient Department (OPD) of the hospitals were screened for uncomplicated malaria. Uninfected healthy children of similar ages as malaria patients were recruited from community schools within the Accra municipality. Informed consent was obtained from parents or guardians of children who qualified for the study. Patients recruited at the hospitals were thus categorized into two main groups, cerebral malaria and uncomplicated malaria, with further subgroups as shown in Figure 3.2. Uninfected healthy controls also constituted one group. Recruitment of study participants was done between May, 2012 and August, 2015.

Samples were obtained at initial clinical presentation (baseline) and at each of the time point as indicated on Figure 3.2. For children presenting with CM, whole blood samples were taken at baseline, at the time of a clinical event (recovery), and at 7 and 14 days post recovery. For children with UM, whole blood samples were obtained at baseline, 7 and 14 days post-baseline. For a UM patient that develops CM, samples were taken at baseline, at the time of conversion to CM, at the time of recovery from CM, 7 and 14 days post recovery. For healthy controls, at baseline, 7 and 14 days). All children who presented with malaria to the hospitals were treated per the routine clinical procedures.

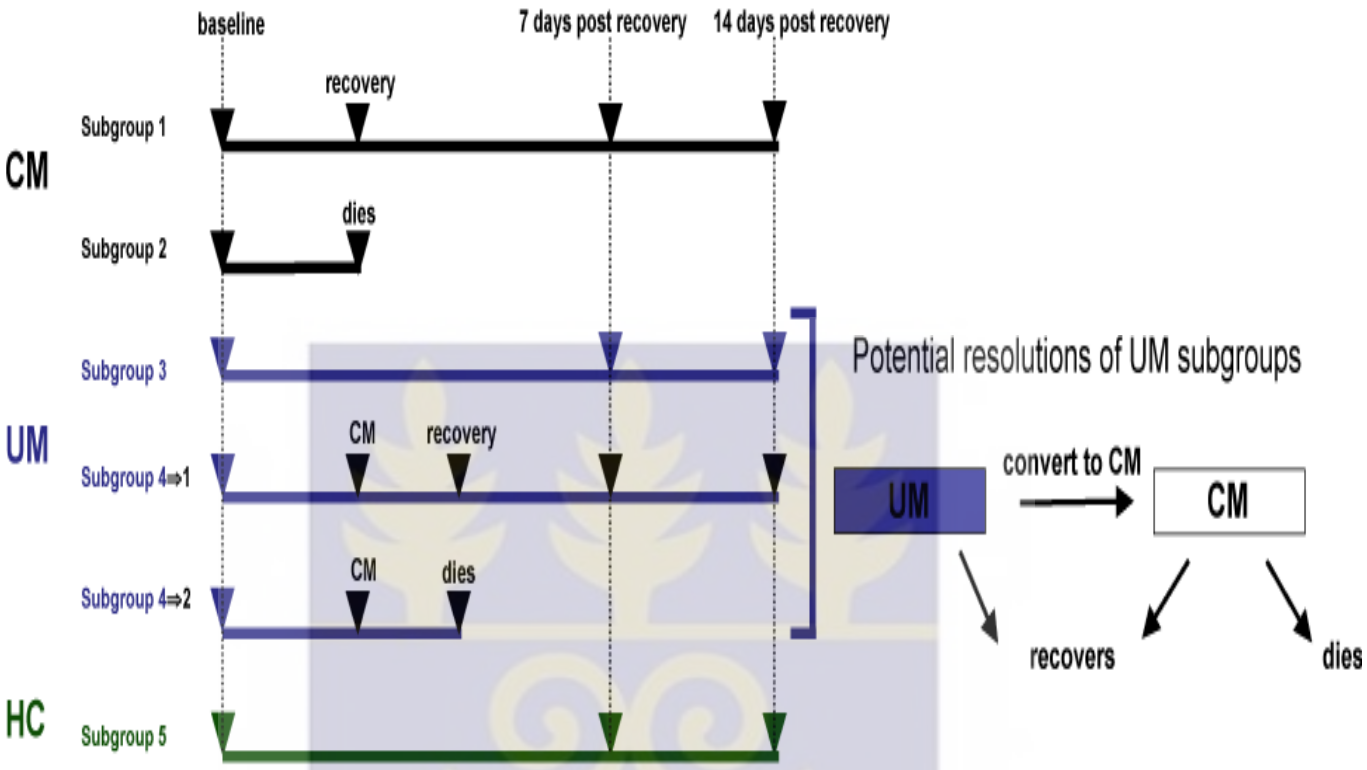


Figure 3. 2 Schematic presentation of the study design

Levels of chemokine/protease (SDF and MMP9), endothelial mediators (Ang-1 and -2), anticoagulant receptors (TM and EPCR) and *P. falciparum* parasite protein, HRP2, were assessed at each of the time points and subgroups. The levels of these biomarkers were also correlated with transition in disease severity or recovery in patients with CM, UM and healthy controls.

For a pilot study on the stability of EPCs and CECs, six male volunteers (30 – 50 years) in the Department of Immunology of NMIMR were recruited after informed consents were obtained. Volunteers had no clinical signs of illness and were not on medication for any medical condition. Blood samples were obtained at one time point and stored in both EDTA and C-C BCT over a seven day period.

3.4 Ethical clearance

Ethical approval for the study was obtained from the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR). Approval from Ghana Health Service and Ghana Education Service was obtained. Study participants were enrolled only after informed consent was obtained from their parents and guardians following their understanding of the objectives of the study (see appendix 2A and 2B for consent forms). Participants could opt out of study at any time since participating was voluntary.

3.5 Inclusion/Exclusion Criteria

3.5.1 Specific inclusion criteria

For clinical malaria, presentation at any of the affiliated hospitals with a history of fever within the previous 24/48 hours or current fever (axillary temperature $\geq 37.5^{O}$C) plus 5 or more *P. falciparum* parasites per HPF (approx. 2500 /µl) and no other obvious cause found for the fever was enough for recruitment into study.

For cerebral malaria, recruitment into the study was based on a patient being unconscious, with a score of $\leq$3 on the Blantyre coma scale (BCS) and being in coma for at least 60 minutes. In addition, patient should have no record of recent severe head trauma or other causes of coma or neurological diseases including meningitis/encephalitis (as assessed by lumbar puncture). Recovery from CM was defined as regaining of full consciousness (BCS> 3). The patient may or may not have neurologic sequelae, which was assessed by study physicians and any deficits were recorded and monitored for the duration of the study. UM was defined as clinical malaria without any WHO criteria for severe malaria such as SMA or respiratory distress. Patients were monitored

briefly in the outpatient clinic at the discretion of the study physician prior to returning home. Unlike CM, UM patients were not hospitalized. Healthy controls were defined as uninfected children assessed by blood films and without any history of malaria treatment two weeks prior to recruitment into study.

3.5.2 Specific exclusion criteria

Participants were excluded from the study if parent, guardian or proxy was unable to give signed informed consent and/or unwilling to comply with requirements of the protocol. Also, evidence of concomitant infection at time of enrollment including meningitis/encephalitis and bacteremia was enough to exclude a participant.

History of any underlying disease that compromised the diagnosis and outcome of the illness including HIV infection (assessed only by history) also excluded participants. Diseases/conditions known to alter levels of EPCs or induce microvascular damage such as recent severe bleeding, sickle cell disease or trait, evidence of bacterial or viral infection, history of diabetes mellitus, cardiovascular disease, hypercholesterolemia, surgery within 1 month, bone fracture within 3 months, major trauma within 1 month (e.g., car accident), blood transfusion within 3 months were also considered as exclusion criteria. Children with severe malaria anaemia that received blood transfusions were also excluded from the study.

3.6 Blood Sample Collection

Per routine clinical procedures, two millilitres (2ml) of venous blood was collected from each patient into EDTA tubes by trained phlebotomists for complete blood count (CBC), blood culture,

sickling test and blood smears for malaria parasite count. In addition 3ml of venous blood were collected into heparinized (1ml) and EDTA (1ml) tubes and blood culture bottles (1ml) for laboratory analysis and immunological assays. For cerebral malaria patients, cerebrospinal fluid (CSF) samples were obtained through lumbar puncture done by trained paediatricians. For healthy controls, a total of 3ml of venous blood were collected into heparinized tubes (1ml) and EDTA tubes (2ml) for laboratory and immunological assays.

For the pilot study on the stability of EPCs and CECs, ten milliliters (10 ml) of venous blood was collected from each of the six volunteers using Butterfly needles (BD); 5 ml of the blood was collected into a 5 ml EDTA and the other 5 ml into a Cyto-Chex BCT tube.

3.7 Sample Processing Time and Storage

Blood samples for routine clinical procedures were taken immediately to the hospital laboratories for evaluation, whiles those for the research study were transported in cold ice chests to the laboratories of the Immunology Department, NMIMR CSF samples were transported to either the Korle Bu Teaching Hospital or the MDS-Lancet laboratory in Accra, for analysis.

At NMIMR, a 400ul aliquot of EDTA treated blood were used for flow cytometry and the rest were separated into RBCs and plasma for storage at -30^{o}C. Heparinized blood was also processed by centrifugation and separated into RBCs and platelet-free plasma. This was done by initial centrifugation at 1000 x g for 15 minutes and separated plasma at 10000 x g for 10 minutes. Both RBCs and platelet free plasma were stored at -30^{o}C for further analysis.

Blood samples in EDTA and C-Chex BCT tube for the pilot study were stored at 4°C for 30 minutes before use in assays. Four hundred microliters (400ul) each of EDTA and C-C BCT treated blood were aliquoted for flow cytometry and the rest stored at 4°C. Flow cytometric analysis was repeated for both stored EDTA and C-C BCT treated blood at day 1 (24hrs), day 2 (48hrs) and day 7.

3.8 Laboratory Evaluations/Assays

Laboratory investigations on samples obtained were carried out at both the Hospital laboratories affiliated to the study and at the Noguchi Memorial Institute for Medical Research. Investigations done at the Hospital laboratories were for routine diagnostic purposes which included blood smears for malaria parasite identification and estimation as well as complete blood counts.

Results from the hospital laboratories were made available to the Clinicians and the study team. Blood culture results and parasite densities obtained on samples at the research laboratories were made available immediately to clinicians to assist in clinical care. For healthy controls, all laboratory investigation were done at NMIMR.

3.8.1 Parasitology

Hospital laboratories prepared blood smears per the routine protocol for the diagnosis of malaria. Briefly, about 10ul of whole blood were obtained from a finger puncture onto a pre-cleaned microscope glass slide and spread uniformly in a circle of diameter 1-2cm (thick film). The smears were left to dry at room temperature and stained with 10% Giemsa for 15 minutes. Stained slides

were dried and examined under a light microscope (at x100) with immersion oil. Results obtained from blood smears were semi-quantitative.

At the research laboratories at NMIMR, thick and thin blood smears were prepared from EDTA treated blood for confirmation of parasitaemia and determination of parasite densities. Blood smears were prepared according to WHO protocol (WHO, 1988; WHO, 1991). Both thick and thin films were prepared on the same slide as shown in Figure 3.2 below.

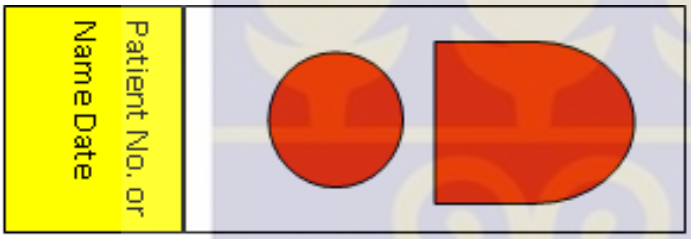


Figure 3. 3 Thick and thin blood film

Briefly, for the thick smears, a drop of about 7µl of blood was placed at one end of a microscope glass slide and evenly spread in a circle to a diameter of about 1cm. For the thin blood film, about 4µl of blood was placed close to the middle of the microscope glass slide. A spreader slide was inclined at an angle of 45^{O} on the drop of blood. The blood was made to spread along the entire width of the spreader slide and pushed forward rapidly and smoothly. The prepared blood films were air-dried thoroughly. The thin blood film was fixed in absolute methanol for species identification. Both films were then stained with freshly prepared 10% Giemsa solution (in Phosphate buffer) and left to stain for 15 minutes, washed and examined under light microscope with immersion oil. Parasite densities were estimated using the WHO guidelines (WHO, 2010).

3.8.2 Haematology

For malaria patients recruited into the study, complete blood counts were done at the hospital laborartory on initial presentation and subsequent reviews. A haemotological analyzer was used to measure: haemoglobin levels, platelets counts, total white blood cell (WBC) counts, total red blood cell (RBC) counts, mean corpuscular volume (MCV), haematocrit (HCT), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin content (MCHC).

Sickling tests were done for all patients and healthy controls recruited for the study. A drop of blood was placed on a clean microscope slide. An equal volume of the 2% sodium metabiosulphite solution was added to the blood and mixed thoroughly. The mixture was covered gently with a cover slip to avoid trapping air bubbles and to give a low oxygen tension and read under a microscope.

3.8.3 Bacteraemia evaluation

Evaluation of bacteremia in patients was done according to Cheesbrough (1984).

Briefly, blood sample (1ml) was obtained aseptically form each patient and added to 50ml broth medium. To reduce the risk of contamination, blood was collected from a peripheral vein by a qualified phlebotomist. Culture medium was kept in an incubator at 37^{o}C and observed daily for seven days for any signs of haemolysis, production of gas, coagulation of the broth and turbidity above the red cell layer. If there was a sign of bacterial growth, a subculture was done on solid media and examined after 24 hours for bacteria growth. However, if there was no sign of bacterial growth, the culture was examined daily for seven days.

Identification of bacteria growth was done by initially doing a Gram stain to differentiate Gram-positive from Gram-negative bacteria.

3.8.4 Flow cytometric analysis

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light (Biosciences, 2000). This technique was used to evaluate the levels of EPCs and CECs in whole blood from the different groups of study participants Using a FACS Calibur flow cytometer (Figure 3.4).

Figure 3. 4 Flow cytometer (FACS Calibur)

3.8.4.1 Processing of whole blood samples for flow cytometry

EDTA treated whole blood samples were used for flow cytometry by following a routine in-house protocol at the Immunology Department, NMIMR. Briefly, panels of six 5ml polystyrene Falcon

tubes (BD) were labeled (1-6) with permanent marker and different volumes of whole blood and Fc receptor blocking agent (IgG chrome) were added as indicated in Table 3.1 below.

Antibodies against the various endothelial receptors considered in the study and their isotypes (positive and negative controls) were also added to the respective panels.

Table 3. 1 Monoclonal antibodies against endothelial receptors

Monoclonal antibodies and Fluorochromes

Tube	Whole Blood (ml)	FITC (3µl)	PE (3µl)	PerCP (3µl)	APC (3µl)
1	50	Unstained			
2	50	M-IgG1 k	M-IgG1	M-IgG1k	M-IgG
3	50	R-IgG 2b	M-IgG1	M-IgG1k	M-IgG
4	50	CD 15	CD 14	CD 4	CD 8
5	100	CD 11b	CD 133	CD 34	CD 31
6	100	CD 45	CD 133	CD 34	CD 309(KDR)

3.8.5 Quantification of cEPC and CEC in whole blood

Circulating EPCs levels were determined by surface staining with receptor-specific fluorescent-labeled antibodies and expressed as percentage of $CD45^{dim}/VEGFR2^{+}/CD34^{+}/CD133^{+}$ cells in total leukocyte gate (G1) as shown in Figure 3.5.

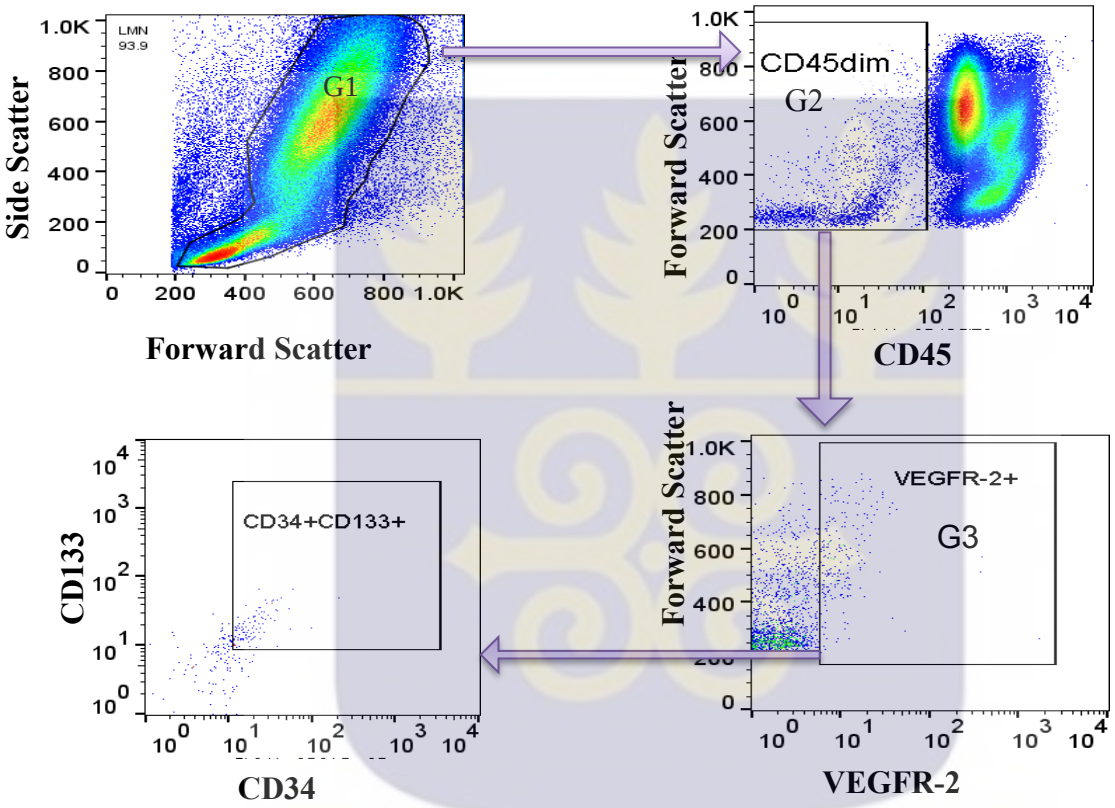


Figure 3. 5 cEPCs gating strategy and estimation

Initial gating on all three major populations of WBCs (Lymphocytes, monocytes and granulocytes) **[G1]**. Gating on $CD45^{dim}$ population from initial gate G1, to exclude cells such as haematopoietic stem cells and leukocytes (**G2)**. CD309 (VEGFR2) population within CD45dim population were gated **(G3)**. Final gating of $CD34^{+}$ and $CD133^{+}$ population from G3 to enumerate cEPCs as $CD45^{dim}/VEGFR2/CD34^{+}/CD133^{+}$.

CEC levels were determined by surface staining with receptor-specific fluorescent-labeled antibodies and expressed as percentage of $CD11b^{-}/CD34^{+}/CD31^{bright}/CD133^{+}$ cells in total leukocyte gate (G1) as shown in Figure 3.6.

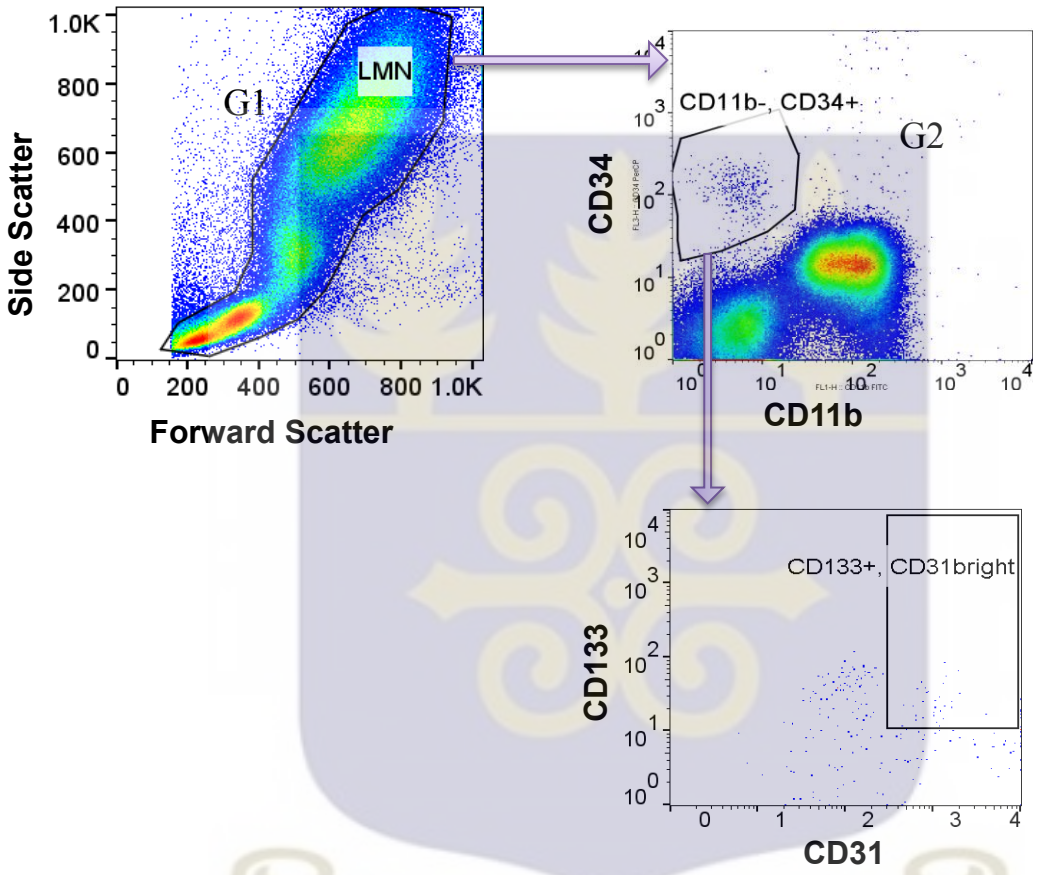


Figure 3. 6 CEC gating strategy and estimation.

Initial gating on all three major populations of WBCs [Lymphocytes, monocytes and granulocytes (LMN)] **(G1)**. Gating on $CD11b^{-}$ and $CD34^{+}$ population from initial gate G1. $CD31^{bright}$ and $CD133^{+}$ population within G2 population were gated to enumerate CECs as $CD11b^{-}/CD34^{+}/CD31^{bright}/CD133^{+}$. CECs were estimated as percentage of $CD11b^{-}/CD34^{+}/CD31^{bright}/CD133^{+}$ cells of initial LMN gate G1.

3.8.6 Evaluation of chemokine/protease and endothelial biomarkers

Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of SDF-1, MMP9, Ang-1 and Ang-2, soluble TM and soluble EPCR as well as parasite protein HRP2.

Measurement of the endothelial biomarkers, Angiopoietin-1 and -2, soluble TM and soluble EPCR were obtained as duo sets from R&D. Coating of plates were done according to the manufacturer's instructions. Ninety six (96)-well polystyrene microtitre plates were coated for each biomarker with 100µl per well of mouse anti-human antibody (capture antigen) specific for the biomarker and incubated overnight at room temperature. The plates were aspirated and washed using 400µl of wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against clean paper towel after three washes to adequately remove excess unbound capture antigen. The plates were then blocked with 300µl per well of reagent diluent [1% Bovine Serum Albumin (BSA) in PBS] and incubated for an hour at room temperature. The plates were then aspirated and washed again.

Hundred microlitres (100µl) of plasma samples (20 times diluted in PBS) were added per well for each of the plates coated for the different biomarkers. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 +/- 50 rpm. The wells were aspirated and washed three times with 400µl of wash buffer using a manifold dispenser. The plates were blotted with clean paper towels at the end of the wash to completely remove any liquid. Hundred microlitres (100µl) of detection antibody diluted in reagent diluent with normal goat serum (NGS) for each biomarker were added to the wells. The plates were covered with a different adhesive strip and incubated for 2 hours at room

temperature on a shaker after which the plates were aspirated and washed three times as described above. Hundred microlitres (100µl) of working dilution of Streptavidin-HRP was added to each of the wells plates. The plates were covered with an adhesive strip and incubated for 20 minutes in the dark at room temperature. The plates were again aspirated and washed three times as described above. Hundred microlitres (100µl) of substrate solution for each biomarker was added to each corresponding well for colour development. The plates were then incubated for 20 minutes at room temperature in the dark. Stop solution (50µl) was added to each well to halt the colour change. The optical density was determined within 30 minutes, using the EL808 BioTek microplate reader set at 450 nm. The levels of each of the endothelial biomarkers were then determined from the optical densities.

3.8.7 Statistical Analysis

Data generated in this study were mostly analyzed by both parametric and non-parametric tests. Kruskal Wallis test and Friedman test were used to determine differences between more than two groups. Dunn's multiple comparison test was employed for pairwise comparison of groups where applicable. A t-test was employed when comparison involved only two groups. Characteristics of study participants were recorded as median with minimum and maximum values. Levels of marker considered in the thesis were presented as mean with measure of dispersion.

P-values of less than 0.05 (two sided) were regarded as statistically significant. Statistical analyses and data presentation were done using GraphPad Prism (San Diego, CA).

CHAPTER FOUR

4 RESULTS

4.1 Background and Demographics of Study Participants

One hundred and fifty three (153) children between one and 12 years were recruited for the study. A total of 21 (13.7%) cases were disqualified based on the exclusion criteria in section 3.4.2. The total number of qualified cases were 132 consisting of 50 (38%) confirmed CM cases, 45 (34%) UM cases and 37 (28%) HC. Table 4.1 shows characteristics of qualified cases recruited for the study. A total of 8 CM resulted in fatalities. No deaths were recorded in UM cases over the four years of the study and none of the cases recruited as UM converted to CM throughout the study.

Median age of healthy controls was higher compared to both CM and UM ($P<0.0007$, Kruskal Wallis and Dunn's multiple comparison). There was no significant difference between the median ages of CM and UM cases ($p>0.999$).

Hb levels were significantly lowest amongst the study groups ($p<0.0001$, Dunn's Multiple comparison test). CM recorded the highest WBC counts compared to both UM and HC ($p<0.0001$, Dunn's Multiple comparison test). No significant difference in the median WBC counts in UM and HC ($p>0.999$) was observed. RBC count in CM was significantly lower than both UM and HC (CM vs UM, $p=0.0012$: CM vs HC, $p<0.0001$, Kruskal wallis and Dunn's multiple comparison). No statistical difference was observed between RBC levels in UM and CM. Kruskal Wallis and Dunn's multiple comparison test shows significant differences ($p<0.0001$) in the HCT levels in the study groups, with CM recording the lowest (26.20%) and HC recording the highest (34.25%).

Platelet count in HC was significantly higher than UM and CM ($p<0.0001$). No statistical significance was observed between the platelet count of UM and CM cases ($p=0.326$, Dunn's Multiple comparison). A significant difference was observed between the median parasite densities of the cerebral malaria (840 parasites/uL/blood) and uncomplicated malaria (27,900 parasites/uL/blood) groups ($P= 0.0023$, t-test). There were significantly more parasite seen in the peripheral blood of the UM than in the CM.

Table 4. 1 General Characteristics of study participants

Characteristics	**Study Groups**			**P value**
	Cerebral Malaria	Uncomplicated Malaria	Healthy Control	
N	50 (38%)	45 (34%)	37 (28%)	
Age (years)	5.3 (1-12)	5.0 (1.5-11)	9 (1-12) *	0.0007
Sex (% male)	62.75$^{\infty}$	56.82	36.36	<0.03
Hb (g/dL)	8.6 (5.9-14.90)$^{\beta}$	10.75 (6-14.10)	12.00 (9.10 -14.20)	<0.0001
WBC (10^3/uL)	10.65 (2.8 – 33.0)	6.70 (2.4 – 29.5)	6.450 (4.1 – 10.6)	<0.0001
RBC (10^6/uL)	3.44 (2.30 – 6.04)	4.30 (2.62 – 5.52)	4.58 (3.5 – 5.66)	<0.0001
HCT (%)	26.20 (18.90 – 43.10)	30.20 (19.00 – 40.20)	34.25 (25.00 – 41.20)	<0.0001
Platelets (10^3/uL)	60.0 (20.0 – 550.0)	105.5 (21.0 – 400.0)	329.5 (209.0 – 573.0) §	<0.0001
Parasite density (parasite/µl)	840 (1-304000)$^{\alpha}$	27900(1680-154000)	-	0.0023 (t-test)

Data represents the median and minimum and maximum values. P value was obtained by Kruskal-Wallis on Ranks. Post-hoc test was done by Dunn's Multiple Test to detect significant differences between paired groups. T-test was used to compare parasite densities of CM and UM. * indicates that the median age of HC was higher than that of CM and UM. $^{\infty}$ indicates there were significantly more males in CM than in UM and HC. $^{\beta}$ Indicates the Hb level in the CM group was significantly lower than UM and HC group ($P < 0.001$) following the Post-Hoc test. § indicates that median platelet count in HC group were significantly higher than UM and CM ($P<0.05$). $^{\alpha}$ median parasite density in CM was lower than that of UM ($p=0.0023$, t-test)

4.2 Stability of cells in C-C BCT

4.2.1 Stability of leukocyte populations in C-C BCT preservative

Whole blood from healthy volunteers were stored in EDTA anticoagulant and C-C BCT blood preservative at 4^{o}C and processed for flow cytometry on days 0 (30 minutes), 2 (48 hours) and 7. There was an overall discrimination of leukocyte populations in C-C BCT compared with that in EDTA-treated peripheral blood stored at 4^{o}C (Figure 4.1). This is evident on day 7 in blood stored in C-C BCT where the three major leukocyte populations could be clearly gated or differentiated.

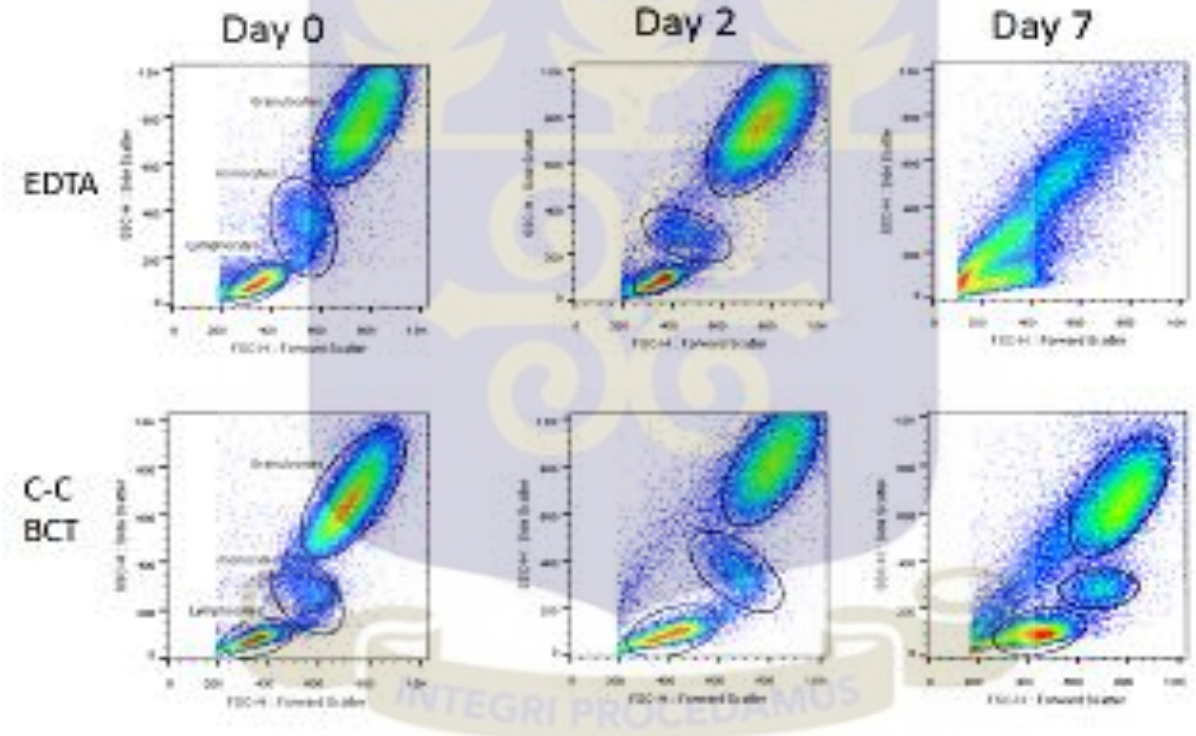


Figure 4. 1 Leukocyte populations in whole blood preserved in C-C BCT preservative and EDTA anticoagulant over a seven day period.

4.2.2 Stability of EPCs in C-C BCT preservative

EPC levels were estimated in adult whole blood preserved in C-C BCT at 4^{o}C over a seven day period. Levels of EPC in C-C BCT at different time points were compared with that in EDTA

anticoagulant at baseline (day 0). Mean cEPC levels were not significantly different between baseline EDTA samples (0.017%, 95% confidence interval 0.006- 0.028) and C-C BCT samples stored at 4^{o}C for day 0 (0.038%, 95% CI 0.005- 0.072), day 1 (0.030%, 95% CI 0.017- 0.042) and day 2 (0.047%, 95% CI 0.019- 0.075) following pairwise comparisons, but the mean baseline EDTA blood cEPC levels were significantly lower (p=0.004) than that in whole blood preserved in C-C BCT for 7 days (0.070%, 95% CI 0.030- 0.10) [Figure 4.2].

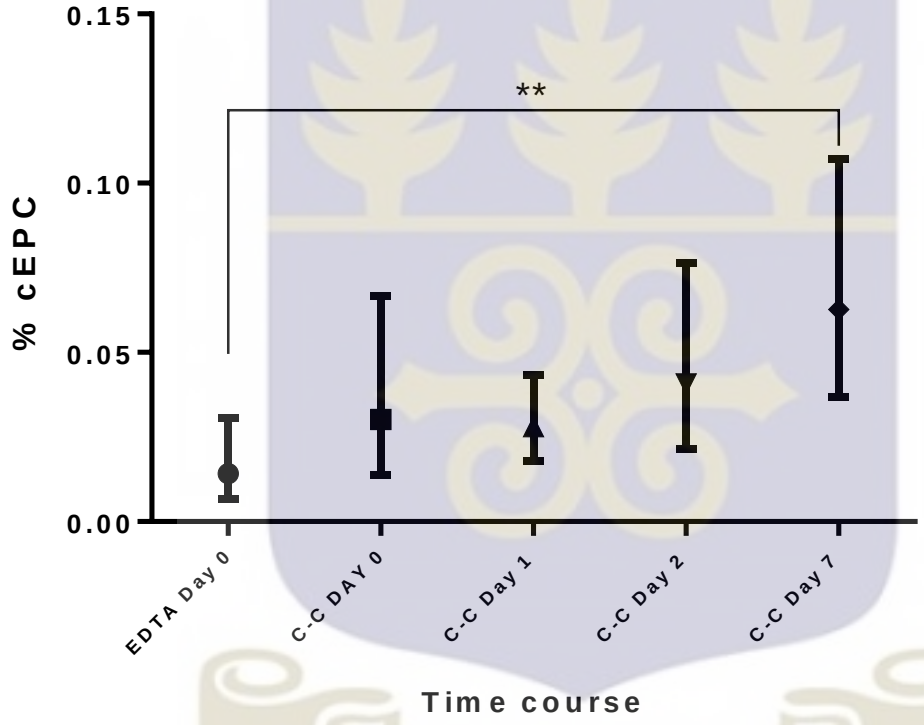


Figure 4. 2 Percentage of cEPC at different time points in whole blood preserved in C-C BCT. ** indicates lower cEPC levels in EDTA compared with C-C BCT day 7 (p<0.01).

4.2.3 Stability of CECs in C-C BCT preservative

CEC levels were estimated in adult whole blood preserved in C-C BCT at 4^{o}C over a seven day period. Levels of CEC in C-C BCT at different time points were compared with that in EDTA

anticoagulant at baseline (Figure 4.3). Mean CEC levels were not significantly different between baseline EDTA samples (0.0026%, 95% CI 0.001- 0.004) and C-C BCT samples stored at 4°C for day 0 (0.0012%, 95% CI 0.00008- 0.003), day 1 (0.002%, 95% CI 0.0001- 0.0035) and day 2 (0.003%, 95% CI 0.002- 0.005) following pairwise comparisons, but the mean baseline EDTA blood CEC levels were significantly higher (p=0.043) than that in whole blood preserved in C-C BCT for 7 days (0.0004%, 95% CI -0.0006- 0.001):

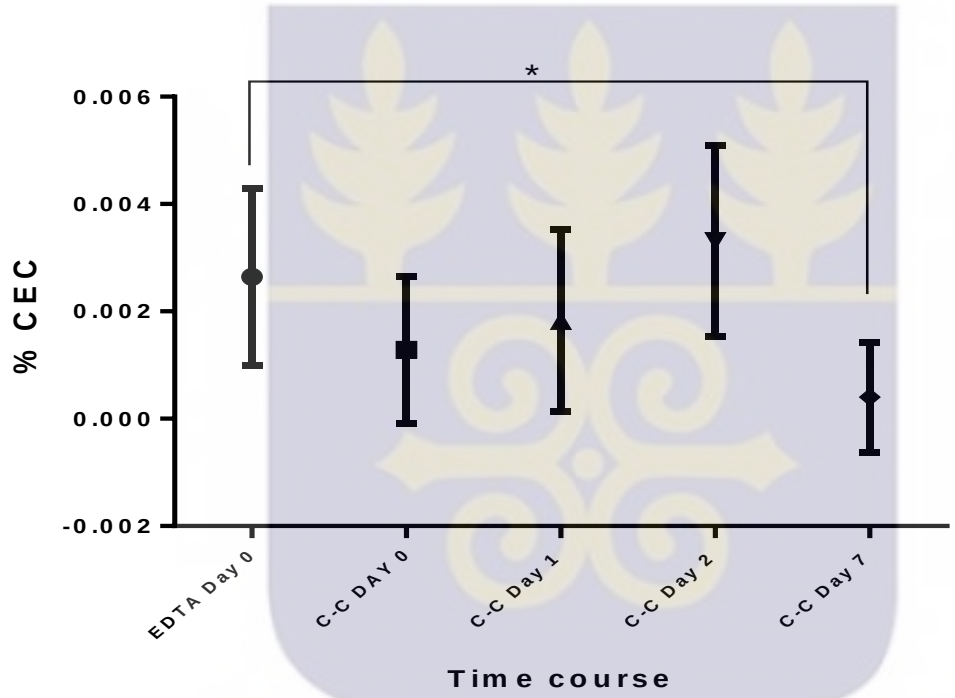


Figure 4. 3 Percentage frequency of CEC at different time points in whole blood preserved EDTA anticoagulant and in C-C BCT. ** indicates higher CEC levels in EDTA compared with C-C BCT day 7 ($p<0.05$).

4.2.4 Stability of common immune markers in C-C BCT

Levels of common immune markers (CD4, CD8, CD14 and CD15) were determined in blood preserved in C-C BCT at 4°C over a seven day period. Levels of these markers at different time points were compared with that in EDTA anticoagulant at baseline as shown in Figure 4.4. No

significant differences were observed between expression levels of CD4, CD8, CD15 over the seven day storage in C-C BCT when compared to EDTA ($p>0.05$, Friedman test and Dunn's multiple comparison) in all cases. However, mean CD14 levels were significantly higher (**$p=0.023$ Dunn's multiple comparison test) on day 7 (13.12%, 95% CI 7.123- 19.11) when compared with EDTA day 0 (4.67%, 95% CI 3.327- 6.016).

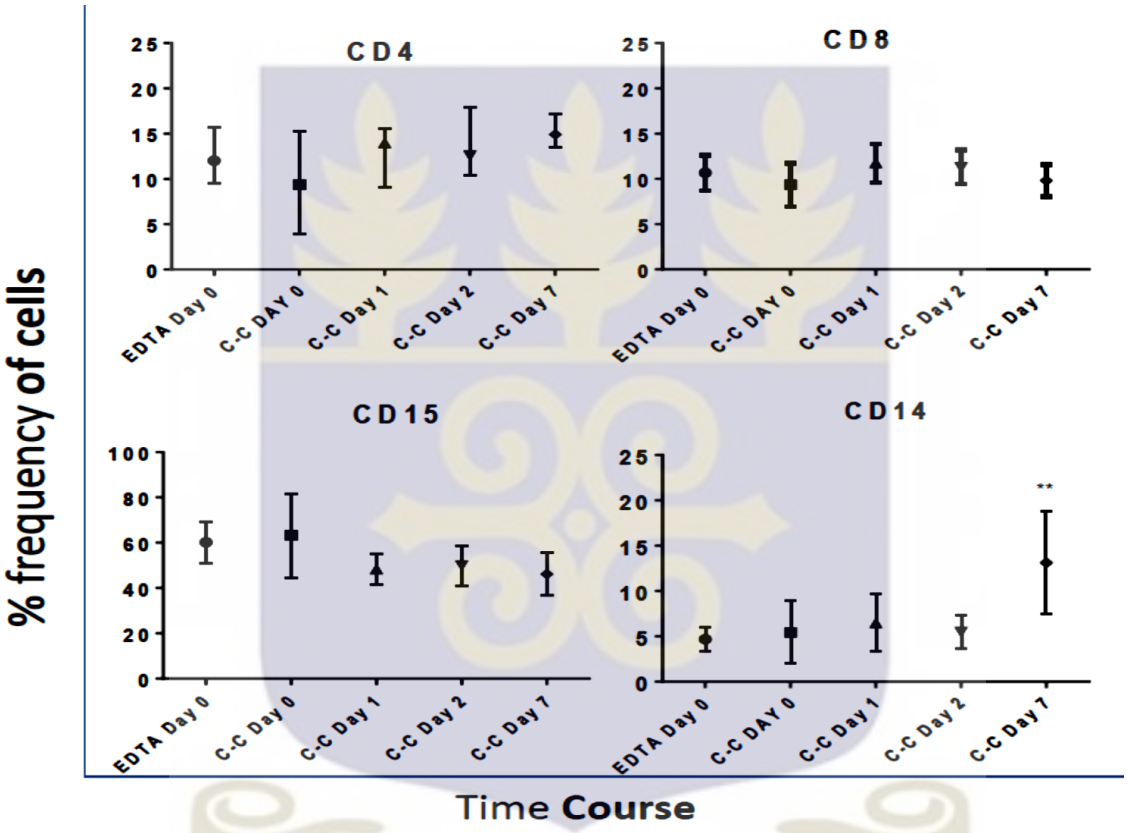


Figure 4. 4 Percentage frequencies of some immune marker at different time points in whole blood preserved in C-C BCT. ** indicates higher CD14 levels compared to EDTA day 0 and first 2 days in C-C BCT.

4.2.5 Stability of endothelial and other markers in C-C BCT

Levels of some endothelial and other markers (CD31, CD133, CD34, CD309, CD11b and CD45) were determined in whole blood preserved in C-C BCT at 4°C over a seven day period. Levels of

these markers at different time points were compared with that in EDTA anticoagulant at baseline as shown in Figure 4.5. Percentage frequency of cells expressing CD133 were significantly higher (**p= 0.041) on day 7 in the preservative (18.98%, 95% CI 14.14 – 23.83) compared to baseline frequencies in EDTA (3.43%, 95% CI 1.66 – 5.183). CD309 levels on Day 7 (2.89%, 95% CI 1.834 – 3.95) in C-C BCT were significantly higher (*p=0.005, Dunn's multiple comparison) compared to baseline frequencies in EDTA (0.76%, 95% CI 0.66 – 2.17). Percentage frequency of CD11b cells were significantly reduced (p=0.02, Dunn's multiple comparison test) on day 2 in C-C BCT (8.032%, 95% CI 5.45 – 10.62) compared to baseline EDTA (20.26% 95% CI 12.13 – 28.39). CD45 frequencies were also significantly reduced (p=0.01, Dunn's multiple comparison test) on day 7 in C-C BCT (95.07%, 95% CI 92.78 – 97.36) compared with EDTA day 0 (98.13%, 95% CI 97.42 – 98.83).

Percentage frequencies of cells expression CD31 and CD34 were significantly stable in C-C BCT over the seven day period and levels were comparable with EDTA day 0.

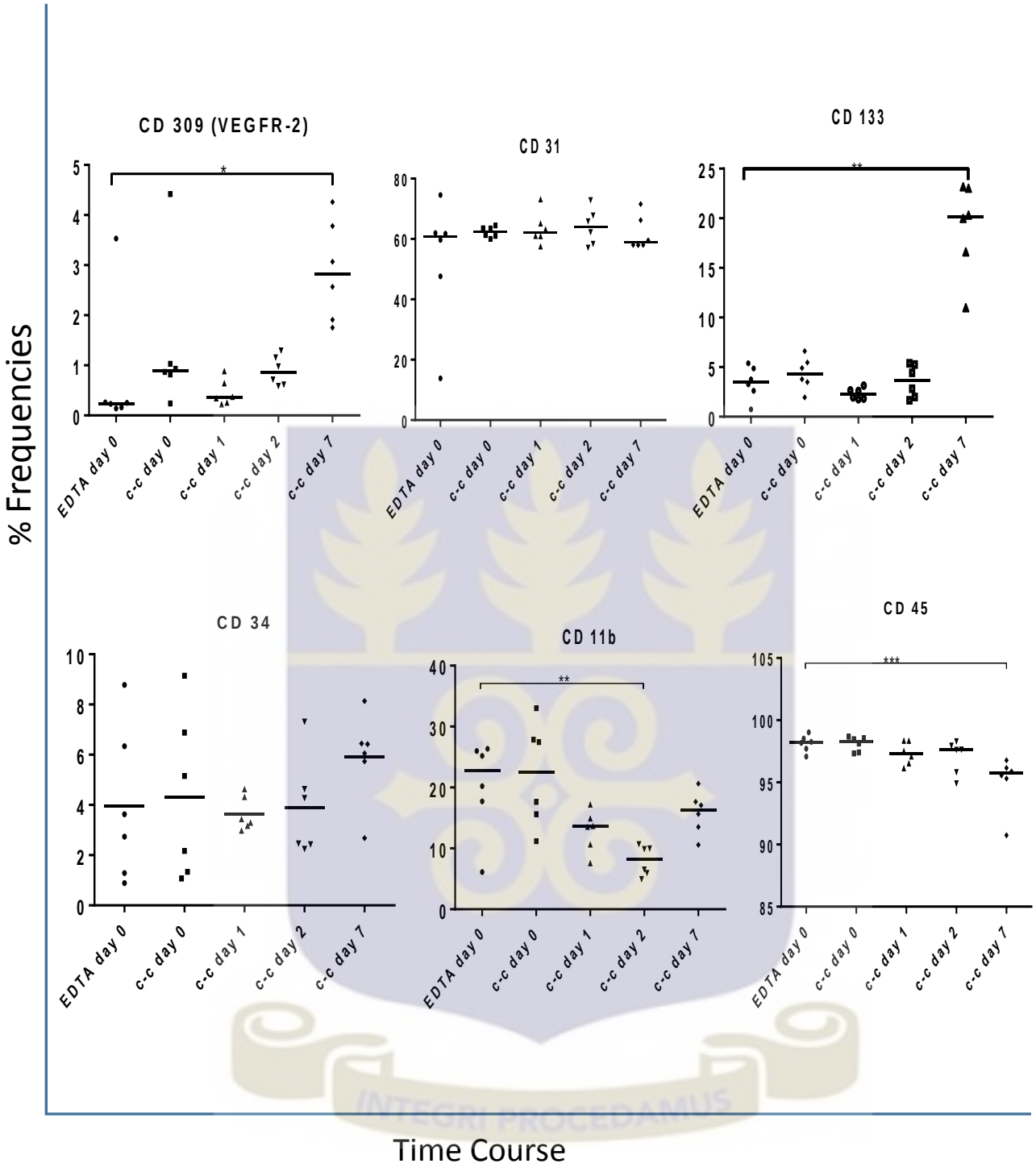


Figure 4. 5 Percentage cell count of individual cell expressing EPC, CEC, haematopoietic and leukocyte receptors in whole blood preserved in Cyto-chex BCT at 4^{o}C for seven days**.**

4.3 Levels of cEPCs and CECs in the Study Groups

4.3.1 cEPC levels in different groups at baseline

cEPC levels were estimated using the gating strategy in Figure 3.5. Comparing the baseline cEPC levels in the three study groups, mean cEPC levels in HC (0.083%) were significantly higher than levels in CM (0.042%, p=0.0071, Dunn's multiple comparison test) but similar to levels in UM (0.140%, p=0.1414). Mean levels in UM were also significantly higher than those in CM (p<0.0001).

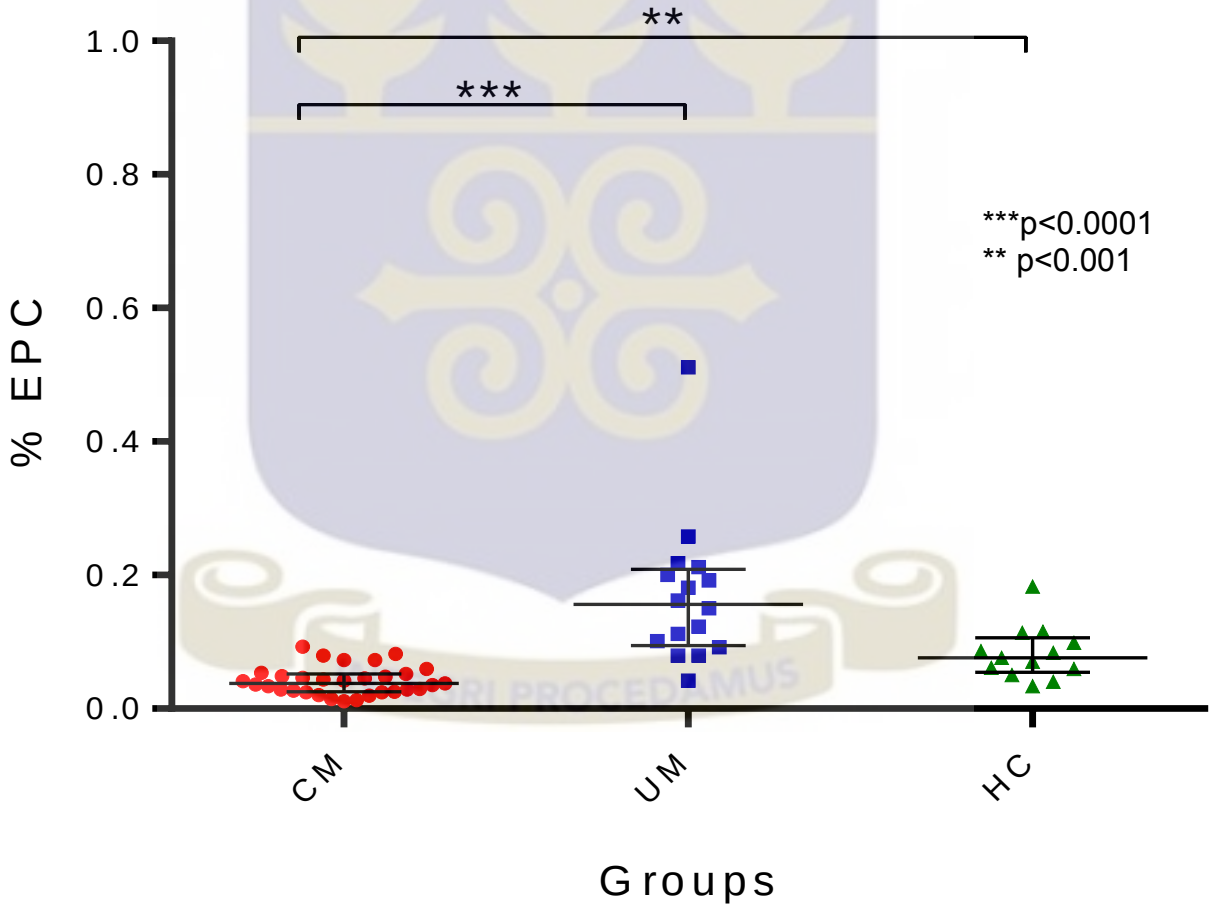
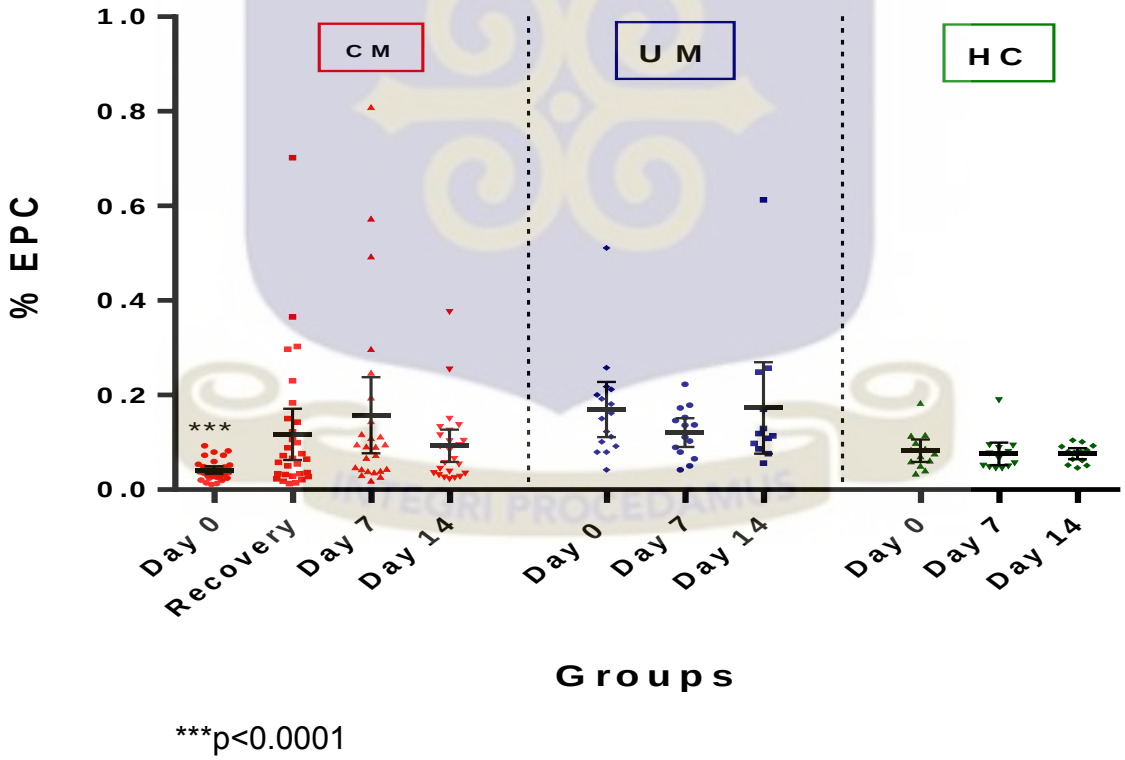


Figure 4. 6 cEPCs levels at initial evaluation in the study groups**.**

4.3.2 Time course estimation of cEPC levels in different groups

cEPC levels were determined during the time course of recovery from malaria infection (CM and UM) as well as in healthy children (Figure 4.7). cEPC levels were elevated during the time course of recovery from CM ($p<0.0001$), peaking at day seven post-recovery indicating repair or replacement of damaged endothelial cells in CM patients. Mean cEPC levels in CM: Day 0= 0.042% [95% confidence interval (CI) 0.034- 0.049], Recovery = 0.117% [95% CI -0.063- 0.171], Day 7= 0.157% [95% CI 0.076-0.238], Day 14 = 0.093% [95% CI 0.059-0.127]. No significant differences were observed in the pairwise comparison within the other study groups [UM (Day 0, day 7 and Day 14) p= 0.4226, HC (Day 0, day 7 and Day 14) p>0.9999)]. Data are presented as mean 95% confidence interval.



***p<0.0001

Figure 4. 7 Time course cEPC levels in study the groups.

4.3.3 CEC levels in different groups at initial presentation

Using Lyse-wash protocol, CECs were quantified as $CD11b^{-}$, $CD34^{+}$, $CD31^{bright}$ and $CD133^{+}$ using flow cytometry gating strategy shown in Figure 3.6. CM showed the highest CEC levels compared to UM or HC (Dunn's multiple comparisons test: CM vs UM **p=0.0037, CM vs HC *p=0.0413, Figure 4.8) suggesting significant endothelial damage in the CM patients. No significant difference was found between uncomplicated and healthy controls (p>0.9999).

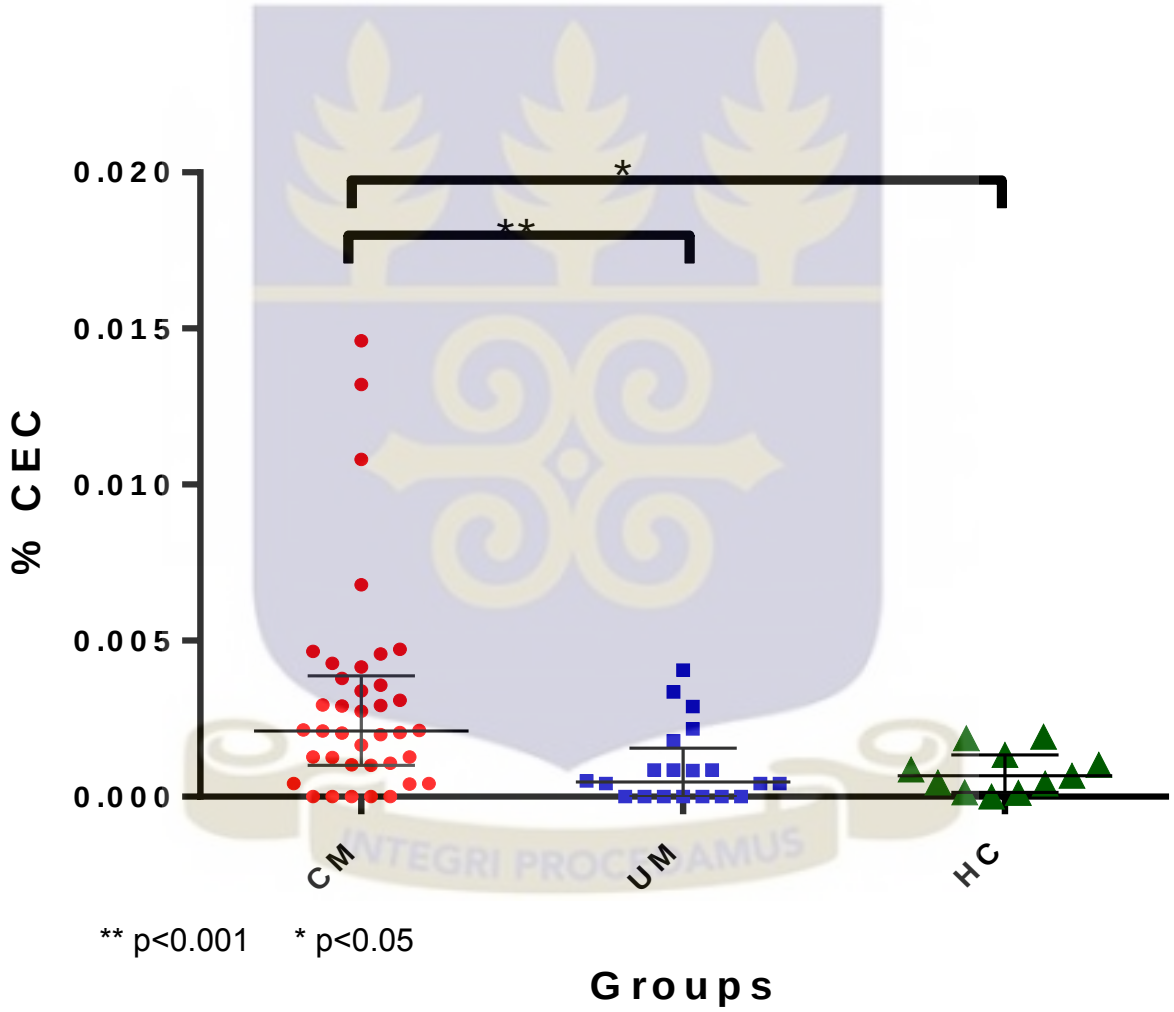


Figure 4. 8 Percentage CECs at initial evaluation in the study groups

4.3.4 Time course evaluation of CECs

CEC levels were determined during the time course of recovery from malaria infection (CM and UM) as well as in healthy children. Higher levels of CECs were observed in CM patients even at fourteen days post recovery from coma (Figure 4.9). UM patients showed a sharp increase and decrease in CEC levels day seven ($p<0.001$) and fourteen ($p=0.002$) post initial clinical evaluation respectively. Mean CEC levels in CM; Day 0= 0.003% (95% CI 0.002- 0.004), Recovery = 0.004% (95% CI -0.003- 0.006), Day 7= 0.006% (95% CI 0.003-0.008), Day 14 = 0.006% (95% CI 0.005-0.009). Mean CEC levels in UM; Day 0= 0.001% (95% confidence interval (CI) 0.0004-0.002), Day 7= 0.009% (95% CI 0.006-0.011), Day 14 = 0.002% (95% CI 0.0009-0.003).

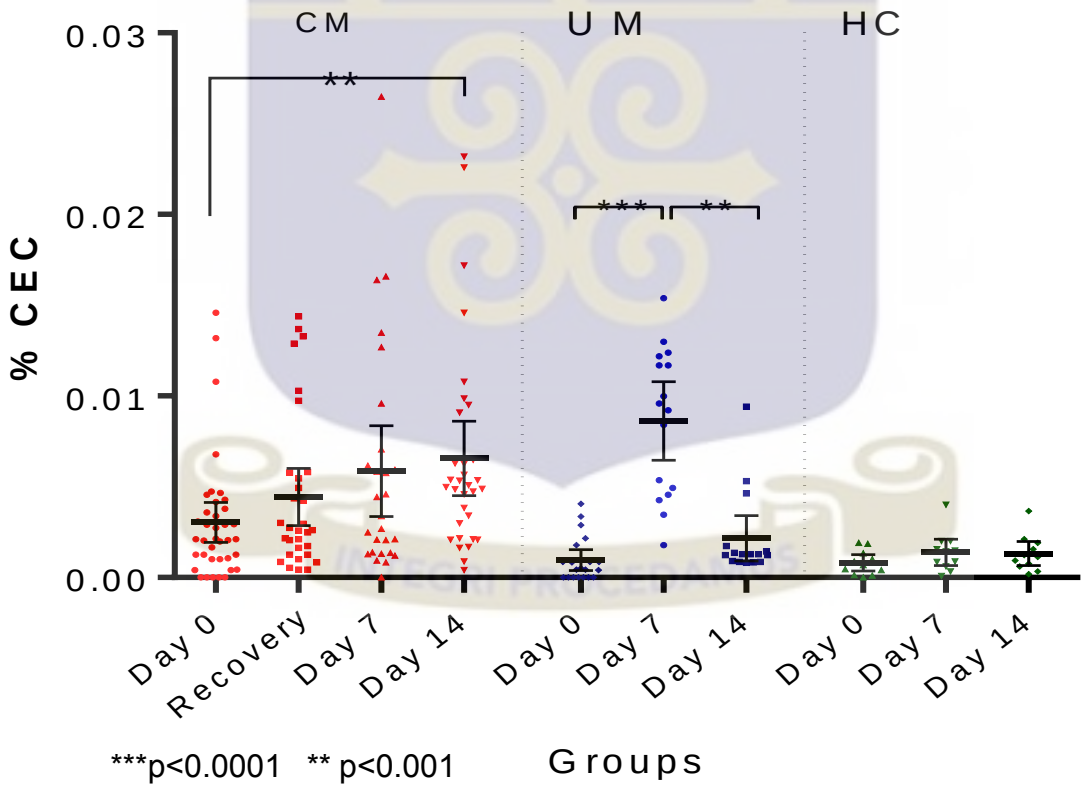


Figure 4. 9 Time course CEC levels in the study groups

4.4 Plasma levels of Ang-1 and Ang-2

Ang-1 and-2 levels were determined during the time course of recovery from malaria infection (CM and UM) as well as in healthy children (Table 4.2). Levels of Ang-2 in the study groups at initial evaluation did not show any statistically significant differences ($p=0.124$, Kruskal Wallis) even though CM patients had higher Ang-2 levels (5260pg/ml) compared to that in UM patients (4041pg/ml) and HC (4909pg/ml) suggesting microvascular leak or damage in CM patients. Ang-2 levels did not also show any significant difference ($p=0.147$) in course of recovery from CM. However, UM showed significant increase ($p=0.016$) on Day 14 after initial clinical presentation.

Ang-1 levels were significantly lower ($p<0.05$) in UM compared to both CM and healthy controls at initial presentation. Plasma levels of Ang-1 reduced ($p=0.04$) during coma but showed a sharp increase ($p<0.001$) seven days after recovery from coma indicating promotion of pro-survival pathways and endothelial cell quiescence. UM also showed a sharp increase ($p=0.005$) in Ang-1 level on day seven after initial presentation of malaria and maintained level on day 14.

The ratio of Ang2:Ang1 showed a significant increase ($p<0.05$) at recovery and a subsequent decrease at days 7 in CM patient suggesting endothelial injury and restoration of endothelial integrity respectively. The ratio of these two endothelial mediators could not differentiate the state of the endothelium as no significant difference was observed between the Ang1:Ang2 ratios of CM, UM and HC ($p>0.5$, Kruskal Wallis).

Table 4. 2 Time course Plasma levels of Ang-1 and -2 in the study groups

Groups	Biomarker	Day 0	Recovery	Day 7	Day 14	P (Kruskal Wallis)
CM	ANG2 (pg/ml)	5260 (4310-6120)	6287 (5275-7299)	4923 (4262-5585)	6021 (4846-7197)	0.147
	ANG1 (pg/ml)	16072 (8215-23928)	8967# (4994-12940)	20609 (15470-25748)	18720 (12968-24471)	***<0.0001
	ANG2:ANG1 (pg/ml)	0.493 (0.32-0.67)	0.895β (0.744-1.05)	0.287 (0.16-0.40)	0.408 (0.29-0.53)	***<0.0001
UM	ANG2 (pg/ml)	4041α (3265-4818)		5354 (4285-6423)	5571 (4861-6280)	**0.017
	ANG1 (pg/ml)	9248∞ (6020-12475)		16451 (11496-21407)	15187 (11468-18906)	**0.005
	ANG2:ANG1 (pg/ml)	0.437 (0.29-0.59)		0.355 (0.24-0.47)	0.453 (0.34-0.57)	0.4026
HC	ANG2 (pg/ml)	4909 (3048-6936)				
	ANG1 (pg/ml)	20675 (17258-24092)				
	ANG2:ANG1	0.318 (0.26-0.38)				

*** p<0.0001 ** p<0.05

#: Levels of Ang-1 is significantly lower (p<0.05) compared to all other time points in CM

β: Ang-2:Ang-1 ratio significantly higher (p<0.05) compared to all time points in CM

α: Significant lower levels of Ang-2 at day 0 compared to day 14 in UM patients

∞: Significant lower Ang-1 levels at day 0 compared with other time points in UM

4.5 Levels of Endothelial Receptors

4.5.1 Baseline levels of soluble EPCR in the study groups

Soluble EPCR levels were measured by ELISA at initial evaluation in the study groups (Figure 4.10). Baseline plasma levels of EPCR in the study groups showed CM patients with the highest levels (20.14pg/ml) compared to that of UM patients (16.82pg/ml) and HC (15.39pg/ml) ($p<0.05$, Kruskal Wallis). Post-hoc comparison of soluble EPCR levels in CM and UM did not reach statistical significance ($p>0.05$, Dunn's multiple comparison test) even though CM showed higher levels. However, levels in CM was significantly higher compared with HC ($p=0.002$, Dunn's multiple comparison test).

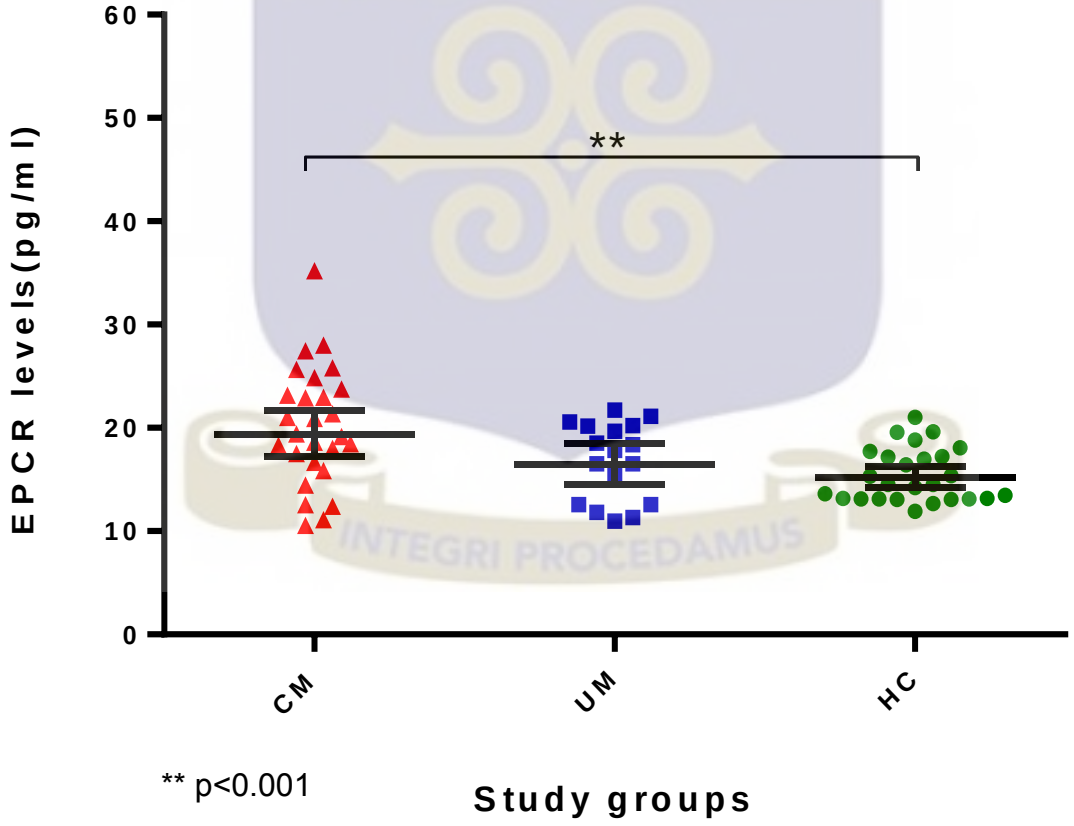


Figure 4. 10 Soluble EPCR levels in study the groups

4.5.2 Time course levels of soluble EPCR in the study groups

Plasma levels of soluble EPCR were determined at different time points in the recovery from malaria and in healthy controls (Figure 4.11). Time course levels did not reach statistical significance in the various study groups (CM, p= 0.31 and UM, p=0.29, Kruskal Wallis).

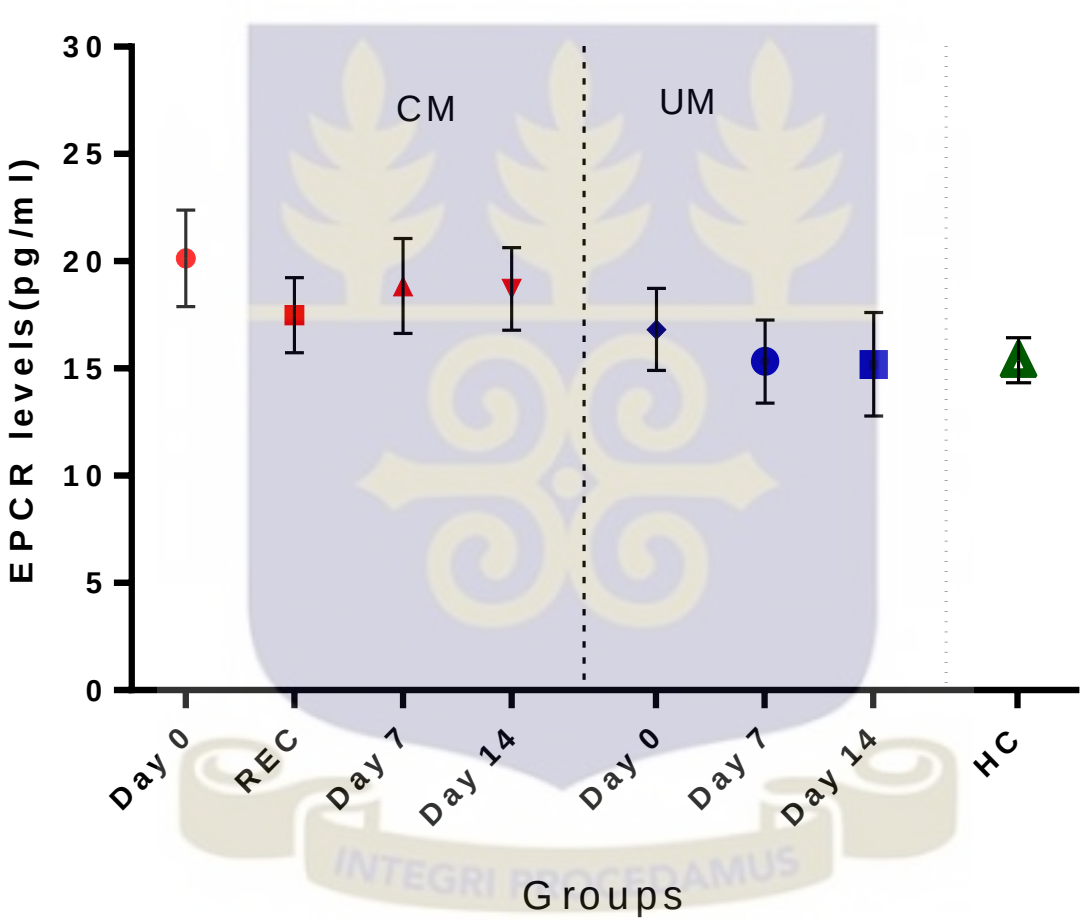


Figure 4. 11 Levels of soluble EPCR at different time points in the study groups

4.5.3 Baseline levels of soluble TM in the study groups

Initial determination of soluble TM was done in the various study groups (Figure 4.12). Levels in CM was significantly higher (8084pg/ml) compared with UM (5785pg/ml) and HC (5076pg/ml)

(CM vs UM, p=0.01: CM vs HC, p<0.0001, Kruskal Wallis and Dunn's multiple comparisons). Levels in HC and UM were not significantly different (p=0.16).

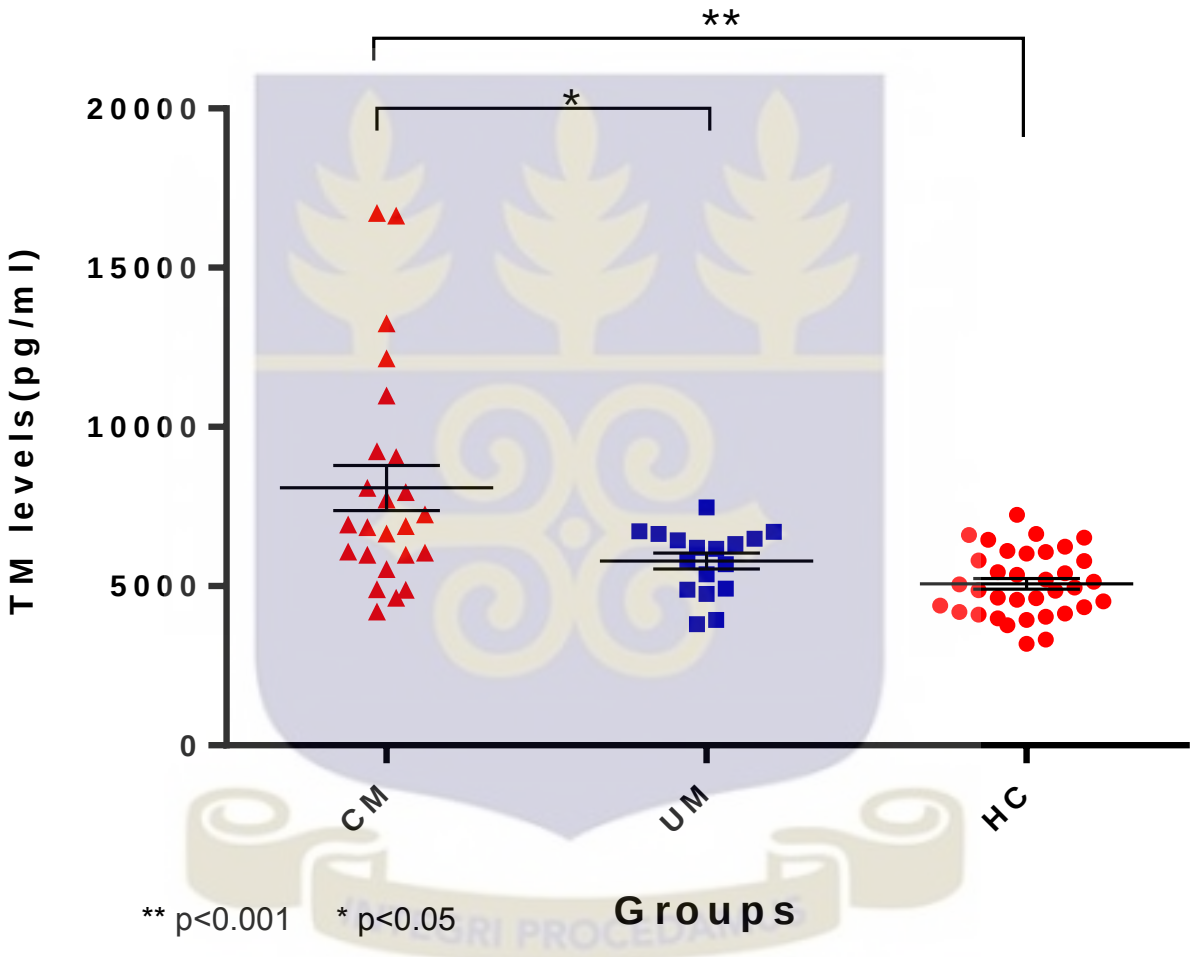


Figure 4. 12 Soluble TM levels in the study groups

4.5.4 Time course levels of soluble TM in the study groups

Follow up plasma levels of soluble TM were determined in CM and UM patients (Figure 4.13). TM levels in CM significantly dropped ($p<0.05$, Kruskal Wallis) after initial clinical presentation (Day 0) compared to subsequent time points (Day 0 vs Rec, $p=0.002$, Day 0 vs Day 7, $p=0.033$, Day 0 vs Day 14, $p=0.0001$, Dunn's multiple comparisons). No significant difference was observed between recovery from coma and fourteen days after that. Similar significant drop in sTM levels was observed in the time course in UM resolution. (Day 0 vs Day 7: $p=0.0013$. Day 0 vs Day 14; $p=0.01$).

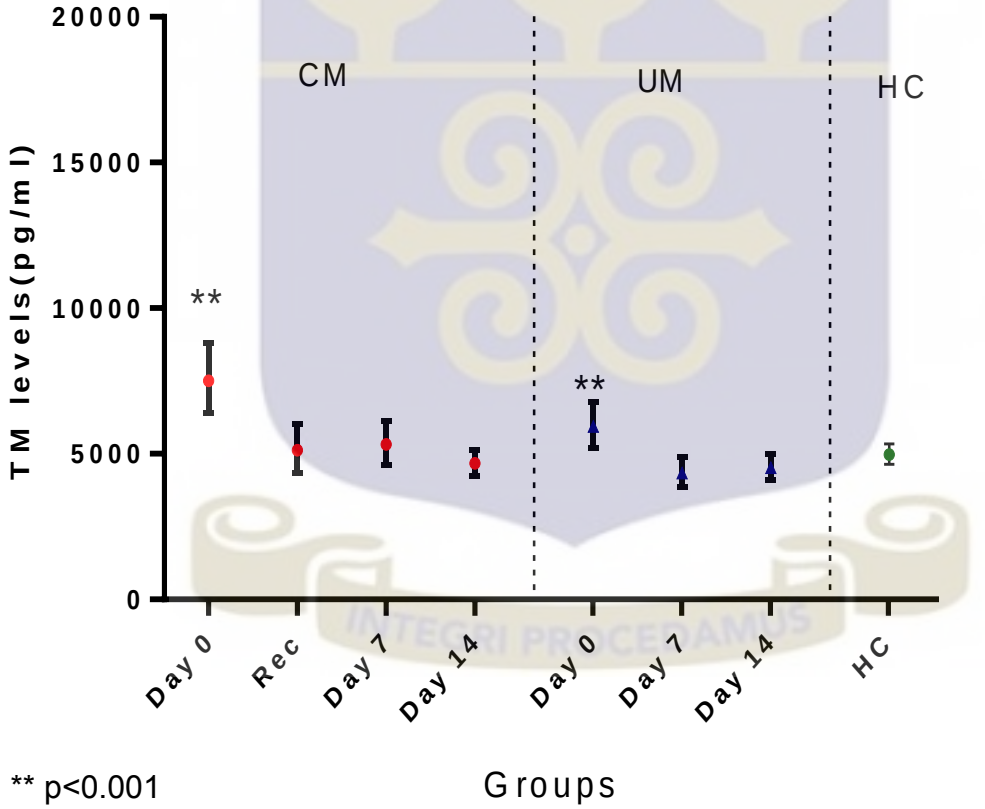


Figure 4. 13 Levels of soluble TM at different time points in the study groups

4.6 HRP2 levels

4.6.1 Baseline levels of HRP 2 in the study groups

HRP2 levels were determined in plasma of children in the various study group at baseline (Figure 4.14). CM showed the highest HRP2 levels (2281ng/ml) [p<0.0001, Kruskal Wallis] compared with both UM (353ng/ml) and HC (15ng/ml) (CM vs UM, ***p<0.0001: CM vs HC,*** p<0.0001 and UM vs HC, **p=0.0088, Dunn's multiple comparison).

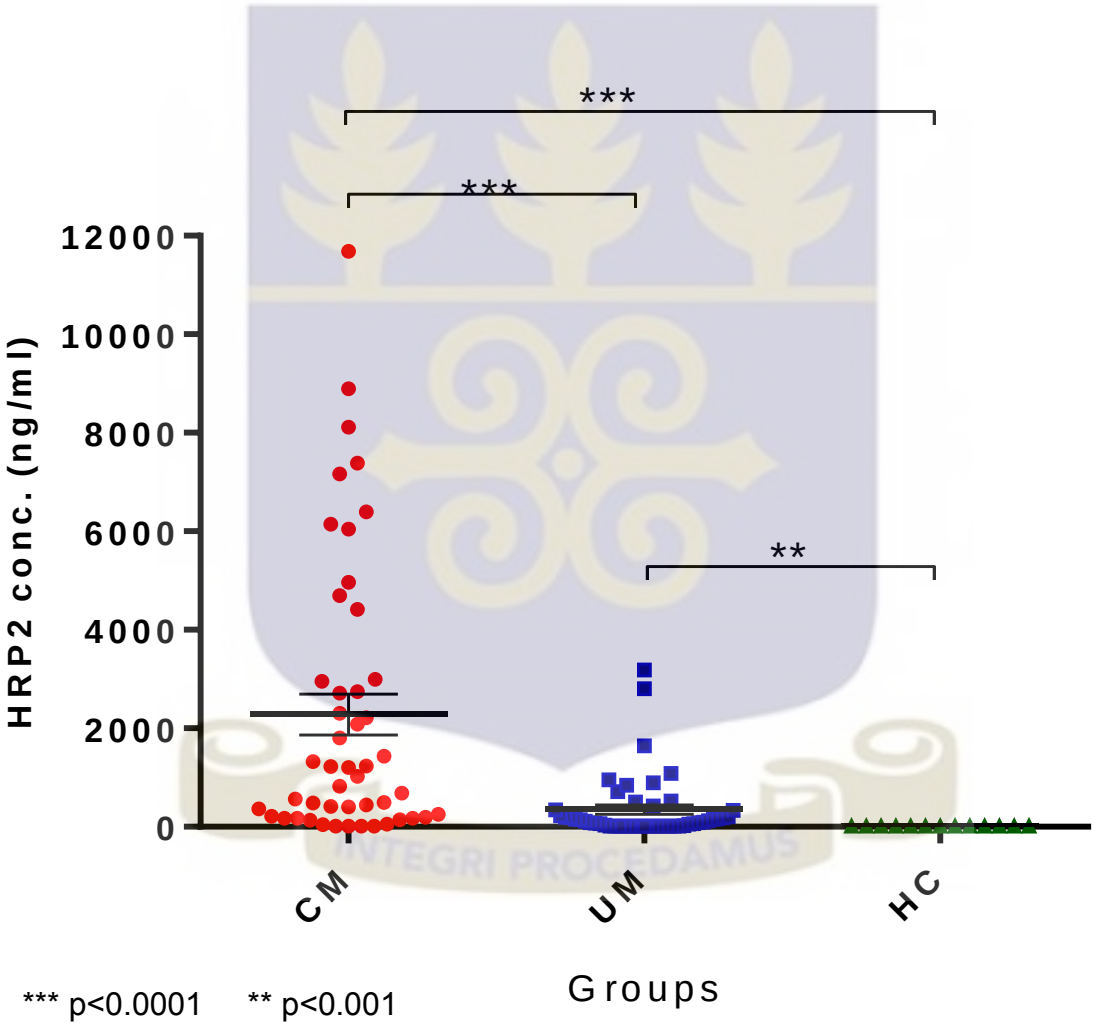


Figure 4. 14 HRP2 levels in the study groups at baseline

4.6.2 Time course levels of HRP2 in the study groups

Time course levels of HRP2 were determined in CM and UM patients (Figure 4.15). HRP2 levels dropped significantly ($p<0.0001$, Kruskal Wallis) in both CM and UM. Levels dropped below detection levels fourteen days after recovery from coma and seven days after initial presentation in CM and UM respectively.

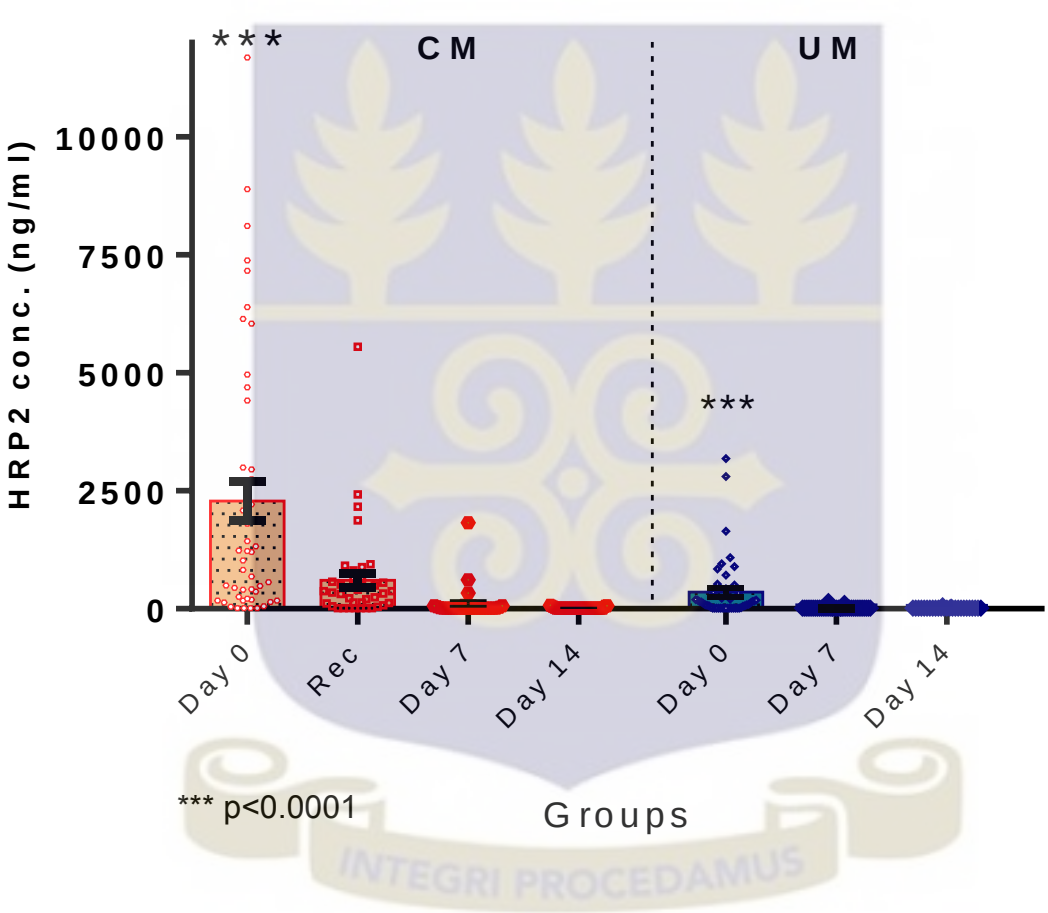


Figure 4. 15 Time course HRP2 levels in the study groups

4.7 Protease/Chemokine Levels

4.7.1 Baseline levels of MMP9 in the study groups

MMP9 levels were determined in the various study group at baseline (Figure 4.16). UM showed the highest MMP9 levels ($p<0.0001$, Kruskal Wallis) compared with both CM and HC (CM vs UM, **$p=0.025$, CM vs HC, $p>0.999$ and UM vs HC, **$p=0.031$, Dunn's multiple comparism).

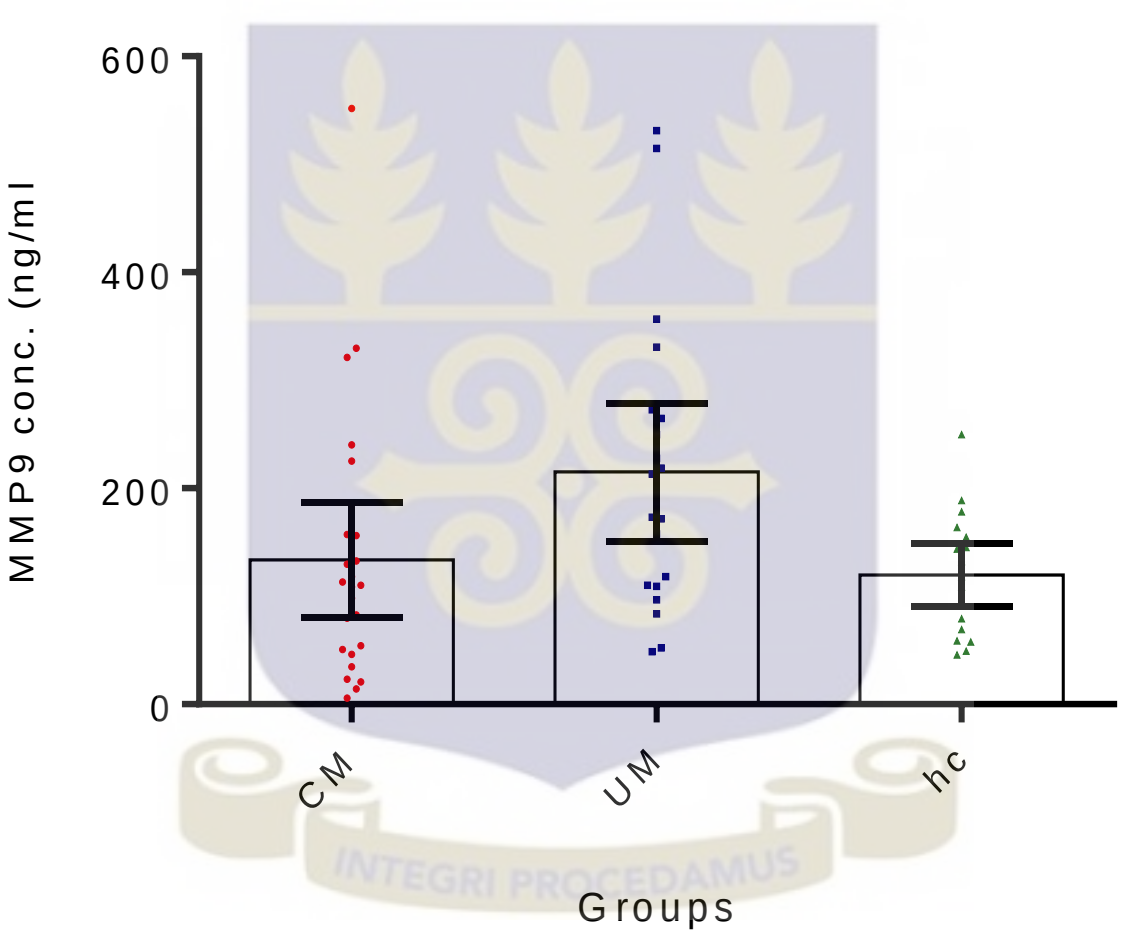


Figure 4. 16 MMP9 levels in the study groups at baseline

4.7.2 Time course levels of MMP9 in the study groups

Time course measurement of MMP9 levels in the various study groups were done (Figure 4.17). Plasma levels of MMP9 in CM patients were not significantly different at initial clinical presentation compared to subsequent time points ($p>0.05$, Kruskal Wallis). UM showed higher MMP9 levels (***$p<0.05$) at initial presentation compared to seven and fourteen days after initial presentation. (Day 0 vs Day 7: $p=0.0013$. Day 0 vs Day 14; $p=0.03$, Dunn's multiple comparisons).

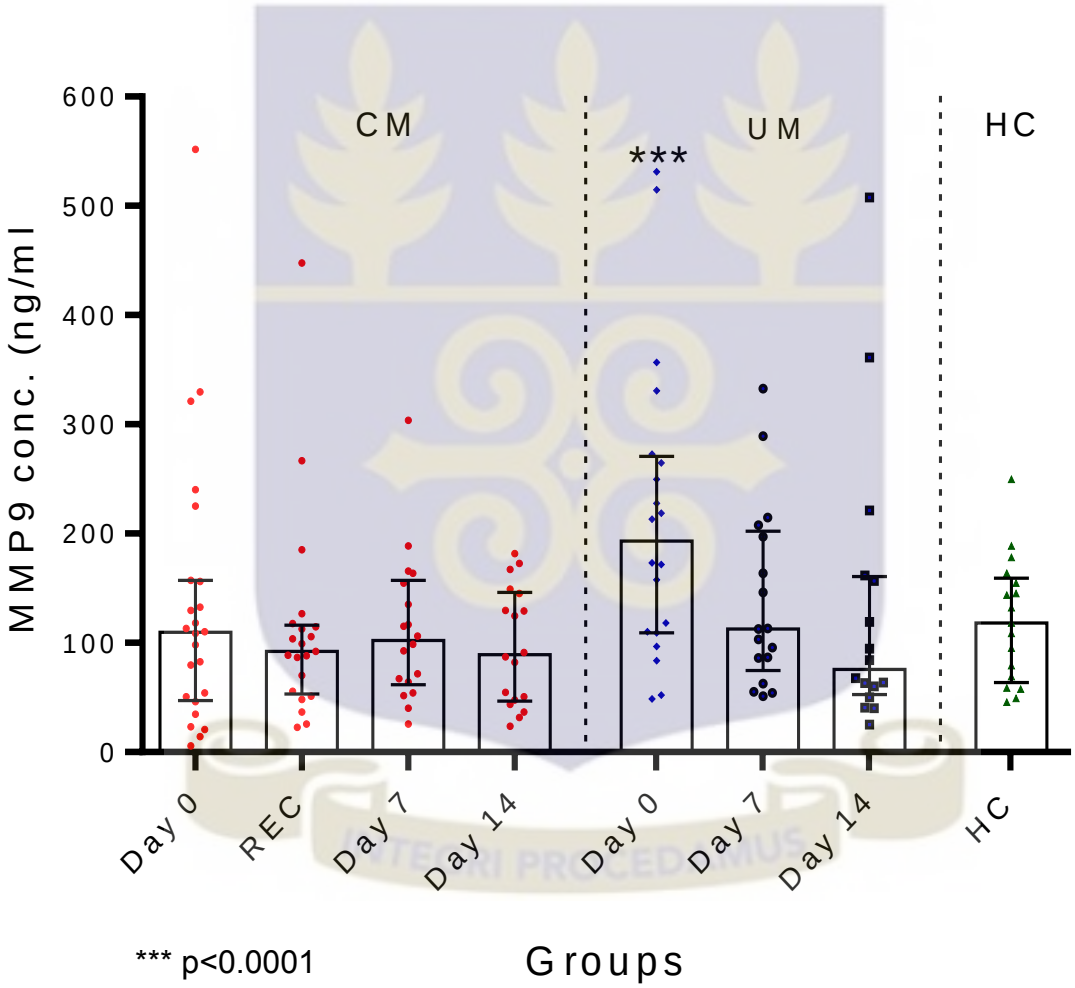


Figure 4. 17 Time course MMP9 levels in the study groups

4.7.3 Baseline levels of SDF-1 in the study groups

SDF-1 levels were determined in the various study group at baseline (Figure 4.18). SDF-1 levels were not significantly different in the initial evaluation between study groups ($p>0.05$, Kruskal Wallis).

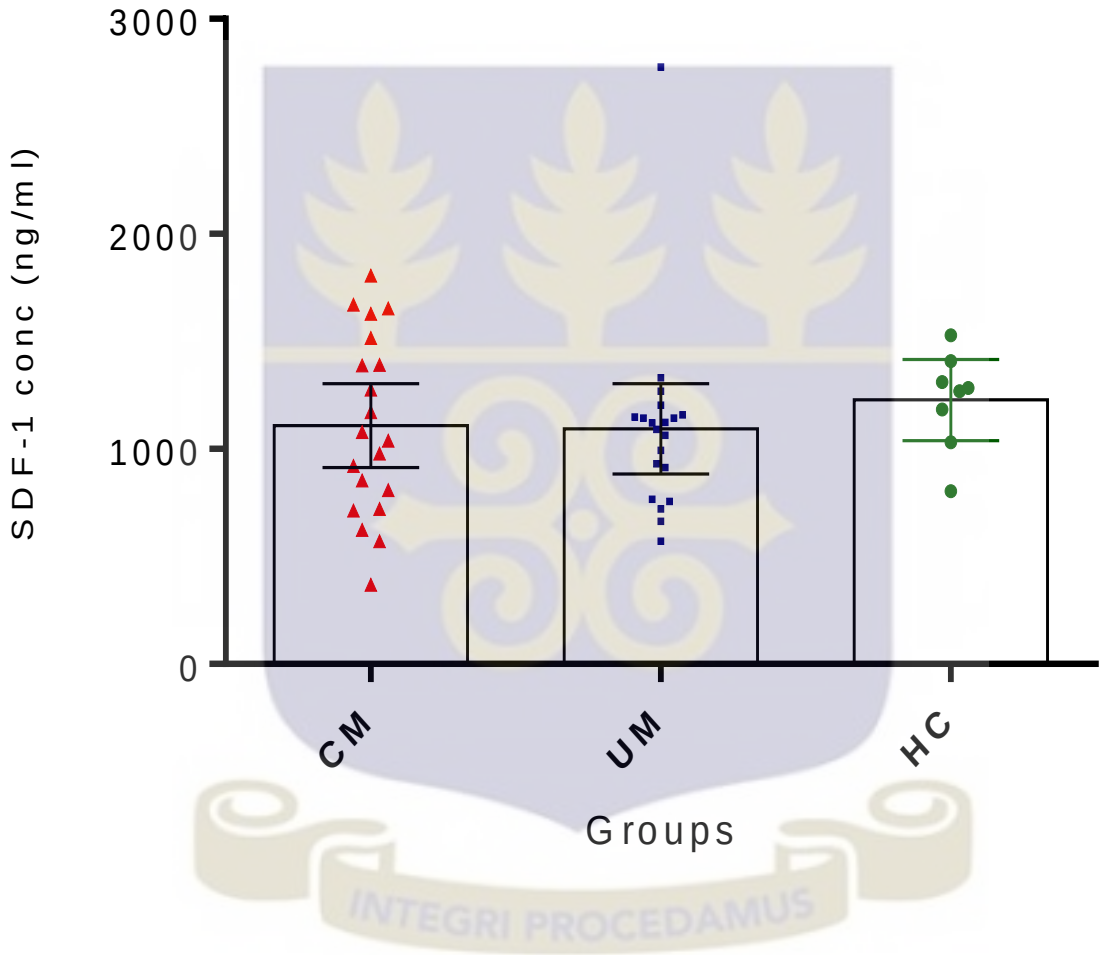


Figure 4. 18 SDF-1 levels in the study groups at baseline

4.7.4 Time course levels of SDF-1 in the study groups

Time course plasma levels of SDF-1 (Figure 4.19) did not show any significantly difference ($p>0.05$, Kruskal Wallis) within and between study groups. Levels in CM and UM were not significantly different from healthy individuals ($p>0.999$, Kruskal Wallis).

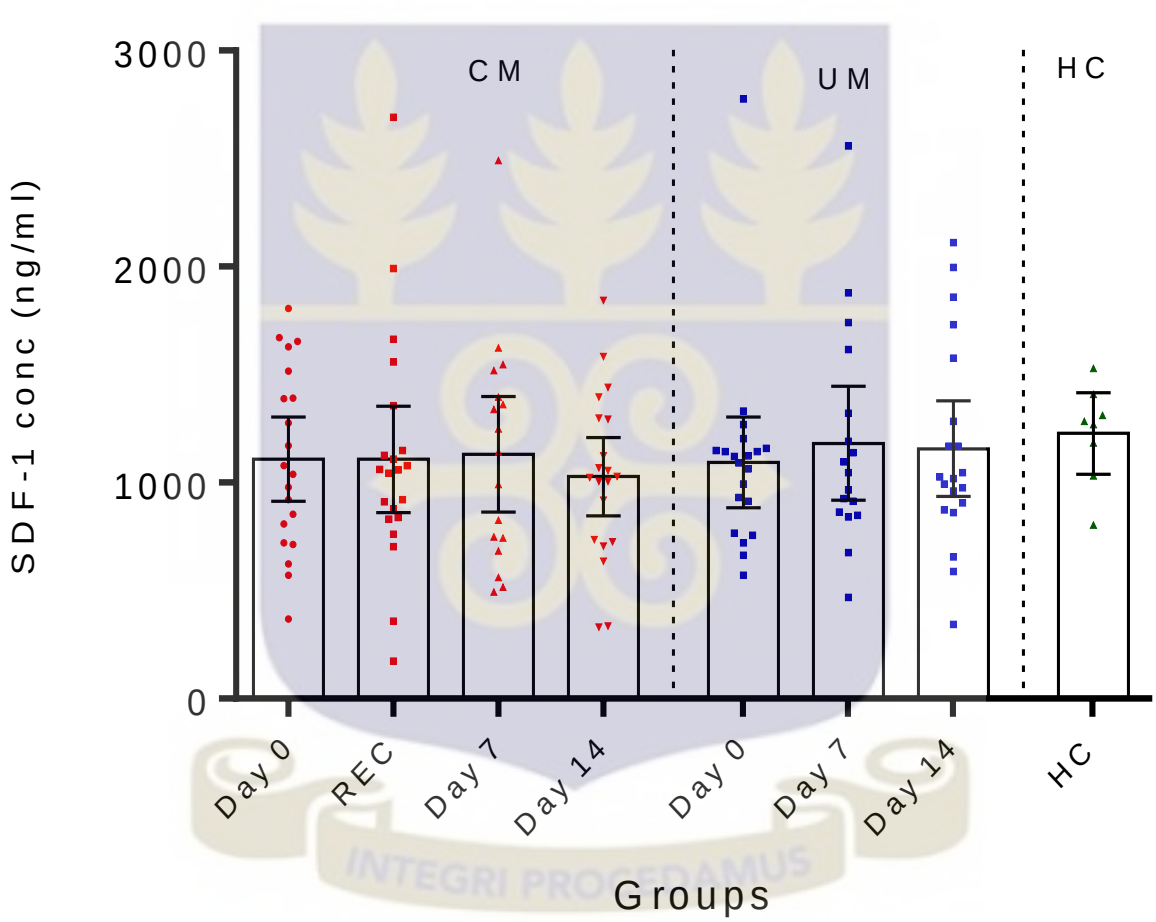


Figure 4. 19 Time course SDF-1 levels in the study groups

CHAPTER FIVE

5 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Sequestration of infected red blood cells (iRBCs) in the brain microvasculature has been shown to be the hallmark of CM and the resultant damage to the endothelium has been postulated as a major initiator of CM (Cooke *et al.*, 2000; Silamut *et al.*, 1999a; Taylor *et al.*, 2004b; Weatherall *et al.*, 2002). The role of cEPCs in the repair of damaged microvasculature has been studied extensively in diseases associated with microvascular damage. The extension of the role of cEPC and factors that affect their levels and function were assessed in this study.

The study was carried out in collaboration from five major referral hospitals in the Greater Accra Region of Ghana. A total of 50 children presenting with CM to these hospitals were recruited and most of these children were aged between a year and twelve years. The number of cases could be indicative of the incidence of CM within this region of Ghana as most cases of CM are referred to these five collaborating hospitals. These hospitals are within a radius of 4 miles from the research centre where analysis of blood samples from the patients was done. The study could not include hospitals from farther distances as processing of samples for rare cells considered in the study need to be done within 2-3 hours after collection.

To assess the possibility of extending the recruitment of study participants to remote centers in the future, the study initially evaluated methods that would allow preservation of samples for longer periods for delayed processing.

CECs and EPCs have been shown as surrogate markers of vascular injury and repair respectively and the successful preservation of these rare cells in biological samples for delayed flow cytometry is very critical for evaluation, particularly in diseases characterized by endothelial damage and interventional clinical trials (Bogoslovsky *et al.*, 2013). It has been recommended that flow cytometric analysis of CECs and EPCs be done within 2-3 hours of collection of samples (Duda *et al.*, 2007). However, not much cryopreservation protocols for EPCs and CECs are available which would fulfill the requirements for multicenter trials. This study therefore evaluated the ability of Cyto-chex BCT (C-C BCT), a commercially available blood cell preservative, to either preserve or stabilize these cells and other immune markers for delayed flow cytometry.

Generally, whole blood preserved in Cyto-chex BCT at 4^0C maintained the overall discrimination of the major leukocyte populations (lymphocytes, monocytes, granulocytes) making gating on the groups possible. This was evident from whole blood stored in C-C BCT at 4^0C for seven days, where lymphocytes, monocytes and granulocytes could be clearly differentiated from each other as was observed in the case of whole blood stored in EDTA on day 0 (Figure 4.3). EDTA is the recommended anticoagulant for the enumeration of CECs and EPC (Duda *et al.*, 2007). EDTA on day seven showed clumped cells, making further evaluation of subpopulation of leukocyte impossible. A study by Warrino *et al.* (2005) also reported the ability to differentiate the various leukocyte populations after seven days of storage of whole blood in C-C BCT at room temperature. Other preservatives such as Transfix (Cytomark, Buckingham, UK) have shown similar leukocyte discriminatory characteristics after seven days of storage (Hoymans *et al.*, 2012).

Flow cytometric analysis of EPCs showed that, these rare cells were stable in whole blood stored in C-C BCT for at least 48 hours (2 days) at 4^{o}C. This is evident in the fact that day 2 levels of EPCs in C-C BCT were not significantly different from that in EDTA at baseline ($p<0.05$) and earlier time points in C-C BCT (Days 0 and 1) [Figure 4.4]. Significant decrease of viable EPC numbers have been shown in blood samples stored in EDTA for more than 24 hours (Masouleh *et al.*, 2010). Cryopreservation of PBMC at -160°C , -80°C and short-term preservation at room temperature have shown decreased EPC numbers (Bogoslovsky *et al.*, 2015). However, the stability of EPCs have also been demonstrated in other stabilizing agents such as the Transfix cellular stabilizing reagents (Hoymans *et al.*, 2012). C-C BCT has therefore shown promise in preserving EPCs.

CECs on the other hand have been used as a marker for endothelial damage and their presence in peripheral blood is indicative of endothelial damage (Dignat-George and Sampol, 2000). Even though their half-life is not known in normal individuals and patients (Erdbruegger *et al.*, 2006), data from this study shows the stability of CECs in C-C BCT for at least 48 hours (Figure 4.5). There was a significant drop (*$p<0.023$) in CEC levels on day 7 in C-C BCT (0.0004%) compared with that in EDTA at baseline (0.0026%). Data from the study therefore suggest the ability of C-C BCT to extend the processing time beyond the 3 hours recommended by Duda *et al.*, (2007).

The stability of some individual receptors expressed on circulating endothelial cells and their progenitors were also evaluated for seven days in C-C BCT. CD309 (VEGFR2) and CD133 which are markers for mature and immature endothelial progenitor cells respectively were stable for at least 2 days in C-C BCT (Figure 4. 5). Mean levels of these cells in the first 48 hours in C-C BCT

were not significantly different from that in EDTA at baseline ($p<0.05$). Other markers for endothelial cells such as CD34 and CD31 were stable for at least seven days in C-C BCT. In other studies, overnight storage at 4°C did not have any effect on $CD34^+$ cell counts and storage in liquid nitrogen for 7 weeks did not affect the percentage of $CD34^+$ cells but was associated with a 26% drop in cell viability (Zubair *et al.*, 2010).

Pan leukocyte markers such as CD45 and CD11b were stable for at least 24 and 48 hours respectively (Figure 4.7). This shows the ability of C-C BCT to stabilize these immune epitopes for delayed analysis. CD45 has been shown to be stable in C-C BCT for seven days at room temperature (Warrino *et al.*, 2005).

Stability of some common immune markers in C-C BCT were evaluated. Immune markers such as CD4, CD8, CD19 and CD16 have all been shown to be stable in C-C BCT for at least seven days after phlebotomy (Warrino *et al.*, 2005). CD4 and CD8 which are also part of the HIV panel of markers were shown to be stable at least seven days in C-C BCT in this study (Warrino *et al.*, 2005). These cells are usually suitable for analysis within 72 hours of collection in either EDTA or Heparin anticoagulant (Mandy *et al.*, 2003). However, in general, cells are considered suspect if they are analyzed after 48 hours of collection (Bergeron *et al.*, 2002). Other receptors such as CD15 mostly found on granulocytes and CD14, also common on monocytes and macrophages were shown to be stable in C-C BCT at least 7 and 2 days respectively. The stability of these common individual receptors in C-C BCT therefore shows promise in delayed analysis. C-C BCT was however not utilized in current study as all affiliated hospitals were within an hour reach by ground transportation.

Based on studies by Gyan *et al*. (2009), which associated decreased cEPC levels to CM in Ghanaian children, this current study hypothesized that recovery from CM is associated with increased cEPC levels and UM patients who progress on the continuum to develop CM will have decreasing levels of cEPCs. Also CM patients who die may have decreasing levels of cEPC. The study therefore evaluated the time course of the host response to *P. falciparum*–induced microvascular damage.

Levels of cEPC were determined at baseline and different times during recovery in CM and UM patients who reported to the five collaborating hospitals and HC from community schools around these hospitals. Pairwise comparison of baseline levels of cEPC in the various study groups indicated lower levels (0.042%) in CM compared to UM (0.140%) and HC (0.083%) [Figure 4.9]. This is consistent with earlier studies by Gyan *et al*. (2009) where cEPCs defined as the dual expression of $CD34^+/VEGFR2^+$ and $CD34^+/CD133^+$ for immature EPC, were higher in both asymptomatic and uncomplicated malaria cases. Several studies have shown that recruitment of EPC represents the body's ability to balance vascular damage with repair (Asahara *et al.*, 1999; Hill *et al.*, 2003; Yoder *et al.*, 2007). A failed attempt to produce and recruit enough EPCs to sites of endothelial injury caused by the sequestration of iRBCs could account for the disease state seen in CM. Levels of cEPCs have been shown to decrease in disease conditions characterized by endothelial damage such as cardiovascular disease, diabetes and hypertension (Vasa *et al.*, 2001; Verma and Anderson, 2002).

Time course evaluation of levels of cEPCs in CM patients showed a sharp increase at recovery (i.e. BCS>3) [0.117%] and at day 7 post recovery (0.157%) compared with levels at baseline

(0.042%) [Figure 4.10]. This trend supports the study hypothesis that CM patients who recover may have increased cEPC levels. The increased levels at recovery and post recovery suggest that the host attempts to repair microvascular damage caused by the activation of the endothelium following sequestration of the malaria parasites. It has been shown that the balance between endothelial injury and repair is critical for the maintenance of endothelial integrity (Sabatier *et al.*, 2009). Endothelial activation has been demonstrated in both mild and severe malaria even though it increases with disease severity (Park *et al.*, 2012). Higher cEPC levels at onset of uncomplicated malaria and the maintenance of levels at sampling time points seem to promote a balance between endothelial damage and repair. UM that converts to CM could be associated with decreasing cEPC levels. However, data on patients prior to conversion to CM could not be obtained in this study. In some *in vitro* studies, CECs have been reported to inhibit the proliferation and migration of EPCs thus affecting the functional capacity of endothelial repair (Holmén *et al.*, 2005).

CECs are a reliable marker of microvascular injury and in most cases high levels of these cells are associated with endothelial activation and damage (Dignat-George and Sampol, 2000). These cells are usually rare in normal individual and higher in individuals who are acutely ill. CM which is characterized by sequestration and subsequent damage to the endothelium saw high levels of CECs at initial presentation compared to UM and HC. This supports the study hypothesis that CM would have higher CECs due the detachment of endothelial cells resulting from endothelial damage. Studies have found a correlation between CECs and other markers of endothelial activation such as von Willebrand factor and plasma tissue factor (Makin *et al.*, 2004a). Levels of these endothelial activation markers have been found to be elevated in CM (de Mast *et al.*, 2007; Mohanty *et al.*, 1997).

Longitudinal quantitation of CECs in other conditions showed that their levels vary according to the clinical evolution with patients in clinical remission or in recovery phases having reduced levels. It is therefore expected that levels of CEC would decrease as patients recover. However, data from this study showed CM patients having increasing levels of CEC even at fourteen days post recovery form coma, contrary to what was expected.

In CM, the endothelium is known to be activated as a result of increased levels of activation markers such as vWF and subsequent endothelial injury or damage. These injured cells are detached from the endothelium, thereby increasing the levels of CECs in peripheral blood as was observed at initial presentation of CM in this study. Not all injured cells are detached immediately and may need to be replaced gradually during clinical remission. This may increase CEC levels in peripheral blood during the time course of recovery from CM as seen in Figure 4.9. The gradual replacement of damaged endothelial cells is further explained by the increased levels of bone marrow derived cEPC in the time course of CM (known to repair and replace damaged ECs in CM time course) [Figure 4.7].

CEC levels in UM patients saw a sharp increase seven days after initial malaria diagnosis (Figure 4.9) suggesting a possible levels of endothelial injury at the onset of *Plasmodium* infection. However, due to high levels of cEPC in UM at baseline (Figure 4.6) there was the ability to balance damage and repair effectively. The presence of the parasite could possibly cause endothelial activation and the endothelial cells affected may have to be replaced with time. The high cEPC in UM permits effective displacement of possible injured cells into circulation resulting in increased CECs levels in UM on day seven after initial presentation. Observation of CEC levels in UM in

this study is consistent with other studies that have shown endothelial disturbance in malaria infection (Boubou, 2000; de Mast *et al.*, 2007; Ohnishi, 1999; Park *et al.*, 2012).

However, microvascular damage has been shown to induce the expression of chemokine/proteases such as SDF-1 and MMP9 (Huang *et al.*, 2009; Tilling *et al.*, 2009). These molecules are involved in the mobilization, proliferation and migration of EPCs to the sites of endothelial damage. Their levels are expected to be higher in conditions associated with endothelial damage such as CM. To mobilize cEPCs for endothelial repair, CM patients were expected to have high levels of SDF-1 and MMP9. However, data from the study could not significantly show any differences in the serum SDF-1 levels at baseline and time course in the three study groups. UM and HC had higher levels (though not significant) than CM contrary to what was observed in earlier studies by Gyan *et al*. (2009).

MMP9 on the other hand was significantly higher in UM compared to CM and HC. Time course data showed a reduction in the MMP9 levels in UM at day seven post initial presentation whiles CM showed no significant difference. Data suggest the attempt by UM patients to mobilize and migrate EPCs at initial presentation. This attempt to mobilize and migrate EPC seems to be lacking or delayed in CM. Levels of these markers represents that of peripheral or venous blood and may be different from levels close to site of damage. CM could have higher levels of MMP 9 and SDF-1 at or close to the site of endothelial damage as EPCs are needed at these sites. However, more studies need to be done to assess the dynamics of these markers and their function in microvascular damage and repair.

Dysregulation of Angiopoietins has been shown in CM (Conroy *et al.*, 2009). In a stable state, the survival and activation of ECs is regulated by Angiopoietin 1 through the Tie-2 receptor whereas Ang-2 opposes this process (Conroy *et al.*, 2012). Ang-2 antagonizes Ang-1 by sensitizing the endothelium to inflammation and increasing the expression of receptors that bind to iRBCs (Shikani *et al.*, 2012). Higher levels of Ang-2 and lower levels of Ang-1 have been observed to correlate with severe malarial infections (Conroy *et al.*, 2009; Lovegrove *et al.*, 2009; Yeo *et al.*, 2008). This study showed higher plasma levels of Ang-2 at initial presentation of cerebral (5260pg/ml) compared to uncomplicated malaria (4041pg/ml) and healthy children (4909pg/ml) even though these were not statistically significantly different (p=0.12, Kruskal Wallis test). Time course level of Ang-2 in CM showed an increase during the coma state and was unstable at subsequent time points. UM on the other hand showed a gradual increase in the time course.

Ang-1 levels were observed to be higher in CM (17021pg/ml) than in UM (9248pg/ml) contrary to earlier studies by Conroy *et al.* (2009) and Lovegrove *et al.* (2009). Levels of Ang-1 seem to be time dependent as there was a sharp drop (8967pg/ml) in the levels at recovery. Therefore analyzing Ang-1 levels at early stage of coma and at late stage of coma in cerebral malaria could show significant differences. This could possibly explain the differences between the Ang1 levels in this study and that in studies by Conroy *et al*. (2009) and Lovegrove *et al*. (2009). Studies relating to brain injury have also reported decreased abundance of Ang-1 and an up-regulation of Ang-2 in the early phase post brain injury (Chittiboina *et al.*, 2013). An increase in Ang-1 levels and a corresponding decrease in Ang-2 seven days post recovery correlated with complete recovery form coma (BCS =5) in the CM patients. The time course levels in Ang-1 in CM also

confirm the antagonistic effects of Ang-1 and Ang-2 as indicated by earlier studies (Conroy *et al.*, 2009; Lukasz *et al.*, 2008; Parikh, 2013).

The ratio of Ang-2 and Ang-1 in this study, did not show any statistical significance ($p>0.05$) at initial clinical evaluation of CM and UM as well as HC. However, a sharp increase (day 0 vs recovery, $p<0.005$, Kruskal Wallis and Dunn's multiple comparism) in the ratio of these two biomarkers in the late stage of coma (i.e. early stage of recovery from coma) could predict some level of endothelial injury in the CM patients. The decrease in the ratio seven days post recovery indicated resolution of the injury. Ratios in UM patients were not significantly different ($p>0.05$) in the time course of disease resolution. Some studies have reported the ability of Ang1:Ang2 ratio to discriminate between severe and uncomplicated malaria (Conroy *et al.*, 2009; Lovegrove *et al.*, 2009). The time course evaluation of these endothelial mediators in CM has demonstrated their importance in the resolution of severe malaria in African children. Lovegrove *et al*. (2009) reported the ability of Ang-1 and Ang-2 to discriminate CM and UM in Thai adults. Data from this study and others have therefore, demonstrated the importance of levels of these two biomarkers in the resolution of severe malaria.

Due to the upregulation of some markers of endothelial damage/dysfunction and brain micro haemorrhages in CM, some studies have suggested a possible clotting disorder (Moxon *et al.*, 2013) which is still being debated. Moxon *et al*. (2013) showed that decreased expression of the anticoagulant and protective receptors TM and EPCR in the brain endothelium make it particularly vulnerable to injury. Cell-surface expression of TM is also known to be reduced when the endothelium is activated, leading to shedding of the molecule and subsequent increase in soluble

forms (Faust *et al.*, 2001). Soluble TM has therefore been proposed as both a diagnostic and prognostic marker of endothelial activation/dysfunction (Page and Liles, 2013). In the current study, soluble TM levels were significantly higher ($p<0.05$) in CM patients compared to UM and HC. Activation of the endothelium has already been established in CM and higher levels of sTM in CM observed may be due to shedding of this anticoagulant which is present in large quantities on the surface of the endothelium in microcirculation. This is consistent with other markers of activation such as CECs and Angiopoietin 2 as discussed earlier and other pro-inflammatory cytokines and anaemia (Maya *et al.*, 2008). Other studies have shown that higher sTM correlated with disease severity (Butthep *et al.*, 2006; Ikegami *et al.*, 1998; Kinasewitz *et al.*, 2004). The current study is consistent with observation in other studies which have reported higher levels of sTM in children with severe than uncomplicated malaria and uninfected healthy controls (Mita-Mendoza *et al.*, 2013; Ohnishi, 1999; Page and Liles, 2013).

Like thrombomodulin, EPCR is expressed on all vascular endothelial cells and is involved in activating protein C by stabilizing the interaction of protein C with the thrombin–thrombomodulin complex (Moxon *et al.*, 2009). It is expressed in lower level in micro-vessels of the brain (Laszik *et al.*, 1997) but upon activation, they are shed from the endothelium, thereby increasing its plasma levels. It has been reported that shedding of this molecule leads to low expression on the endothelium and increase plasma levels makes the brain vulnerable (Moxon *et al.*, 2013), thus implicating EPCR in the pathology associated with CM. The current study show that the means levels of sEPCR in CM (20.14pg/ml) was higher (even though not statically significant, $p>0.05$, Kruskal-Wallis test) compared with UM (16.82pg/ml) and HC (15.39pg/ml). Other studies have reported reduced expression and increased plasma levels of these EPCR in bacterial sepsis (Faust

et al., 2001), dengue haemorrhagic fever (Cabello-Gutiérrez *et al.*, 2009) and Crohn's disease (Scaldaferri *et al.*, 2007).

The definition of CM includes the inability of a child or patient to localize a painful stimulus, presence of peripheral asexual *P falciparum* parasitaemia with no other identified causes of encephalopathy. However, in malaria-endemic regions such as Ghana, asymptomatic parasitaemia is common and children in coma who show positive peripheral parasitaemia are often initially considered as having CM. On the other hand, some children or patients in coma without peripheral parasitaemia (as a result of parasite sequestration) may not be considered as having CM. These false positives and false negatives often results in misdiagnosis and subsequent implications for patient care. Retinopathy which consist of two unique features: patchy retinal whitening and focal changes of vessel colour has been shown to be highly specific for encephalopathy of malarial etiology (Beare *et al.*, 2011). However, detection of retinopathy requires highly trained personnel and expensive equipment (Seydel *et al.*, 2012). A more user-friendly biomarker, HRP2, has been shown to predict iRBC sequestration and a quantitative measure of plasma levels of this *P. falciparum* specific protein have been evaluated and used to discriminate CM from other forms of malaria and also CM and non-malaria comatose conditions (Kariuki *et al.*, 2014; Seydel *et al.*, 2012).

The current study also evaluated the plasma levels of HRP2 in CM, UM and HC and showed that CM patients had the highest levels of HRP2 (2281ng/ml [95% CI 1453-3109]) compared with UM (353ng/ml [95% CI 162-543]). Healthy controls did not have any detectable HRP2. This result is consistent with other studies that detected higher HRP2 levels in CM compared with UM (Kariuki

et al., 2014; Seydel *et al.*, 2012). Seydel *et al*., 2012, upon determining the plasma levels of HRP2 in Malawian children who had histological evidence of sequestration (through autopsy), proposed a cutoff of >1700ng/ml as sensitive and specific for CM. Even though 42% of CM cases recruited for this current study had HRP2 levels above the cutoff proposed by Seydel *et al*., 2012, the study could not confirm this cutoff in Ghanaian children as autopsies were not conducted on cases that died. The level of endemicity may affect the cutoff levels of HRP2 and therefore estimation of cutoff values in different endemic areas may be very critical.

5.2 Conclusion

Combined measurements of the endothelial indicators evaluated in this study offers a non-invasive and user friendly approach to assess endothelial integrity in CM patients and also offers biomarker strategies that could predict who is at risk of developing CM. These indicators also show the potential to monitor response to treatment by assessing the balance between damage and repair as an index of the endothelial integrity.

This study has shown that low cEPC, high CEC, high sTM, and high HRP2 levels were associated with cerebral malaria. An increase in cEPC levels has been shown to be very critical in the resolution of coma in CM patients. CECs and cEPCs levels could predict degree of endothelial activation and/or damage in CM. Lower levels of MMP9 in CM indicate reduced mobilization and proliferation of EPC in CM.

This study also showed Angiopoietins as promising endothelial mediators and good biomarkers predicting endothelial damage and repair. Soluble TM levels were elevated in CM as a result of

shedding from the endothelium and therefore have been shown as good prognostic and diagnostic markers in predicting endothelial damage in CM. Soluble EPCR has also shown promise as biomarker in predicting CM and therefore its role in CM pathogenesis cannot be overlooked.

This study confirms HRP2 as a biomarker capable of differentiating CM from UM and other non-malaria comatose conditions. The utility of HRP2 as an alternative to retinopathy and a more user friendly marker in confirming CM is highly supported by this study.

C-C BCT has shown the ability to preserve rare cells such as EPC, CECs and other immune markers. Extension of this technique is therefore possible for the preservation of biological samples from remote settings where flow cytometers may not be readily available.

5.3 Recommendations

Further studies that would assess the migratory capacity and the functionality of EPCs and other markers involved in the repair of damaged endothelium and techniques that could assess levels of markers at the site damage (especially, the brain vasculature in CM) are highly recommended.

Data from this studies show peripheral or systemic levels of this markers. Further studies targeting levels of these markers and several others at sites of endothelial damage would be of great benefit to the understanding of the pathogenesis of cerebral malaria.

Therapies that would increase levels of EPCs and other markers which have shown promise in the resolution of CM could be considered. Consideration of EPCs in malaria vaccines cannot also be overemphasized.

HRP2 levels may differ according to the prevalence of parasitaemia in a population and with the associated degree of immunity in that population. Therefore, HRP2 threshold that differentiate CM from non-malaria comatose conditions in various malaria endemic areas would be very useful.

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APPENDICES

Appendix 1: Buffers and Reagents

A. Buffers for ELISA (Buffer preparation was the same for all the biomarkers)

I. Coating buffer — 90g of NaCl plus 10.9g of Na_2HPO_4 (dibasic) plus 3.2g of NaH_2PO_4 (monobasic) in 1000ml of double distilled water.

II. Washing buffer — 0.05% Tween 20 in PBS.

III. Blocking buffer — 1% BSA plus 0.05% Tween 20 in PBS.

IV. Reagent diluent — 1% BSA in PBS.

B. Giemsa Buffer for parasite staining

Na_2HPO_4	1.0g
KH_2PO_4	0.7g
Distilled water	1 litre

(Adjusted pH, 7.25; Temp. 30.5^0C)

C. Sickling test Buffer

$Na_2S_2O_5$ — 2% of $Na_2S_2O_5$ in distilled water.

D. Antibody and Fluorochromes

Item	Fluorochrome	**Supplier:**
Mouse Anti-Human CD15	FITC	BD Pharmingen
Mouse Anti-Human CD 14	PE	BD Pharmingen
Human CD 45 Antibodies	FITC	Becton Dickinson
Human CD133/2 (AC141) Antibodies	PE	Miltenyi Biotec (MACS)
Human CD 34 Antibodies	PerCP	Becton Dickinson
Human CD 31 Antibodies	APC	Miltenyi Biotec (MACS)
Human CD 11b Antibodies	FITC	Miltenyi Biotec (MACS)
Anti-Human CD 309 (VEGF R2/KDR)	APC	Miltenyi Biotec (MACS)
ChromPure mouse IgG1 Whole molecule Size 5.0mg		Jackson ImmnoResearch Labs

Appendix 2A: Consent for Children with Malaria

Title: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben Gyan, PhD

Address: Department of Immunology, NMIMR, Box LG 581, Legon

Information: (To be read or translated to parents/guardians in their own mother tongue)

Dear Volunteer,

This consent form contains information about the research entitled *Circulating endothelial cells and the pathogenesis of malaria.* In order to be sure that you are informed about being in this research, we are asking you to read (or have read to you) this Consent Form. You will also be asked to sign it (or make your mark in front of a witness). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand.

Why this study is planned

Your child is being asked to participate in the above study in order to find out factors in the blood that may be of risk to severe malaria. Malaria is caused by a germ that is passed from one person to the other by the bite of a mosquito that carries the malaria germ. Malaria is a very serious health problem in Ghana, as it is in many African countries. We do not know why some children become severely ill from malaria or why some of those children die from malaria. To understand this problem we need to study children who come to the hospital with severe malaria and compare them to children who have less severe malaria, and to other children who are feeling well. The purpose of the study is to find out what factors they already have in their blood that may make

them severely sick when they have malaria. If we can find the answer to this question, we hope to be able to suggest new ways of controlling such severe sicknesses in malaria.

General Information and your part in the study

For a child to qualify to be part of this study that child should be between the ages of 1 and 12 years. If your child/ward agrees to be in the study, we will collect venous blood sample for laboratory diagnosis and 2 ml (teaspoonful) for our research at the time of admission, 7 days and 14 days after recovery. If you miss a scheduled follow-up visits (7 days and 14 days after recovery), we may contact you at home by phone, or in person to schedule another visit and to see if you still want to take part in the research. When this contact is made you will not be identified as being in this research.

Possible Benefits

There are no direct benefits to your child from this study. However, his/her participation may help us develop better malaria treatment. He/she will not be paid for participation in this study but you will be reimbursed with an amount of fifteen Ghana cedis for your time and travel during the follow up visits.

Possible Risks

The amount of blood collected is harmless, although there may be a slight pain and bruising at the bleeding site. All subjects will receive appropriate treatment as necessary. Sterile techniques and disposable, single-use equipment will be used at all times.

Withdrawal from study

We would like to stress that this study is strictly voluntary. Should the child decide not to participate; it will have no consequences for him/her. Should the volunteer, at any point during the study, decide that he/she do not wish to participate any further, you are free to terminate the participation, effective immediately. Any such decision will be respected without any further discussion. Your decision will not affect the health care you would normally receive.

Visits

If the child misses a scheduled visit, we may contact you at home by phone, or in person to schedule another visit and to see if you still want to take part in the research. When this contact is made you will not be identified as being in this research.

Confidentiality

All information gathered would be treated in strict confidentiality. We will protect information about your child taking part in this research to the best of our ability. The child will not be named in any reports. However, the staff of [list all groups that may access the research records] may sometimes look at his/her research records. If you have any questions, please feel free to ask the physician in charge. Someone from the IRB or Ethical Committee might want to ask you questions about being in the research, but you do not have to answer them. A court of law could order medical records shown to other people, but that is unlikely.

Contacts: If you ever have any questions about the research study or study-related problems, you may contact Prof. Ben Gyan of the Noguchi Memorial Institute for Medical Research (0244

726016) at any time. For questions about the ethical aspects of this study or your rights as a volunteer, you may contact Dr. Chris Dadzie, Chairman, Institutional Review Board, NMIMR, University of Ghana (0302 501178/9) or Chairman of the Ghana Health Service Ethical Committee (Tel. 0302 681109)

Your rights as a participant

This research has been reviewed and approved by the NMIMR IRB and Ghana Health Service Ethical Committee. An IRB or Ethical Committee is a committee that reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant you may contact [Dr. Chris Dadzie, Tel 0302-501-178/179 or Chairman of the Ghana Health Service Ethical Committee (Tel. 0302 681109)

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title *Circulating endothelial cells and the pathogenesis of mala*Figure 1.1 Model of the development and resolution of cerebral malaria.....27

ria has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree my child/ward to participate as a volunteer.

---------- ----------

Date Signature or thumbprint of volunteer

If volunteer's Parent/Guardian cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer's Guardian/Parent has agreed to take part in the research.

---------- ----------

Date Signature or thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

---------- ----------

Date Signature of person who obtained consent

Appendix 2B: Consent for Healthy Controls

Title: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben Gyan, PhD

Address: Department of Immunology, NMIMR, Box LG 581, Legon

Information: (To be read or translated to parents/guardians in their own mother tongue)

Dear Volunteer,

This consent form contains information about the research entitled *Circulating endothelial cells and the pathogenesis of malaria.* In order to be sure that you are informed about being in this research, we are asking you to read (or have read to you) this Consent Form. You will also be asked to sign it (or make your mark in front of a witness). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand.

Why this study is planned

Your child is being asked to participate in the above study in order to find out factors in the blood that may be of risk to severe malaria. Malaria is caused by a germ that is passed from one person to the other by the bite of a mosquito that carries the malaria germ. Malaria is a very serious health problem in Ghana, as it is in many African countries. We do not know why some children become severely ill from malaria or why some of those children die from malaria. To understand this problem we need to study children who come to the hospital with severe malaria and compare them to children who have less severe malaria, and to other children who are feeling well. The purpose of the study is to find out what factors they already have in their blood that may make

them severely sick when they have malaria. If we can find the answer to this question, we hope to be able to suggest new ways of controlling such severe sicknesses in malaria.

General Information and your part in the study

For a child to qualify to be part of this study that child should be between the ages of 1 and 12 years. If your child/ward agrees to be in the study, we will collect venous blood sample for laboratory diagnosis and 2 ml (teaspoonful) for our research initially and 7 days and 14 days later. If you miss a scheduled follow-up visits (7 days and 14 days) in your school, we may contact you at home by phone, or in person to schedule another visit and to see if you still want to take part in the research. When this contact is made you will not be identified as being in this research.

Possible Benefits

There are no direct benefits to your child from this study. However, his/her participation may help us develop better malaria treatment. He/she will not be paid for participation in this study but you will be reimbursed with an amount of fifteen Ghana cedis for your time and travel during the follow up visits.

Possible Risks

The amount of blood collected is harmless, although there may be a slight pain and bruising at the bleeding site. All subjects will receive appropriate treatment as necessary. Sterile techniques and disposable, single-use equipment will be used at all times.

Withdrawal from study

We would like to stress that this study is strictly voluntary. Should the child decide not to participate; it will have no consequences for him/her. Should the volunteer, at any point during the study, decide that he/she do not wish to participate any further, you are free to terminate the participation, effective immediately. Any such decision will be respected without any further discussion. Your decision will not affect the health care you would normally receive.

Visits

If the child misses a scheduled visit, we may contact you at home by phone, or in person to schedule another visit and to see if you still want to take part in the research. When this contact is made you will not be identified as being in this research.

Confidentiality

All information gathered would be treated in strict confidentiality. We will protect information about your child taking part in this research to the best of our ability. The child will not be named in any reports. However, the staff of [list all groups that may access the research records] may sometimes look at his/her research records. If you have any questions, please feel free to ask the physician in charge. Someone from the IRB or Ethical Committee might want to ask you questions about being in the research, but you do not have to answer them. A court of law could order medical records shown to other people, but that is unlikely.

Contacts: If you ever have any questions about the research study or study-related problems, you may contact Prof. Ben Gyan of the Noguchi Memorial Institute for Medical Research (0244

726016) at any time. For questions about the ethical aspects of this study or your rights as a volunteer, you may contact Dr. Chris Dadzie, Chairman, Institutional Review Board, NMIMR, University of Ghana (0302 501178/9) or Chairman of the Ghana Health Service Ethical Committee (Tel. 0302 681109)

Your rights as a participant

This research has been reviewed and approved by the NMIMR IRB and Ghana Health Service Ethical Committee. An IRB or Ethical Committee is a committee that reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant you may contact Dr. Chris Dadzie, Tel 0302-501-178/179 or Chairman of the Ghana Health Service Ethical Committee (Tel. 0302 681109)

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title *Circulating endothelial cells and the pathogenesis of malaria* has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree my child/ward to participate as a volunteer.

---------- ----------

Date Signature or thumbprint of volunteer

If volunteer's Parent/Guardian cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer's Guardian/Parent has agreed to take part in the research.

---------- ----------

Date Signature or thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

---------- ----------

Date Signature of person who obtained consent

Appendix 3A: Assessment Forms/Questionnaires for Cerebral Malaria Cohort

Endothelial progenitor cells and the pathogenesis of malaria

CEREBRAL MALARIA COHORT

INITIAL ASSESSMENT/QUESTIONNAIRE

Initial Clinical Presentation

I.1 Study ID number |__|__|__|__|__|__|

I.2 Date of Admission___/___/___

1.2a Date of recruitment into study……..……__/___/___

I.3 Time of recruitment into study ___:_(AM or PM)

I.4 Name……_____

1.5 Hospital name (circle) PML__Ridge__La General___ Tema General ___LEKMA

I.5a Hospital Folder #..…… |__|__|__|__|__|__|

General Exclusion Criteria

G.1 Pre-existing neurological disease (1=yes, 2 =no) |__|

G.2 Recent severe head trauma (within 3 months)(1 = yes 2 = no) |__|

G.3 Other cause of coma e.g. DM (1 = yes, 2 = no) |__|

G.4 Blantyre Coma Score>3 (1=Yes, 2=No) …… |__|

G.5 Blantyre Coma Score >3 **within 5 min of correction for hypoglycemia**(1=Yes,2=No)|__|

G.6 Duration of coma < 60 mins (1=Yes, 2=No) …… |__|

G.7 Other febrile illness (1=Yes, 2=No) …… |__|

G.8 Recent severe bleeding i.e. within 3 months (1=yes 2=no) |__|

G.9 Other causes of anaemia including SCD and Sickle Cell trait (1= yes, 2=no) |__|

G.10 Obvious clinical evidence of bacterial infection (1=yes, 2=no) |__|

G.11 Obvious clinical evidence of viral infection (1=yes, 2=no) |__|

G.12 History of Diabetes Mellitus (1=yes, 2=no) |__|

G.13 History of cardiovascular disease (1=yes, 2=no) |__|

G.14 History of Hypertension (1=yes, 2=no) |__|

G.15 History of increased cholesterol (1=yes, 2=no)…… |__|

G.16 History of surgery within 1 month? (1=yes, 2=no) |__|

G.17 History of bone fracture within 3 months (1=yes, 2=no) |__|

G.18 History chronic viral infection (chronic weight loss, diarrhea) (1=yes, 2=no) |__|

NOTE –exclude HIV patients

G.19 Major trauma (car accident, etc.) within 1 month (1=yes, 2=no) |__|

G.20 Transfusion within past 3 months (1=Yes, 2=No) |__|

G.21 Severe pallor (1=yes, 2=no)…… |__|

If the answer to any of the above questions is yes (1), do not recruit into study!

G.22 *****CONSENT REQUIRED****** please indicate obtained, 1=Yes 2=No.....|__|

Name of staff who obtained consent._____

G.23 Ethnic origin |__|

(Akan=1, Ga-Adangme=2, Ewe=3, Hausa=4, Frafra=5, Dagomba=6

Non-Ghanaian=7, Other=8 Specify _____)

G.24 Area of residence/Direction to your house

_____

_____ ****Cell phone #_____

G.25 Sex (1=M, 2=F)|__|

G.26 Age (Last half year passed) |__|__|.|__|

G.27 Referral on the basis of a lab report positive for malaria parasites (1=Yes, 2=No) |__|

G.28 History of a febrile illness in the preceeding 2 weeks (1=Yes, 2= No)|__|

G.29 Duration of symptoms before presentation (Days same day = 1) |__|

G.30 History of other antimalarial for this attack (1=yes, 2=no, 9=DK*) |__|

G.31 If yes specify: ……

G.32 Reported cola urine (1=Yes, 2=No 9=DK) |__|

G.33 Observed cola urine (1=Yes, 2=No) |__|

G.34 Reported convulsions (1=Yes, 2=No 9=DK) |__|

G.35 Already seen at this hospital for this attack? (1=Yes, 2=No) |__|

G.36 If yes initial antimalarial prescribed (**……**)

Physical exam, vital signs and laboratory results

P.1 Best Motor Response (0-2) |__|

P.2 Best Verbal Response (0-2) |__|

P.3 Eye Movements (0-1) |__|

P.4 Total Coma Score (0-5) |__|

P.5 Duration of Coma (0=no coma, 1=0-60 mins, 2=60+ mins) |__|

P.6. Observed Convulsions (1=Yes, 2=No)|__|

P.7 Alar flare (1=Yes, 2=No) |__|

P.8. Chest (subcostal, intercostal) Recession (1=Yes, 2=No) |__|

P.9 Abnormally deep breathing (1=Yes, 2=No) |__|

P.10 Use of Accessory muscles (supraclavicular/suprasternal recessions) (1=Yes, 2=No)|__|

P.11 Fast breathing (1-4yr>40/min, >5yr>30/min) (1=yes, 2=no) |__|

P.12 Respiratory Distress (1=Yes, 2=No)…… |__|

P.13 *Peripheral O_2 saturation (**for all patients with resp. distress**) |__|__|

P.14 Temperature. |__|__|.|__|

P.15 Weight (in kgs) |__|__|.|__|

P.16 Height (in cms) |__|__|__|

P.17 Blood Pressure (mmHg) |__|__|__|/|__|__|

P.18 Pulse |__|__|__|

P.19 State of hydration (1=normal, 2=impaired, i.e., ↓ skin turgor or dry mouth) |__|

P.20 Recruited into study (1=Yes, 2=No) |__|

Name:_____ Study id no. |__|__|__|__|__|__|

Samples (Please tick when taken):

S.1 ___ EDTApurple top (EPC sample)
S.2 ___ EDTA purple top (FBC-full blood count)
S.3 ____ Heparin tube(s)
S.4 ____ Blood Culture
S.5 _____PAX-gene tube (RNA)
S.6 staff=nurse/MD filling out data

S.5 ___CXR (for respiratory distress)
S.6 ___CSF (for CM)

Results initial studies at recruitment:

R.1aDate sample obtained (____/____/____)

R.1 RBS: Glucometer (mmol/L)..…… |__||__|.__|

R.2 Haemoglobin (Hb). |__|__|.|__|

R.3 WBC ($X10^9/\mu L$)…… |__|__|.|__|

RBC ____($X10^6/\mu L$) Hemoglobin (Hb)_____(g/dL) HCT____ (%) MCV_____(fL)
MCH_____(pg) MCHC____(g/dL) PLT_____ ($X10^3/\mu L$)

R.4 Blood film species (1=p.f.,2=p.m.,3=p.o., 4=p.v.,5=p.f.+p.m.,6=p.f.+p.o.)|__|

R.5 Parasite density (per µL)_____

R.6 Relative abundance (negative smear, 1+, 2+, 3+, 4+)……|__|

***Inform team if smear is negative and that repeat must be verified as positive within 48 hours for continued inclusion in the study.**

R.7 Asexual stage, density per µl |__|__|__|__|__|__|__|

R.8 Sickling status (1=positive, 2=negative) |__|

NOTE- If positive then exclude the from study

R.9 Blood culture results (1=positive, 2=negative |__|

R.10 If positive organism cultured_____

NOTE- If positive then exclude from the study

Name:_____ Study id no. |__|__|__|__|__|__|

Endothelial progenitor cells and the pathogenesis of malaria

INPATIENT CM MONITORING CHART

See footnote(s)/codes end of form		**Day0 *date_____**	**Day 1 *date_____**	**Day 2 *date_____**	**Day 3 *date_____**
M.1 Asexual parasite count (per µL) AM/date (If initial smear negative)**Ψ**					
2 AM	M.27 Coma score†				
	M.28 Temp.ºC				
	M.29 BP				
	M.30 Pulse/Resp Rate	P___ /RR___	P___ /RR___	P___ /RR___	P___ /RR___
	M.31 Staff name				
6 AM	M.2 Coma score†				
	M.3 Temp. ºC				
	M.4 BP				
	M.5 Pulse/Resp Rate	P___ /RR___	P___ /RR___	P___ /RR___	P___ /RR___
	M.6 Staff name				
10 AM	M.7 Coma score†				
	M.8 Temp. ºC				
	M.9 BP				
	M.10 Pulse/Resp Rate	P___ /RR___	P___ /RR___	P___ /RR___	P___ /RR___
	M.11 Staff name				
2 PM	M.12 Coma score†				
	M.13 Temp.ºC				
	M.14 BP				
	M.15 Pulse/Resp Rate	P___ /RR___	P___ /RR___	P___ /RR___	P___ /RR___
	M.16 Staff name				
6 PM	M.17 Coma score†				
	M.18 Temp. ºC				
	M.19 BP				

	M.20 Pulse/Resp Rate	P___ /RR___	P___ /RR___	P___ /RR___	P___ /RR___
	M.21 Staff name				
10 PM	M.22 Coma score†				
	M.23 Temp ^{o}C				
	M.24 BP				
	M.25 Pulse/Resp Rate	P___ /RR___	P___ /RR___	P___ /RR___	P___ /RR___
	M.26 Staff name				
M.32Clinical developments 1=yes 2=no § use codes below, indicate time, name of staff making the entry, with additional information on notes page.		1=Y, 2=N____ code_____ time_____ staff_____	1=Y, 2=N____ code_____ time_____ staff_____	1=Y, 2=N__ code_____ time_____ staff_____	1=Y, 2=N__ code_____ time_____ staff_____
M.33 If change in status (recovery/worsening) indicate if blood samples taken (1=Y, 2=N) and check off which: FBC/EPC= purple top tubes Heparin tube(s)		1=Y, 2=N____ FBC/smear__ EPC_____ Heparin_____ Time samples obtained____	1=Y, 2=N_____ FBC/smear____ EPC_____ Heparin_____ Time samples obtained_____	1=Y, 2=N__ FBC/smear_ EPC_____ Heparin___ Time samples obtained____	1=Y, 2=N__ FBC/smear_ EPC_____ Heparin____ Time samples obtained____

Ψ If initial blood smear and follow up smears are *negative* at day 2 (48hours) i.e. three consecutively negative smears then exclude patient from the study (assume patient does not have cerebral malaria – see protocol for meeting exclusion criteria (notify Study team, primary MD, etc.) †**Blantyre score of 4 or 5** Blood samples to be obtained (EPC/FBC i.e. 2 purple top tubes AND heparin tube when patient recovers OR deteriorates

Name:_____ Study id no. |__|__|__|__|__|__|

Endothelial progenitor cells and the pathogenesis of malaria

CEREBRAL MALARIA COHORT

HOSPITAL (INPATIENT) COURSE SUMMARY

CS.1a **Date of discharge*_____CS.1b MD/RN completing summary_____**

Clinical Course (Refer to page prior pages for clinical notes/information)

CS.1 Highest temperature first 24 hours |__|__|.|__|

CS.2 Time for fever to settle (hrs) |___|___|___|

(from admission till first time temp. falls to <37.5°C for at least 48 hours)

CS.3 Time for coma score to reach 5 (hrs) |___|___|___|

CS.4 Antimalarial changed before discharge? (1=Yes, 2=No)……. |__|

CS.5 If yes; what was it changed to

CS.6 Day of change (Day 0 = Admission day) |__|

CS.7 Any other drugs (1=Yes, 2=No) |__|

CS.8 If yes, specify _____

CS.9 Blood C/S (1=positive‡, 2=negative, 3=not investigated, 9=missing) . |__|

CS.10 If positive organism cultured _____

CS.11 Antibiotics prescribed (1=yes, 2=no)……|__|

CS.12 Name of antibiotic given_____

CS.13 CSF C/S (1=positive‡, 2=negative, 3=not investigated, 9=missing) . |__|

CS.14 If positive organism cultured=_____

CS.15 Antibiotics prescribed (1=yes, 2=no)……..|__|

CS.16 Antibiotic given 1=ceftriaxone 2=other, give name_____|__|

CS.17 HB ≤ 5 g/dL during admission (1=Yes, 2=No) |__|

CS.18 Blood transfusion (1=Yes, 2=No) |__|

CS.19 If yes day of transfusion (day 0=day of admission) |__|

CS.20 Coca cola urine (1=Yes, 2=No) |__|

CS.21 Respiratory distress (1=Yes, 2=No) |__|

CS.22 Total number of convulsions |__|

CS.23 Duration of longest convulsion (minutes) |__|

CS.24 Died (1=Yes, 2=No) |__|

CS.25 Interval between admission and death (hours) (10=NA) |__|__|__|

CS.26 Postmortem done (1=Yes, 2=No) (10=NA) |__|

CS.27 Additional / Differential Diagnoses _____

Detailed information regarding address/contact_____

CS.28 Patient to continue in the study (1=Yes, 2=No)..……|__|

CS.29 If excluded give reason _____

CS.29 Scheduled days/times for return:

CS.30 7 days post discharge=_____14 days post discharge=_____

CS.31 Name MD who discharge patient/completed form_____

Name:_____ Study id no. |__|__|__|__|__|__|

CEREBRAL MALARIA COHORT
OUTPATIENT ASSESSMENT/QUESTIONAIRE

****NOTE: EPC, FBC, parasite smear (2 purple tops) and 2 heparin tubes required for each follow up visit**	7 DAYS POST RECOVERY Tick if visit is late ☐ QA1aDate_____ QA1b Staff_____	**14 DAYS POST RECOVERY** Tick if visit is late☐ QA1a Date_____ QA1b Staff_____
HISTORY SINCE DISCHARGE OR LAST ASSESSMENT		
QA.1 Illness/change since discharge or last assessment (1=Yes, 2=No).		
QA.2 If yes; §use codes at end of form/describe if not listed, provide additional information on notes page or "10" if not applicable		
QA.3 Fever (1=Yes, 2=No)		
QA.4 Medical care/Hospitalization (1=Yes, 2=No)		
QA.5 If yes, was the medical care or hospitalization malaria related 1=Yes, 2=No		
QA.6 Convulsion or impaired consciousness (1=Yes, 2=No)		
QA.7 Severe bleeding (1=Yes, 2=No)		
QA.8 Trauma (1=Yes, 2=No) QA.9 If yes, indicate type (eg car accident)_____		
QA.10 Surgery (1=Yes, 2=No) QA.11 If yes, indicate type_____		
QA.12 New antibiotics taken (1=Yes, 2=No) QA.13 If yes specifiy;_____ ___		
QA.14 New antimalarial taken (1=Yes, 2=No)		
CLINICAL ASSESSMENT		
Q15. Temperature ^{o}C		
Q16. BP (mm Hg)		
Q17. Pulse		

Q19. Weight (in kgs)		
Q19aHeight (in cms)		
Q20. Neurologic sequelae at last assessment (1=Yes, 2=No)		
Q21. Resolution of neurologic sequelae since last assessment (1=Yes, 2=No, 10=not applicable)		
Q22. If yes indicate deficit type*, if other specify;_____		
Q23. If no, indicate if deficit has improved (1=Yes, 2=No, 3=not applicable) Q24. Describe improvement_____		
Q25. Blood sample obtained (1=Y, 2=N). Check off which: FBC and EPC= purple top tubes (1 each)	1=Y, 2=N____ FBC/smear__ EPC_____ Heparin___	1=Y, 2=N____ FBC/smear__ EPC_____ Heparin___
Q26. Parasite density (per microlitres) Asexual stage, density per µl		
Q27. WBC $X10^9$/L		
Q28. HB (g/dL)		
Q29. Platelet count		

Appendix 3B: Assessment Forms/Questionnaires for Uncomplicated Malaria Cohort

Endothelial progenitor cells and the pathogenesis of malaria

UNCOMPLICATED MALARIA COHORT

INITIAL ASSESSMENT/QUESTIONNAIRE

Initial Clinical Presentation

IU.1 Study ID number.....|__|__|__|__|__|__|__|
IU.2 Date of assessment..... ___/___/___
IU.3 Time of assessment ..... ___:___hrs AM/PM
IU.4 Name.....
IU.5 Hospital name/ (circle)PML____Ridge___La General__TemaGeneral___LEKMA___
IU.6 Hospital Folder #.....|__|__|__|__|__|__|__|

General Exclusion Criteria

GU.1 Blantyre Coma Score<5 (1=Yes, 2=No) ..... |__|
GU.2 Blood film negative for *P. falciparum* asexual forms (1=Yes, 2=No) ..... |__|
GU.3 Recent severe bleeding i.e. within 3 months (1=yes 2=no) .....|__|
GU.4 Other causes of anaemia including SCD and Sickle Cell trait (1= yes, 2=no) ... |__|
GU.5 Obvious clinical evidence of bacterial infection (1=yes, 2=no) .....|__|
GU.6 Obvious clinical evidence of viral infection (1=yes, 2=no) .....|__|
GU.7 History of Diabetes Mellitus (1=yes, 2=no).....|__|
GU.8 History of cardiovascular disease (1=yes, 2=no).....|__|
GU.9 History of Hypertension (1=yes, 2=no) .....|__|
GU.10 History of increased cholesterol (1=yes, 2=no)..... .....|__|
GU.11 History of surgery within 1 month? (1=yes, 2=no).....|__|
GU.12 History of bone fracture within 3 months (1=yes, 2=no).....|__|
GU.13 History chronic viral infection (chronic weight loss, diarrhea) (1=yes, 2=no)....|__|
NOTE –exclude HIV patients
GU.14 Major trauma (car accident, etc.) within 1 month (1=yes 2=no) .....|__|
GU.15 Transfusion within past 3 months (1=Yes, 2=No).....|__|
GU.16 Moderate or Severe pallor (1=yes, 2=no).....|__|
GU.17 Reported Convulsions (1=yes, 2=no).....|__|
GU.18 Reported Cola urine (1=yes, 2=no).....|__|
GU.19 Respiratory distress (1=yes, 2=no).....|__|
GU.20 Requires hospitalization (1=yes, 2=no) .....|__|

If the answer to any of the above questions is yes, do not recruit into study!

GU.21 *****CONSENT REQUIRED****** please indicate obtained, 1=Yes 2=No ..|__|
Name of staff who obtained consent._____
GU.22 Ethnic origin.....|__|

(Akan=1, Ga-Adangme=2, Ewe=3, Hausa=4, Frafra=5, Dagomba=6
Non-Ghanaian=7, Other=8 Specify _____)

GU.23 Area of residence/Direction to house (eg Any popular Spot or street etc) _____ Cell phone #_____

GU.24 Sex (1=M, 2=F).....|__|

GU.25 Age (Last half year passed).....|__|__|.|__|

GU.26 Referral on the basis of a lab report positive for malaria parasites (1=Yes, 2=No)|__|

GU.27 History of a febrile illness in the preceding 2 weeks (1=Yes, 2= No).....|__|

GU.28 Duration of symptoms before presentation (Days, same day = 1).....|__|

GU.29 History of other antimalarial for this attack (1=yes, 2=no, 9=DK) .....|__|

GU.30 If yes specify:

GU.31 Observed cola urine (1=Yes, 2=No) .....|__|

NOTE- If cola urine=Yes (1) do not recruit into the study!

Physical Exam, Vital Signs and Laboratory Results

PU.1 Temperature (At time of blood collection).....|__|__|.|__|

PU.2 Weight (in kgs) .....|__|__|.|__|

PU.3 Height (in cms) ..... |__|__|__|

PU.4 Blood Pressure (mmHg) .....|__|__|__|/|__|__|

PU.5 Pulse..... |__|__|__|

PU.6 Best Motor Response (0-2).....|__|

PU.7 Best Verbal Response (0-2).....|__|

PU.8 Eye Movements (0-1) .....|__|

PU.9 Total Coma Score (0-5) .....|__|

NOTE – if < 5 notify study principles for possible inclusion in CM study group-
Do not enroll in UM if<5

PU.10 Alar flare (1=Yes, 2=No).....|__|

PU.11 Chest (subcostal, intercostal) Recession (1=Yes, 2=No).....|__|

PU.12 Abnormally deep breathing (1=Yes, 2=No) .....|__|

PU.13 Use of Accessory muscles (supraclavicular/suprasternal recessions) (1=Yes, 2=No) .....|__|

PU.14 Fast breathing (1-4yr>40/min, >5yr>30/min) (1=yes, 2=no).....

PU.15 Respiratory Distress (1=Yes, 2=No).....|__|

PU.16 State of hydration (1=normal, 2=impaired, ie↓ skin turgor or dry mouth) .....|__|

PU.17 Spleen size (cm below costal margin). .....|__|__|

PU.18 Liver size (cm below costal margin)….....|__|__|

PU.19 Antimalarial (you) prescribed (**i.e., received by patient today**)…… |__|

Samples (Please tick when taken):

SU.1 ___ EDTA purple top(EPC) _____ SU.5 _____PAX-gene tube (RNA)

SU.2 ___ EDTA purple top (FBC/CBC) SU.3 ___ Blood C/S SU.4 __ Heparin tube(s)

Results day of recruitment :

RU.1 Haemoglobin (Hb).....|__|__|.|__|

RU.2 WBC ($X10^9$/L).....|__|__|.|__|

RBC ____($X10^6$/µL) Hemoglobin (Hb)____(g/dL) HCT____ (%) MCV____(fL)

MCH_____(pg) MCHC____(g/dL) PLT_____ ($X10^3$/µL)

RU.13 Platelet count.....

RU.14 Blood Film species (1=p.f.,2=p.m.,3=p.o., 4=p.v.,5=p.f.+p.m.,6=p.f.+p.o.) .|__|

RU.15 Parasite Density (per microlitres) (NOTE- if <2500/µl then exclude from the study)

RU.16 Asexual stage, density per µl .....|__|__|__|__|__|__|__|

RU.17 Sickling status (1=positive, 2=negative) .....|__|

(NOTE- If positive then exclude from the study)

RU.18 Blood culture results (1=positive, 2=negative) .....|__|

RU.19 If positive organism cultured_____

NOTE- If positive then exclude from the study

Name:_____ Study id no. |__|__|__|__|__|__|

Endothelial progenitor cells and the pathogenesis of malaria

UNCOMPLICATED MALARIA COHORT

FOLLOW-UP ASSESSMENT/QUESTIONNAIRE

****NOTE: FBC, parasite smear & EPC (2 purple tops) and heparin tube(s)required for each follow up visit**	**7 DAYS POST RECRUITMENT**	**14 DAYS POST RECRUITMENT**
HISTORY SINCE DISCHARGE OR LAST ASSESSMENT	Tickif visit is late☐ OU.1aDate_____ OU.1b Staff_____	Tick if visit is late☐ OU.1a Date_____ OU.1b Staff_____
OU.1 Illness/change since discharge or last assessment (1=Yes, 2=No).		
OU.2 If yes; §use codes at end of form/describe if not listed, provide additional information on notes page		
OU.3 Fever (1=Yes, 2=No)		
OU.4 Medical care/Hospitalization (1=Yes, 2=No)		
OU.5 If yes, was the medical care or hospitalization malaria related 1=Yes, 2=No)		
OU.6 Convulsion or impaired consciousness (1=Yes, 2=No)		
OU.7 Severe bleeding (1=Yes, 2=No)		
OU.8 Trauma (1=Yes, 2=No) OU.9 If yes, indicate type (eg car accident)_____		
OU.10 Surgery (1=Yes, 2=No) OU.11 If yes, indicate type_____		
OU.12 New antibiotics taken (1=Yes, 2=No) OU.13 If yes specifiy;_____		
OU.14 New antimalarial taken (1=Yes, 2=No)		
OU.15 If yes, specify_____		
CLINICAL ASSESSMENT		
OU.16 Temperature ^{o}C		

OU.17 BP (mm Hg)		
OU.18. Pulse		
OU.9. Weight (in kgs)		
OU.20 Neurologic sequelae at last assessment (1=Yes, 2=No)		
OU 21 Resolution of neurologic sequelae since last assessment (1=Yes, 2=No, 10=not applicable)		
OU.22 If yes indicate deficit type*……..		
OU.23 If no, indicate if deficit has improved (1=Yes, 2=No, 3=not applicable) OU.24 Describe improvement_____		
OU.25. Blood sample obtained (1=Y, 2=N). Check off which: FBC and EPC= purple top tubes (1 each) Heparin=red top tube	1=Y, 2=N_____ FBC/smear_____ EPC_____ Heparin_____	1=Y, 2=N_____ FBC/smear_____ EPC_____ Heparin_____
OU.26 Parasite density (per microlitres) Asexual stage, density per µl		
OU.27 WBC $X10^9/L$		
OU.28 HB (g/dL)		
OU.29 Platelet count		

§Indicate all changes in clinical status

Appendix 3C: Assessment Form/Questionnaire for Healthy Volunteer Cohort

Endothelial progenitor cells and the pathogenesis of malaria

HEALTHY CONTROLS

INITIAL STUDY QUESTIONNAIRE

IHC.1 Location of Recruitment_____ OR

IHC.2 Hospital Folder #.....|__|__|__|__|__|__|__|

IHC.3 Date of recruitment……___/___/____

IHC.4 Time of recruitment……_____:_____AM/PM

General Exclusion Criteria

GC.1 Recent severe bleeding e.g. within 3 months (1=yes, 2=no) .....|__|

GC.2 Other causes of anaemia including SCD and Sickle Cell trait (1= yes, 2=no) ..... |__|

GC.3 Obvious clinical evidence of bacterial infection (1=yes, 2=no) .....|__|

GC.4 Obvious clinical evidence of viral infection (1=yes, 2=no) .....|__|

GC.5 History of diabetes mellitus (1=yes, 2=no) .....|__|

GC.6 History of cardiovascular disease (1=yes, 2=no) .....|__|

GC.7 History of hypertension (1=yes, 2=no) .....|__|

GC.8 History of increased cholesterol (1=yes, 2=no)…… ..... |__|

GC.9 History of surgery within 1 month? (1=yes, 2=no).....|__|

GC.10 History of fever within last 2 weeks (1=yes, 2=no) .....|__|

GC.11 History of treatment for malaria within the last two weeks(1=yes, 2=no).....|__|

GC.12 History of cola urine within the last 2 weeks (1=yes, 2=no).....|__|

GC.13 History of history convulsions within the last 2 weeks (1=yes, 2=no) .....|__|

GC.14 History of bone fracture within 3 months (1=yes, 2=no).....|__|

GC.15 History chronic viral infection (chronic weight loss, diarrhea) (1=yes, 2=no) ....|__|

NOTE – exclude HIV patients

GC.16 Major trauma (car accident, etc.) within 1 month (1=yes, 2=no).....|__|

GC.17 Transfusion within past 3 months (1=yes, 2=no).....|__|

Severe pallor (1=yes, 2=no)…….. |__|

If the answer to any of the above questions is yes, do not recruit into study!

GC.18 *****CONSENT REQUIRED****** please indicate obtained, 1=Yes…… |___|

Name of staff who obtained consent._____

GC 18b. Name of medical professional taking history/physical_____

Name:_____ ID no. |__|__|__|__|__|__|

GC.19 Area of residence:_____
_____Cell phone #_____
GC.20 Ethnic origin .....|__|
(Akan=1, Ga-Adangme=2, Ewe=3, Hausa=4, Frafra=5, Dagomba=6
Non-Ghanaian=7, Other=8 Specify _____)
GC.21 Sex (1=M, 2=F) .....|__|
GC.22 Age (Last half year passed) .....|__|__|.|__|
GC.23 Temperature .....|__|__|.|__|
GC.24 Blood Pressure (mmHg) .....|__|__|__|/|__|__|
GC.25 Pulse ..... |__|__|__|
GC.26 Weight (in kgs) .....|__|__|.|__|
GC.26a Height (in cm) ..... |__|__|__|
GC. 27 **Samples (Please tick when taken):**
___ EDTA purple top (EPC sample) _____PAX-gene tube (RNA)
___ EDTA purple top (FBC/CBC-full blood count) ___ Heparin tube

Results on day of recruitment:
GC.28 Hemoglobin (Hb). |__|__|.|__|
GC.29 WBC ($X10^9$/L).....|__|__|.|__|
GC.30 RBC_____($x10^6/\mu L$)
GC.30a Hb_____ (g/dL)
GC.31 HCT_____ (%)
GC.32 MCV_____ (fL)
GC.32a MCH_____(pg)
GC.32b MCHC_____(g/dL)
GC.33 PLT_____ _____($x10^3/\mu L$)
GC.34 LYMPH%_____(%)
GC.35 MXD%_____(%)
GC.38 NEUT%_____(%)
GC.38a LYM#_____($x10^3/\mu L$)
GC.38b MXD#_____($x10^3/\mu L$)
GC.38c NEUT#_____($x10^3/\mu L$)
GC.39 RDW(SD) _____(fL)
GC.39a RDW-CV_____(%)
GC.40 PDW_____(fL)
GC.40a MPV_____(fL)
GC.40b P-LCR_____(%)
GC.41 Blood Film species (1=p.f.,2=p.m.,3=p.o., 4=p.v.,5=p.f.+p.m.,6=p.f.+p.o.) ...|__|

GC.42 Parasite density (per μL)

GC.43 Asexual stage, density per μl ..... |__|__|__|__|__|__|__|

GC.44 Sickling status (1=positive, 2=negative) ..... |__|

NOTE- if positive then exclude from the study

HEALTHY CONTROLS

FOLLOW-UP ASSESSMENT/QUESTIONNAIRE

	7 DAYS POST INITIAL ASSESSMENT	**14 DAYS POST INITIAL ASSESSMENT**
HISTORY SINCE LAST ASSESSMENT*	Date_____	Date_____
QC.1 Illness since last assessment (1=Yes, 2=No) QC.2 If yes; describe (diarrhea, cough, infection etc)		
QC.3 Fever (1=Yes, 2=No)		
QC.4 Medical care/Hospitalization (1=Yes, 2=No)		
QC.5 If yes, was the medical care or hospitalization malaria related(1=Yes, 2=No)		
QC.6 Severe bleeding (1=Yes, 2=No)		
QC.7 Convulsion or impaired consciousness (1=Yes, 2=No)		
QC.8 Trauma (1=Yes, 2=No) QC.9 If yes, indicate type (eg car accident)_____		
QC.10 Surgery (1=Yes, 2=No) QC.11 If yes, indicate _____		
QC.12 Antibiotics (1=Yes, 2=No) QC.13 If yes specifiy;_____		
QC.14 Antimalarials (1=Yes, 2=No)		
QC.15 If yes‡, specify;		
CLINICAL ASSESSMENT		
QC.18 Temperature °C,		
QC.19 BP (mm Hg)		

QC.20 pulse		
QC.21 Weight (in kgs)		
QC.22 Blood samples obtained (indicate test EPC, FBC, heparin: 1=Yes, 2=No, 3=ND – if not state why)		
QC.23 Parasite density (per microlitres) QC.24 Asexual stage, density per µl		
QC.25 WBC $X10^9/L$		
QC.28 HB (g/dL)		

*‡IF PATIENT REFERRED FOR MEDICAL CARE DUE TO MALARIA ALERT for possible inclusion in malaria arm but cannot be included as a healthy control.