

UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

**ANTIBODY RESPONSES TO VARIANT SURFACE ANTIGENS AND BIOMARKERS
OF ENDOTHELIAL DAMAGE AND REPAIR IN THE DEVELOPMENT OF
CEREBRAL MALARIA**

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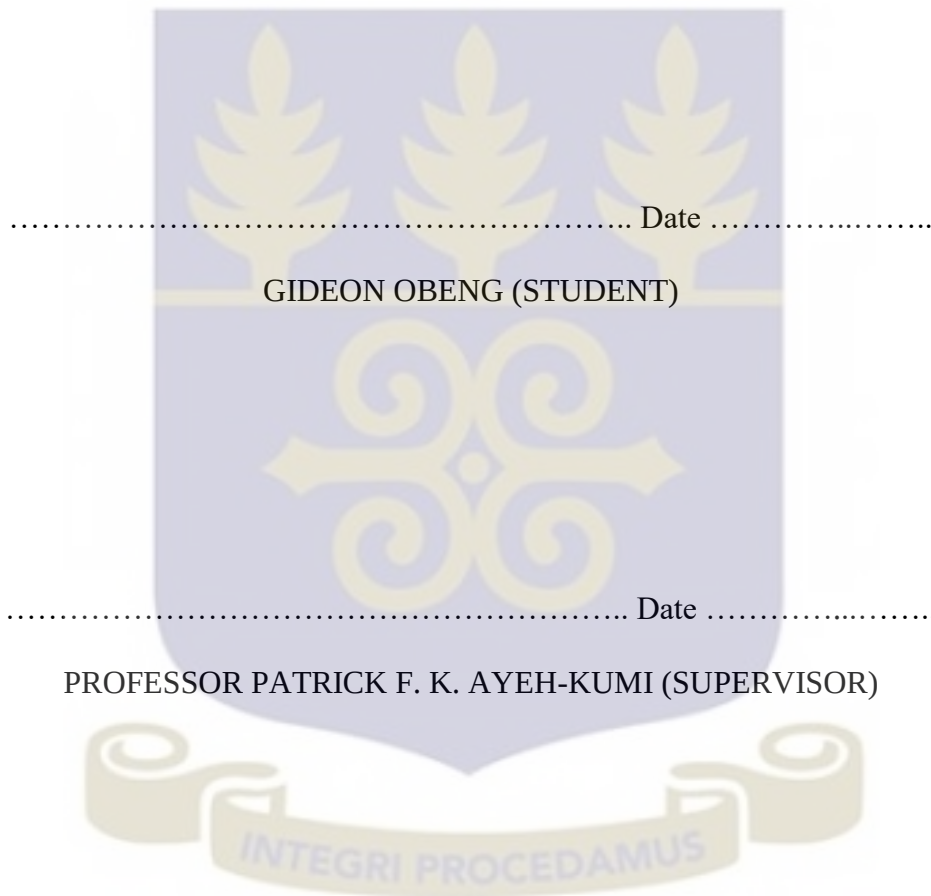
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DECLARATION

I hereby declare that this is the product of my own research undertaken under the supervision of Professor Patrick F. K. Ayeh-Kumi and Professor Ben A. Gyan and that references made to other people's work have been duly acknowledged. I also declare that this work has neither been presented in whole nor in part for another degree elsewhere.



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DEDICATION

This thesis is dedicated to all those who have trusted and believed in me during these testing times especially my amazing parents, Mr. and Mrs. Obeng and my truly wonderful siblings, Juliana Obeng, Evans Fiifi Obeng and Christiana Ama Praba Obeng.



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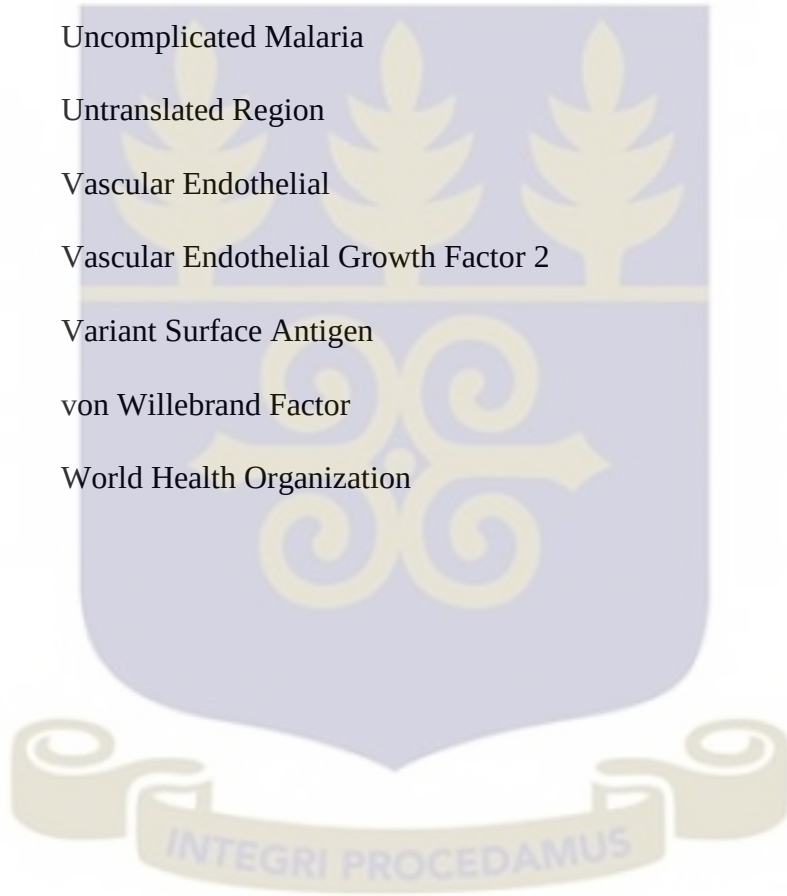


LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
APC	Activated Protein C
APC	Allophycocyanin
BCS	Blantyre Coma Score
BD	Becton Dickenson
BSC	Biosafety Cabinet
CD	Cluster of Differentiation
CEC	Circulating Endothelial Cell
CIDR	Cysteine Rich Inter-Domain Region
CM	Cerebral Malaria
CPD	Citrate Phosphate Dextrose
CPM	Complete Parasite Medium
CSF	Cerebrospinal Fluid
DBL	Duffy Binding Like Domain
DC	Domain Cassette
EC	Endothelial Cell
EDTA	Ethylene Diamine Tetra-Acetate
EPC	Endothelial Progenitor Cell
EPCR	Endothelial Protein C Receptor
FACS	Florescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Flourescein Isothiocyanate

HC	Healthy Control
H-L	Hosmer-Lemeshow
ICAM 1	Intercellular Adhesion Molecule 1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
KAHRP	Knob Associated Histidine Rich Protein
LEKMA	Ledzokuku Krowor Municipal Assembly
MACS	Magnetic Activated Cell Sorting
MFI	Median Fluorescence Intensity
NO	Nitric Oxide
NOI	Nitric Oxide Intermediates
NOS	Nitric Oxide Synthase
PAR-1	Protease Activated Receptor 1
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PECAM 1	Platelets Endothelial Cell Adhesion Molecule 1
PerCP	Peridinin Chlorophyll
<i>PfEMP1</i>	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein 1
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
RBC	Red Blood Cell
RCF	Relative Centrifugal Force

RPMI-1640	Roswell Park Memorial Institute-1640
SD	Standard Deviation
SMA	Severe Malaria Anemia
TF	Tissue Factor
TM	Thrombomodulin
TNF	Tumor Necrosis Factor
UM	Uncomplicated Malaria
UTR	Untranslated Region
VE	Vascular Endothelial
VEGFR-2	Vascular Endothelial Growth Factor 2
VSA	Variant Surface Antigen
vWF	von Willebrand Factor
WHO	World Health Organization



ABSTRACT

Background: The pathogenesis of cerebral malaria (CM) is still recognized as multifaceted, despite the plausible explanation provided by the high degree of sequestration of parasitized erythrocytes in major organs of the body. Postmortem studies have demonstrated the presence of hemorrhage, deposition of fibrin and clots in blood vessels of the body where sequestration has been observed. These findings support a potential association between sequestration, endothelial damage and repair, in the development of cerebral malaria. Considering the evidence, it is plausible that associated biomarkers of these pathogenetic features may be related in the development of CM. The aim of this study was to determine the association between the development of CM and the levels of circulating endothelial cells (CECs), endothelial progenitor cells (EPCs) and antibody responses to variant surface antigens (VSA).

Materials and methods: Seventy two children including nineteen CM patients, fifteen severe malaria anemia (SMA) patients, ten uncomplicated malaria (UM) patients and twenty eight healthy controls (HC) were recruited from hospitals within Greater Accra region. Whole blood sample obtained with consent from the patients was separated into plasma and erythrocytes. Five parasite isolates including three obtained from CM patients and one each from an UM and a SMA patient were successfully cultured and the erythrocytes expressing VSAs were separated. Plasma from the seventy two patients was then tested against VSAs expressed by each parasite isolate and the levels of plasma anti-VSA antibody responses were determined using flow cytometry. Additionally, levels of CECs and EPCs were determined from whole blood using flow cytometry.

Results: For the CM parasite isolates tested, there were significant differences in the levels of anti-VSA_{CM} antibody responses between the groups (ANOVA: CM₁, $p = 0.048$; CM₂, $p = 0.006$; CM₃, $p = 0.042$). Post hoc analysis revealed that, levels of antibody responses to CM₃ isolate were higher in the CM group than the HC. Conversely, levels of antibody responses to CM₂ were higher in the SMA group than the HC group. For CM₁ isolate, there were no differences in the antibody levels between the groups. For the non-CM parasite isolates, the levels of antibody responses were different between the groups (ANOVA: VSA_{UM}, $p = 0.008$; VSA_{SMA}, $p < 0.0001$). Levels were higher in the CM group than the other study groups following post hoc analysis. Taken the above results together, the levels of antibody responses were higher in the CM group than the other groups suggesting that CM may have induced the production of cross reactive antibodies with broad spectrum of recognition. For CECs, there were no significant differences in the levels between the study groups. (ANOVA, $p = 0.088$). However, levels of EPCs were lower in the CM group compared to the UM group (ANOVA, $p < 0.05$). Logistic regression analysis revealed that a unit increase in the levels of EPCs decreases the odds of a UM patient developing CM by 0.09 when the levels of CECs and anti-VSAs were held constant. Conversely, there was no association between the development of CM and the levels of CECs and anti-VSA antibody responses when the effects of the other biomarkers were held constant.

Conclusion: There was a negative association between the levels of EPCs and the development cerebral malaria. Despite the results, it may be premature to make conclusions regarding the potential associations between development of CM and these biomarkers evaluated merely based on this study. This is particularly true when one considers the potential effects of small sample sizes and potential confounding variables that were not measured in this study.

CHAPTER ONE

1 BACKGROUND

1.1 Introduction

Cerebral malaria is a complication of malaria which is caused by *Plasmodium falciparum*. It is marked by the presence of neurological abnormalities including coma which usually lasts for an hour or more with no other explanation for the altered consciousness. Cerebral malaria is recognized as a disease of children particularly in sub-Saharan Africa where children below the ages of five suffer the heaviest burden. In Asia and other endemic areas the occurrence of the disease is not restricted to children but may be diagnosed in all categories of age groups (WHO, 2000). The case fatality of the disease in sub-Saharan Africa has been estimated to be approximately twenty percent which suggests that two out of every ten cerebral malaria cases results in death despite adequate treatment (Birbeck *et al.*, 2010; Dondorp *et al.*, 2010). What is more, thirty percent of survivors suffer post disease neurological deficits which may be permanent (Birbeck *et al.*, 2010).

Despite the huge investment in cerebral malaria research, the pathogenetic mechanisms of the disease still remains obscure (Monteiro *et al.*, 2014). Until recently, pathogenesis of cerebral malaria had been explained by independent models including but not limited to the sequestration of parasitized erythrocytes in the blood vessels of the brain, which consequently results in ischemia and downstream repercussions including tissue damage (MacPherson *et al.*, 1985; Berendt *et al.*, 1994). Other schools of thought had also suggested that development of the disease was primarily mediated by systemic inflammatory responses which may explain the multiple organ pathologies associated with this clinical syndrome (Clark *et al.*, 1991; Clark *et al.*,

1994). Others had linked the complex and systemic nature of the disease to dysregulation and defects in the hemostatic pathway (Moxon *et al.*, 2009). However, despite the significant contribution of these independent theories in explaining the pathogenesis of the disease, it has become evident that no single feature of the disease is adequate to fully explain the pathogenesis (van der Heyde *et al.*, 2006). Indeed, studies have demonstrated that, these features are related and may actually contribute to the development of the disease in a synergistic fashion. Sequestration has been associated with inflammation and coagulation in cerebral malaria (Moxon *et al.*, 2013; Moxon *et al.*, 2014). Hemorrhages, aggregation of platelets, deposition of fibrin, thrombi at sites of parasite sequestration in tissues, have been shown in postmortem studies involving cerebral malaria children (Milner Jr *et al.*, 2014). Not only do these evidences support a strong association between sequestration and coagulation in the pathogenesis of the disease but also suggest a potential association between sequestration and endothelial damage which may be crucial in the pathogenesis. It is plausible that sequestration may be a trigger which sets off a positive feedback mechanism resulting in endothelial damage and a resultant response to this damage by the body.

Sequestration of parasite infected erythrocytes in the microvessels of the body is a product of an intricate interaction between the host and *P. falciparum*. It results from interactions between proteins expressed on the surfaces of erythrocytes infected by the parasite and host receptors on the endothelial surface (MacPherson *et al.*, 1985; Silamut *et al.*, 1999; Turner *et al.*, 2013). These antigens bind specifically to these receptors sequestering these infected erythrocytes in the blood vessels. Consequently, sequestration may shield parasites from immune clearance in the spleen (Mebius *et al.*, 2005; Milner Jr *et al.*, 2014) while also resulting in the formation of clumps of cells or a growing mass of parasite infected and uninfected erythrocytes which may obstruct

blood flow in the capillaries. This results in ischemia which may eventually cause damage to the endothelium and tissues downstream of the capillaries.

These antigens which mediate sequestration are parasite encoded and produced at a period within the erythrocytic cycle of the asexual cycle of *P. falciparum* when the parasite is primarily intracellular. They are proteins of high molecular weight and diversity expressed by mature and metabolically active forms of the parasite (Leech *et al.*, 1984; Magowan *et al.*, 1988; Su *et al.*, 1995; Rask *et al.*, 2010). The marked variation in the structure of these proteins, coupled with their extreme diversity and ability to bind cognate antibodies, warrants the name Variant Surface Antigens (Su *et al.*, 1995; Bull *et al.*, 1998; Rask *et al.*, 2010).

The most characterized of these variant surface antigens is the class of proteins called *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*PfEMP 1*). *PfEMP 1* ranges in weight between 150 and 350 kDa and is encoded by the var gene family of approximately 60 members (Baruch *et al.*, 1995; Su *et al.*, 1995; Rask *et al.*, 2010). Var genes have been characterized into Groups A, B, C, B/A and B/C primarily based on homology, position and orientation of sequences in the 5' untranslated region (UTR) of the gene. Sub groups of the var genes as well as the proteins encoded by these genes have been associated with distinct disease pathologies including cerebral malaria (Avril *et al.*, 2012; Claessens *et al.*, 2012; Lavstsen *et al.*, 2012). More significantly, identities of receptors mediating endothelial binding of VSAs which have been associated with cerebral malaria have also been described (Turner *et al.*, 2013).

While these VSAs may be extremely diverse, only a subset has been associated with complicated forms of the disease (Jensen *et al.*, 2004; Lavstsen *et al.*, 2012). Antibodies induced by these VSAs, which have been extensively characterized in children and adults alike, have provided

evidence for acquired immunity to malaria (Dodoo *et al.*, 2001; Ofori, 2002; Rask *et al.*, 2010). These antibodies have been shown to have the capacity to agglutinate parasite infected erythrocytes expressing these antigens on their surface (Aguar *et al.*, 1992). Consequently, it has been shown that these antibodies otherwise called anti-VSA antibodies can inhibit cytoadherence of parasitized erythrocytes to the endothelium and as a result inhibit sequestration (Ricke *et al.*, 2000). Indeed, high levels of anti-VSA antibody responses have been associated with protection against the disease and reduced susceptibility to re-infections (Dodoo *et al.*, 2001). Thus, it is evident that levels of anti-VSA antibody responses are crucial in the pathogenesis of the disease. Whether or not levels of these anti-VSA antibody responses may be associated with other biomarkers of the disease in contributing to the pathogenesis is yet to be fully explored.

The endothelium is a monolayer of endothelial cells lining the blood vessels. It is separated from underlying connective tissues by the basement membrane. The endothelium serves as a barrier between blood and the underlying tissues while also allowing the passage of some molecules and cells across it (Aird, 2007). More significantly, the endothelium provides surface receptors which also mediate sequestration of parasitized erythrocytes. Increased levels of von Willebrand factor (Hollestelle *et al.*, 2006) and presence of hemorrhages, on one hand, and deposition of fibrin and thrombi in blood vessels and tissues in cerebral malaria patients, (Milner Jr *et al.*, 2014) on the other hand provide ample evidence that the endothelium cycles between phases when it is significantly damaged and phases when it undergoes repair respectively. What is more, the presence of these features provides support for a potential association between sequestration, and endothelial damage and repair in the development of cerebral malaria.

Endothelial damage is explained by the loss of endothelial cells to the blood circulation as a result of their disintegration from the endothelium. The ability to estimate these blood borne

endothelial cells which are more appropriately referred to as circulating endothelial cells (CECs) provide another potentially useful marker for endothelial damage (Goon *et al.*, 2004; Blann *et al.*, 2005). High levels of CECs have been demonstrated in acute phases of diseases like sickle cell disease (Solovey *et al.*, 1997), vasculitis (Nakatani *et al.*, 2003) and myocardial infarctions (Mutin *et al.*, 1999) in which endothelial damage is a key mediator of the pathogenesis. It is possible that development of cerebral malaria may be associated with high levels of CECs given that there is plausible evidence for the presence of endothelial damage in the disease (Milner Jr *et al.*, 2014).

Endothelial damage is repaired by neighboring endothelial cells at the site of damage (Hanahan *et al.*, 1996; Holash *et al.*, 1999) and endothelial progenitor cells which augment this process. Endothelial progenitor cells are cells which have their origin in the bone marrow. Together with these endothelial cells, these cells share the properties of being able to proliferate, differentiate, migrate and incorporate into sites of damage (Asahara *et al.*, 1997). The production of these cells from the bone marrow may be mediated by multiple factors but key among them may be the stimulus provided by endothelial damage (Gill *et al.*, 2001). EPCs like CECs are rarely found in the blood of the healthy host. Thus, high levels of these cells in the blood may suggest possible endothelial damage and repair (Erdbruegger *et al.*, 2006).

Given that sequestration may be enhanced by low levels of anti-VSA antibodies which have been shown to inhibit sequestration and the likelihood that this process may result in damage to the endothelium and subsequently may require replacement of lost endothelial cells, it is not far-fetched to predict that development of cerebral malaria may be associated with low levels of anti-VSA antibodies, high levels of CECs and low levels of EPCs. The purpose of this study is to investigate the relationship between these biomarkers and the development of cerebral malaria

while also exploring the potential usefulness of these biomarkers as diagnostic and/or prognostic indicators.

1.1 Problem Statement

According to the world malaria report 2015, an estimated 200 million cases of malaria were diagnosed in 2014 in sub-Saharan Africa alone. Out of this estimated number, half a million fatalities were recorded with children below the ages of five years suffering the heaviest burden (WHO, 2015). With an estimated incidence rate of 1120/100 000/year (Bremner, 2001) and case fatality of approximately 20 percent within the sub region (Birbeck *et al.*, 2010), cerebral malaria still remains a disease of clinical and economic importance.

In Ghana, admissions and deaths due to malaria reduced by an estimated 9 percent and 30 percent respectively between 2013 and 2014 (WHO, 2015). While these figures may indicate an improvement in the management of the disease, the threat and the burden of the disease is still enormous with the inability to diagnose the disease early enough and lack of indicators to predict who is at risk of post disease neurological abnormalities. Currently, the estimated percentage of children who develop cerebral malaria post disease neurological sequelae is around 30 percent (Birbeck *et al.*, 2010).

1.2 Justification

Despite the massive investment in cerebral malaria research, the pathogenetic mechanisms mediating the disease is still incompletely understood (Monteiro *et al.*, 2014). Evidence of a potentially pathogenetic role for endothelial damage and repair particularly at the sites of sequestration is only beginning to come to light. The aim of this study is to determine an association between the development of cerebral malaria and levels of anti-VSA antibodies,

circulating endothelial cells and endothelial progenitor cells. This study will potentially contribute to the knowledge base of the pathogenesis of the disease with respect to sequestration and, endothelial damage and repair.

These potential biomarkers described above may be of utility in predicting children who are at risk of developing cerebral malaria and post disease neurological deficits. Moreover, these biomarkers may be potentially used as diagnostic and prognostic indicators of the disease following proper validation. These risk, prognostic and diagnostic indicators may improve the early detection of the disease which may consequently improve management of the disease and the chances of making a full recovery from this clinical syndrome.

1.3 Hypothesis

Development of cerebral malaria is associated with low levels of anti-VSA antibody responses, high levels of circulating endothelial cells and low levels of endothelial progenitor cells.

1.4 Aim

The aim of this study is to determine an association between the development of cerebral malaria and the levels of anti-VSA antibodies, levels of circulating endothelial cells and levels of endothelial progenitor cells

1.5 Specific objectives

1. To determine the levels of anti-VSA antibody responses among the study subjects
2. To determine the levels of circulating endothelial cells and endothelial progenitor cells among the study subjects

3. To determine the association between the development of cerebral malaria and, the levels of anti-VSA antibodies, levels of circulating endothelial cells and levels of endothelial progenitor cells.



CHAPTER TWO

2 LITERATURE REVIEW

2.1 Clinical definition of cerebral malaria

Cerebral malaria like severe anemia malaria and respiratory distress is a complication of malaria which is invariably caused by *Plasmodium falciparum*. Cerebral malaria is common in endemic regions of the world especially sub-Saharan Africa where children bear the heaviest burden. However, in other endemic regions, it is not uncommon to see adults affected. Cerebral malaria is recognized as a clinical syndrome and thus is defined by a group of specific but related signs and symptoms which includes but not limited to the demonstration of *P. falciparum* parasitemia and the presence of coma with a Blantyre coma score of less than three and lasting for at least an hour. Additionally, presence of coma must be explained by *P. falciparum* infection alone and not by other causes or conditions which may be malaria and/or non malaria related. Conditions such as hypoglycemia, convulsions, severe anemia, hyperpyrexia, acidosis, meningitis among others may independently cause coma and therefore should be firmly ruled out as a cause of coma despite the demonstration of parasitemia (WHO, 2000).

Pathological studies involving children diagnosed with cerebral malaria have suggested a revision of this present clinical definition to include signs and symptoms of retinopathy (Taylor *et al.*, 2004; Beare *et al.*, 2006; Milner Jr *et al.*, 2014). These studies put misdiagnosis of the condition at twenty three percent. These studies have subsequently suggested that inclusion of four pathologic features of the retina during cerebral malaria would vastly enhance the definite diagnosis of the condition. These abnormal features which can be determined through ophthalmoscopy include retinal hemorrhages, papilledema, vessel changes and retinal whitening

(Taylor *et al.*, 2004; Beare *et al.*, 2006). Retinal whitening and vessel changes are specific to cerebral malaria while the remaining two may be found in other diseases but their presence together with retinal whitening or vessel changes may indicate poor prognosis with high risk of death. Thus, the absence of malaria retinopathy may suggest an indefinite diagnosis of cerebral malaria and/or an alternative cause of coma or death even with the demonstration of *P. falciparum* parasitemia. Also in these studies, patients diagnosed with cerebral malaria using malaria retinopathy as diagnostic index also showed significant cerebral sequestration of parasitized erythrocytes which is a key feature in the pathogenesis of the disease (Lewallen *et al.*, 2000; Beare *et al.*, 2006).

2.2 Pathogenesis of cerebral malaria

Despite being recognized as a multi-organ disease, the brain appears to be the most affected and therefore several hypotheses to explain the pathogenesis have mostly centered on the neurological abnormalities associated with the disease. Several plausible mechanisms to explain disease development have been proposed but the overwhelming consensus is that no single pathogenic feature is adequate to explain this clinical syndrome (van der Heyde *et al.*, 2006). Notable among these mechanisms/features are the sequestration of parasitized erythrocytes in the microvessels of major organs of the body (MacPherson *et al.*, 1985; Pongponratn *et al.*, 2003; Milner Jr *et al.*, 2014; Milner *et al.*, 2015), inflammatory responses resulting in multi organ failure (van der Heyde *et al.*, 2006), and defects in hemostasis (Moxon *et al.*, 2009; Moxon *et al.*, 2014).

2.3 The role of sequestration in cerebral malaria pathogenesis

Sequestration is a product of intricate upstream interactions mediated predominantly by proteins expressed on the surfaces of parasite infected erythrocytes and receptors on the endothelium (Turner *et al.*, 1994; Avril *et al.*, 2012; Moxon *et al.*, 2013). This typical binding of infected erythrocytes to the endothelium is commonly referred to as cytoadhesion. Cytoadhesion results in the formation of a growing mass of cells which are held firmly in place on/by the endothelium. Consequently, infected cells may bind other infected and uninfected erythrocytes increasing the growing mass of cells via processes referred to as autoagglutination (Roberts *et al.*, 2000) and rosetting (Mercereau-Puijalon *et al.*, 2008) respectively.

Rosetting appears to be associated with severe malaria given that it is yet to be demonstrated in parasites implicated in uncomplicated malaria (Rowe *et al.*, 1995). Despite being considered a severe disease phenomenon other studies have specifically demonstrated the ability of cerebral malaria parasites to cause rosetting (Carlson *et al.*, 1990). Rosetting has also been shown to increase parasite density in severe malaria (Rowe *et al.*, 2002).

Sequestration is also facilitated by a process termed platelet mediated clumping of infected cells. Platelets contribute to the formation of this growing mass of cells by initially binding the infected erythrocytes contributing to the size of the mass (Moxon *et al.*, 2011) and also by acting as a link between infected erythrocytes and the endothelium (Wassmer *et al.*, 2004). Platelets express on their surfaces CD 36 (Wassmer *et al.*, 2004) and gC1qR/HABP1/p32 receptors which can bind ligands on infected erythrocytes. The gC1qR/HABP1/p32 receptor has also been shown to be expressed on endothelial surfaces, hence may bind infected erythrocytes directly to the endothelium also facilitating sequestration (Biswas *et al.*, 2007). Platelet mediated clumping has been associated with severe disease including cerebral malaria (Wassmer *et al.*, 2008).

Evidence of sequestration in the microvessels of body has been overwhelmingly presented in numerous studies demonstrating this feature as a significant contributor to the pathogenesis of the disease. Sequestration has been observed in the brain, lungs, kidney, the gut and other organs. With the exception of the brain and regardless of the high magnitude of sequestration observed in the gut, there is seemingly no obvious pathology associated with the phenomenon in the other organs of the body (MacPherson *et al.*, 1985; Silamut *et al.*, 1999; Moxon *et al.*, 2013; Milner Jr *et al.*, 2014).

2.3.1 Significance of sequestration in the pathogenesis of cerebral malaria

In cerebral malaria, neurological abnormalities which appear to be pathognomonic are thought to result from the obstruction of blood flow leading to hypoperfusion of tissues downstream of that vessel. Hypoperfusion may eventually lead to ischemia which may potentially result in limited supply of oxygen, production of lactic acid and endothelial damage.

Microvascular obstruction of blood flow has also been linked to red blood cell deformability (Dondorp *et al.*, 2004). Red blood cell deformability is a phenomenon which enable erythrocytes in blood to go through smaller capillaries of the vascular bed without been impeded despite their relatively bigger sizes than the capillaries they traverse. Normal erythrocytes have a diameter ranging between 6-8 microns but can traverse capillaries with diameters as small as 3 microns without been impeded (Mohandas *et al.*, 1993). Thus, cells with reduced deformability become rigid (Glenister *et al.*, 2002) and may obstruct blood flow by plugging capillaries and forming clumps (Dondorp *et al.*, 2004).

Reduced deformability of cells has been observed among infected and uninfected erythrocytes alike. A parasite protein called knob associated histidine rich protein (KAHRP) is inserted in the

membrane of the erythrocyte during parasite development and this is believed to account for the reduced deformability in infected cells (Glenister *et al.*, 2002). For uninfected cells however this does not happen obviously and that oxidative damage from increased oxidation of uninfected cells during malaria has been ascribed to reduced deformability (Griffiths *et al.*, 2001). Reduced deformability has been associated with severe disease and has been shown to have a strong predictive value of mortality (Dondorp *et al.*, 1997; Cooke *et al.*, 2004).

Sequestration besides enabling the parasite achieve better replication rates also enable it avoid circulation through the spleen and consequently avoiding death via action of phagocytes and destruction of abnormal erythrocytes in the spleen (Sherman *et al.*, 2003; Mebius *et al.*, 2005; Milner Jr *et al.*, 2014).

2.3.2 Variant surface antigens and receptors mediating sequestration

Cytoadherence of parasite infected erythrocytes to the endothelium which facilitates sequestration is mediated by antigens expressed on erythrocyte surfaces and receptors on the endothelium (MacPherson *et al.*, 1985). These antigens have been traditionally referred to as variant surface antigens (VSAs) reflecting their highly diverse nature, expression on the surfaces of erythrocytes and ability to bind cognate antibodies.

VSAs are produced during the erythrocytic cycle of the parasite where it exhibits high metabolism and replication. This is also the stage where the signs and symptoms associated with the disease are evident. This stage of the life cycle indicated in yellow in figure 2.1 begins when merozoites are released into the blood stream upon the rupture of the matured schizonts at the latter stages of the exo-erythrocytic cycle which occurs in the liver. These merozoites invade fresh erythrocytes and enter the trophic phase where they are highly metabolic. This stage of the

parasite is termed the trophozoite. The parasites mature and undergo 3-5 rounds of nuclear divisions without cytokinesis in a process termed schizogony. It is during this stage that the parasites begin to produce these variant surface antigens. These proteins are produced within the cytoplasm of the parasite and are transported to the membrane of the erythrocyte where portions are displayed on the surface of the erythrocyte. This process is intensified resulting in the production of more variant surface antigens as the parasite matures further into a schizont. The matured schizont which at this stage occupies the whole cytoplasm of the erythrocyte ruptures and releases 8-32 immature parasites into the circulation called merozoites. These merozoites invade new erythrocytes and begin the whole cycle again. The whole cycle from the merozoite phase to the schizont stage may take 24-48 hours depending on the strain of the parasite and conditions within the host.

Several gene families within the parasite genome have been shown to encode VSAs which are structurally distinct (Scherf *et al.*, 2008). The most popular of these gene families is the *var* gene family which encodes *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*PfEMP 1*). The other gene families known to encode VSAs include *Stevor*, *Rif*, *Pfmc- 2 TM* and *Surf* gene families. The proteins they encode include stevor (Kaviratne *et al.*, 2002), rifins (Kyes *et al.*, 1999), *Pfmc- 2 TM* (Sam-Yellowe *et al.*, 2004) and surfins respectively (Winter *et al.*, 2005). Unlike *PfEMP 1* which has been extensively studied and characterized, the exact biological roles of the others are yet to be fully elucidated (Scherf *et al.*, 2008).

PfEMP 1 has a molecular weight which ranges between 150 and 350 KDa. It is a highly complex protein and comprises of at least two distinct structural domains called duffy binding like domains (DBLs) and cysteine rich interdomain regions (CIDR) (Rask *et al.*, 2010).

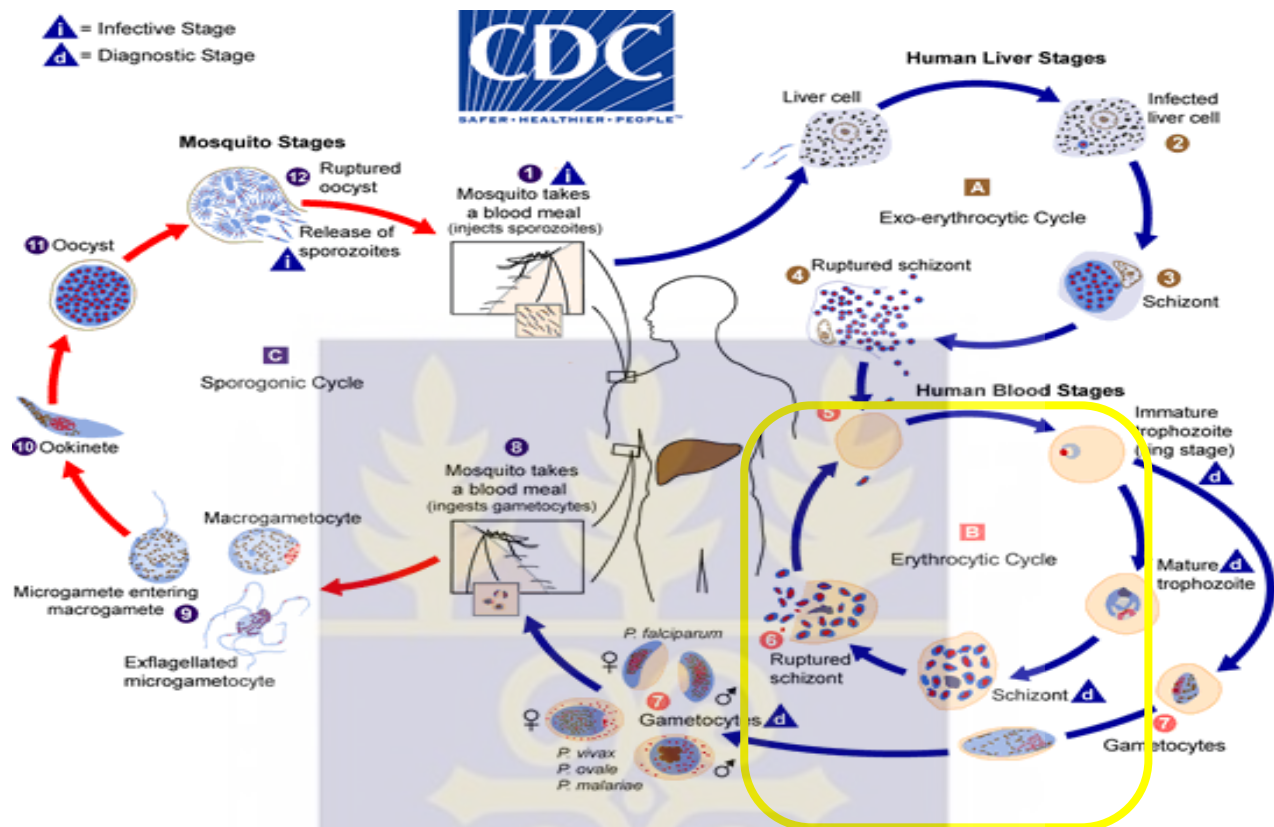


Figure 2.1: Life cycle of *Plasmodium falciparum*

The blue arrows show the asexual phase of the cycle while the red arrows the sexual phase which occurs in the definite host, the mosquito. The yellow rounded rectangular box shows the phase of the asexual cycle (erythrocytic cycle) where variant surface antigens are produced. These antigens are produced by the mature trophozoites and the schizonts.

Some sequences within these domains have shown broad similarities and therefore have been further divided into several subclasses. Some of these subclasses have been conserved over time despite the high level of recombination during parasite replication. These conserved subclasses have been referred to as domain cassettes (DC), of which 23 have been characterized so far (Rask *et al.*, 2010).

A few of these characterized DCs have been associated with disease syndromes and specific binding phenotypes. DC 2 has been associated with placental malaria and a specific binding phenotype (Salanti *et al.*, 2003). Parasites isolates expressing this DC have been shown to bind chondroitin sulphate A receptors on the placenta in a variant specific manner (Fried *et al.*, 1996; Salanti *et al.*, 2003). Other DCs including 8 and 13 have also been linked with severe malaria and a specific binding phenotype (Lavstsen *et al.*, 2012). DC 8 and DC 13 bind endothelial protein C receptors (EPCR) (Lavstsen *et al.*, 2012). EPCR is expressed on the endothelium in large quantities in major vascular beds of the body but in the brain, however, it has been shown to be expressed in very low quantities. Recent studies have linked the loss of these receptors which mostly occurred on sites of heavy sequestration to coagulation and inflammation in cerebral malaria (Moxon *et al.*, 2013). DC 4 and DC 5 have also been shown to bind intercellular adhesive molecule 1 (ICAM 1) and platelet endothelial cell adhesive molecule 1 (PECAM 1) respectively (Bengtsson *et al.*, 2013; Berger *et al.*, 2013). Both receptors have been shown to be expressed in the brain and other parts of the body. ICAM 1 has also been shown to be upregulated during cerebral malaria (Turner *et al.*, 1994).

The *var* genes which encode *Pf*EMP 1 is characterized into Groups A, B, C, B/A and B/C based on the homology, position and orientation of sequences in the 5' untranslated region (UTR) of the gene. Group A and B genes encode VSAs involved in severe malaria (Jensen *et al.*, 2004;

Lavstsen *et al.*, 2012) while Groups B and C have been shown to encode VSAs which bind CD 36, DC 8 and DC 13 which mediate severe malaria pathogenesis have also been shown to belong to groups B/A and A respectively (Turner *et al.*, 2013).

2.3.3 Antibodies to variant surface antigens

Variant surface antigens (VSAs) are highly immunogenic and hence, have the ability to induce the production of antibodies in the host. These antibodies have been suggested to specifically inhibit cytoadherence and consequently sequestration of parasite infected erythrocytes in the lumen of the micro blood vessels (Ricke *et al.*, 2000). Thus, associations have been made between the levels of anti-VSA antibodies on one hand and susceptibility to infections and protection from disease on the other. (Dodoo *et al.*, 2001; Avril *et al.*, 2012; Claessens *et al.*, 2012). Anti-VSA antibodies bind VSAs and may specifically target epitopes on domain cassettes producing variant specific anti-DC antibodies. These antibodies have been shown to protect against disease syndromes mediated by these domain cassettes (Berger *et al.*, 2013). High levels of anti-DC 5 antibodies have been suggested to protect against febrile malaria episodes and that these antibodies are acquired early in life in areas where the transmission of malaria is high (Claessens *et al.*, 2012; Lavstsen *et al.*, 2012). These studies suggest that age and intensity of transmission may be determinants of levels and composition of a person's repertoire of antibodies. Indeed, studies have demonstrated that levels of antibodies and more so the spectrum of antibodies increase as a person ages (Bull *et al.*, 1998; Bull *et al.*, 1999; Ofori, 2002). This suggests that children in endemic regions may have lower levels and a narrower spectrum of antibodies than adults in the same area. This may partly explain why children are more susceptible to *P. falciparum* infections than adults.

A common theme in malaria regarding VSAs is that, the disease is caused by parasite isolates expressing VSAs for which the host has no corresponding antibodies (Bull *et al.*, 1998; Bull *et al.*, 1999; Ofori, 2002) and that these parasites preferentially express VSAs mediated in severe disease earlier while VSAs implicated in uncomplicated disease are expressed a bit later in life (Nielsen *et al.*, 2002; Cham *et al.*, 2009). Thus, in children, severe disease is invariably common and that immunity to these syndromes is thought to be acquired early in life. This phenomenon of acquisition of natural immunity suggests that in areas of high malaria transmission this process of acquisition of antibodies mediating immunity may be rapid (Snow *et al.*, 1998; Vestergaard *et al.*, 2008).

Anti-VSA antibodies are generally accepted as been variant specific (Bull *et al.*, 1998) but this notion has been challenged by several other studies where evidence of the existence of cross reactive antibodies have been shown. VSAs of particular parasite isolates have been shown to induce antibodies that recognized VSAs of several other genetically distinct parasite isolates (Giha *et al.*, 1999; Ofori *et al.*, 2002; Chattopadhyay *et al.*, 2003; Elliott *et al.*, 2007).

While it is evident that VSAs have the capacity to induce the production of antibodies, the levels of these anti-VSA antibodies associated with specific disease syndromes have been unclear. On one hand, studies have demonstrated the association of severe malaria syndromes with low levels of anti-VSA antibodies (Tebo *et al.*, 2002) while on the other hand, high levels of anti-VSA antibody responses have been linked with severe disease (Theander *et al.*, 2006).

2.4 The role of inflammatory mediators in the pathogenesis of cerebral malaria

Systemic immune response mediated by cytokines and/or immune effector molecules have been suggested to be significant in the pathogenesis of cerebral malaria (Clark *et al.*, 1994; van der

Heyde *et al.*, 2006). Strong arguments for the role of pro-inflammatory cytokines particularly tumor necrosis factor (TNF) (Kwiatkowski *et al.*, 1990; Brown *et al.*, 1999; Akanmori *et al.*, 2000; Gimenez *et al.*, 2003) and the immune effector molecule nitric oxide in contributing to this multi-organ pathology have been made (Clark *et al.*, 2003). Other mediators of the inflammatory response which have been shown to act in a similar fashion include the pro-inflammatory cytokines; interleukin 1 (IL-1) (Brown *et al.*, 1999) and interferon gamma, and effector molecules such as nitric oxide intermediates (NOI) (Weiss *et al.*, 1998; Nmorsi *et al.*, 2010). Other studies have also shown the contribution of anti-inflammatory cytokines to the pathogenesis of severe malaria (Lyke *et al.*, 2004).

TNF is produced by monocytes, macrophages and lymphocytes, either via the direct induction by parasites and their products (Pichyangkul *et al.*, 1994) *P. falciparum* is known to produce hemozoin as a by-product of metabolism and while also found within the infected erythrocyte is another molecule called Glycosylphosphatidylinositol (GPI) otherwise known as the malaria toxin. This GPI has been shown to directly stimulate the production of TNF α and IL-1 from monocytes (Vijaykumar *et al.*, 2001; Krishnegowda *et al.*, 2005). However, the role of hemozoin as a potent stimulator of immune response is not clear. Macrophages containing significant amount of hemozoin were seen in the brain and other organs in postmortem studies of children who died of cerebral malaria (Milner Jr *et al.*, 2014). While studies have shown the capacity of this molecule to induce macrophage production of TNF there have also been evidence of inhibition of primary functions of macrophages by this same molecule (Metzger *et al.*, 1995).

Studies in Gambia and Ghana independently demonstrated high levels of TNF in cerebral malaria patients compared with uncomplicated malaria patients. These studies also showed a

significant rise in the levels of these receptors for this molecule (Kwiatkowski *et al.*, 1990; Akanmori *et al.*, 2000).

Nitric oxide is a molecule produced when L-arginine is converted to L-citrulline. This reaction is catalysed by nitric oxide synthase (NOS) which has three isoforms. These are inducible NOS, endothelium NOS and neuronal NOS (Stuehr *et al.*, 2006). NO is a popular vasodilator molecule and a potent neurotransmitter (Tuteja *et al.*, 2004) thus the overproduction of this molecule has been associated with coma in cerebral malaria (Clark *et al.*, 2003). NO has also been shown to inhibit endothelial activation and platelet aggregation (Laroux *et al.*, 2000; Loscalzo, 2001). Postmortem studies of cerebral malaria patients have demonstrated inducible NOS in cerebral vessels (Clark *et al.*, 2003). NO intermediates were also found to be elevated in CSF of children who died of cerebral malaria (Weiss *et al.*, 1998) and have also been shown to be potent killers of malaria parasites (Rockett *et al.*, 1991).

Interferon gamma is another pro-inflammatory cytokine known to be a potent activator of macrophages and other immune cells. Interferon gamma acts to induce the production of other cytokines and effector molecules including TNF and NOI (Clark *et al.*, 1997). Other cytokines produced by macrophages under the influence of interferon gamma are IL-1 and IL-6. Inducible NOS has also been shown to be produced by macrophages (Clark, 1987).

2.5 The role of hemostasis in the pathogenesis of cerebral malaria

Hemostasis is an intricate process which results in the formation of a blood clot and the subsequent cessation of blood flow into surrounding tissues following damage to blood vessels. The formation of the clot which seals any opening created in the vessels as result of the damage is a sum of the synergistic processes of platelet aggregation and the conversion of fibrinogen to

fibrin which provides a base to strengthen the platelet aggregate (Palta *et al.*, 2014). Following damage to a vessel wall, platelets adhere to cells adjacent to the opening of the vessel wall via their binding to receptors. The platelets become activated following this process and this sets off a feedback mechanism which results in the release of a number of molecules notably thromboxane and von willibrand factor from platelets and the endothelium. These molecules function predominantly in attracting and aggregating platelets at the site of the opening in the vessel wall. The platelet aggregate forms a plug-like structure which eventually seals the opening in the vessel (Heemskerk *et al.*, 2002). Following the escape of blood into surrounding tissues via the opening created by the damage to the vessel wall, blood coagulating factors specifically factor VII is exposed to tissue factor (TF) expressed by surrounding tissues. TF binds factor VII and results in the activation and amplification of a series of proteolytic reactions and the resultant formation of factor X (Mackman, 2004). Factor X then binds activated factor V and becomes activated resulting in the conversion of prothrombin to thrombin and the subsequent production of fibrin from fibrinogen. Fibrin forms a meshwork around the platelet aggregates holding it firmly in place. Platelet aggregation and production of fibrin occur rapidly and simultaneously to stop bleeding into tissues and thereby restore the normal flow of blood through the vessels (Heemskerk *et al.*, 2002; Palta *et al.*, 2014).

The clot formed at the site of injury is removed and subsequently replaced by tissue through the action of fibrinolytic system and blood inhibitors of coagulation like anti-thrombin which dissolves clots and maintain blood free of clot respectively (Ezihe-Ejiofor *et al.*, 2013). Another system, the anticoagulant system which is mediated by activated protein C and facilitated by thrombomodulin receptor and endothelial protein C receptor also ensure that blood does not contain clots by preventing its formation in the first place (Mosnier *et al.*, 2007; Bouwens *et al.*,

2013). Clots can block tiny vessels and thus obstruct blood flow through them. Thus, for hemostasis to ensure that there is uninterrupted and normal blood flow through the vessels, a fine balance between factors that favor the formation of clots and factors that preclude their formation, should be maintained (Palta *et al.*, 2014).

In cerebral malaria, postmortem studies have described the presence of hemorrhages, fibrin, thrombi and accumulation of platelets in the capillaries of the brain and some major organs of the body in children (Milner Jr *et al.*, 2014). These suggest that hemostasis is poorly regulated in cerebral malaria and may contribute to the pathogenesis.

Studies have also shown that thrombocytopenia, high levels of vWF, prolonged prothrombin time (PT) and partial thromboplastin time (PTT) are all features observed in cerebral malaria patients. These features specifically suggest a defect in the pathways mediating coagulation (Moxon *et al.*, 2009). Evidence of a potentially defective anticoagulant system has also come to light in recent studies on cerebral malaria pathogenesis. Loss of anticoagulant receptors such as endothelial protein C receptor and thrombomodulin receptors on the endothelium of blood vessels in the brain coupled with high levels of soluble forms in the CSF and plasma were demonstrated in these studies. This invariably points towards a defective anticoagulant pathway in cerebral malaria pathogenesis. Indeed, a model of the development of cerebral malaria mediated by a potentially defective anticoagulant pathway has been proposed by the same investigators (Moxon *et al.*, 2013).

Endothelial protein C receptor (EPCR) and thrombomodulin (TM) are constitutively expressed on the surfaces of the endothelium in most organs of the body but in the brain the quantity expressed is relatively low. These receptors particularly EPCR mediate the protein C pathway

which is divided into two main pathways: the anticoagulant pathway and the cytoprotective pathway. The anticoagulant pathway functions to prevent thrombosis in blood while the cytoprotective pathway functions to prevent inflammation, apoptosis, and endothelial barrier dysfunction. Protein C circulates in the body in an inactive conformation and is activated through the binding of thrombin. Activation of protein C is facilitated by the binding of TM to thrombin. Activated protein C (APC) in the presence of protein S acts as a catalyst to proteolytically break down activated factor V and VIII. The proteolytic consumption of these clotting factors and the binding of thrombin by TM effectively shuts out the coagulation pathway ensuring that thrombosis is prevented in blood. This anticoagulant pathway mediated by APC is greatly enhanced when it binds EPCR. The binding of EPCR also confers anti-inflammatory and anti-apoptotic properties to the endothelium while also protecting endothelial barrier loss and leakage. EPCR mediate the cytoprotective pathway by proteolytically cleaving protease activated receptor 1 (PAR -1) and setting off a series of reactions that leads to enhanced cell survival, anti-inflammatory effects and enhanced endothelial barrier protection (Mosnier *et al.*, 2007; Bouwens *et al.*, 2013).

In the pathogenesis of cerebral malaria, a theory has been proposed to explain pathological observations in the brain such as hemorrhage, fibrin and thrombi which incidentally correlates with diagnostic indices particularly retinal hemorrhage. Loss of TM and EPCR in the brain has been shown in cerebral malaria patients. Given that there is already low constitutive expression of TM and EPCR in the brain, the theory proposes that loss of these receptors lead to loss of anticoagulant and cytoprotective effects mediated by TM and EPCR leading to hemorrhage, deposition of fibrin and thrombi in the microvessels. This theory also explains the plausible roles of other features of cerebral malaria pathogenesis like sequestration, inflammation, endothelial

damage in pathological observations of the disease. Loss of receptors and marked fibrin deposition were associated with heavy sequestration of parasite infected erythrocytes in the brain suggesting that sequestration might have induced the loss of TM and EPCR. In the absence of EPCR which is a key mediator of the cytoprotective pathway, thrombin activates PAR-1 (Coughlin, 2005) and upregulates the expression of key pro-inflammatory molecules such as IL-6 and TNF with pleiotropic functions. These cytokines particularly TNF is a potent activator of the endothelium and this may result in the production of vWF and microparticles. Microparticles are vesicle-like membranous structures formed from the plasma membrane of cells (Chironi *et al.*, 2009). Increased numbers have been shown to be associated with endothelial activation and cerebral malaria (Combes *et al.*, 2004). They have been shown to express TF on their surfaces and this is crucial in thrombosis (Hrachovinová *et al.*, 2003).

2.6 Endothelial damage

The endothelium is the innermost layer of blood and lymphatic vessels separating blood and/or lymph from the surrounding connective tissues (Aird, 2007). The endothelium is comprised of a monolayer of endothelial cells of varying structure. These cells exhibit a high degree of structural variation to the extent that neighboring endothelial cells within the same vessel wall may be different. They have been shown to vary in size and thickness, to shape and the composition of cytoplasmic organelles (Muro *et al.*, 2004) and molecules expressed on their surfaces. Depending on the pattern of arrangement, presence or absence of holes and gaps within/between individual/adjacent cells and the underlying basement membrane, the endothelium has been described as continuous or discontinuous and with or without holes (fenestrations). For a continuous endothelium, endothelial cells as well as the underlying basement membrane are tightly linked to each other without gaps. A continuous endothelium

may be fenestrated or not with the latter referred to as discontinuous fenestrated endothelium. The marked variation in structure of endothelial cells between vessel walls in different tissues and organs reflect the complexity of the endothelium and their myriad of mediating functions. These functions include but are not limited to the regulation of hemostasis, movement of blood cells and molecules across vessel walls to target tissues and organs, and regulation of immune response (Aird, 2007).

The endothelium is widely recognized as metabolically active and given its plethora of physiological functions one will expect an actively proliferating tissue. This is usually not the case as the endothelium has been shown to be generally quiescent in healthy individuals (Aird, 2007). A significant alteration in endothelial functions during disease has been shown to result in necrosis and/or apoptosis of endothelial cells. These dysfunctional endothelial cells may detach from the endothelium precipitating a need for replacement and restoration of tissue integrity. In the healthy host, this process of endothelial cell turnover is nearly non-existent with the turnover of cells estimated to be less than one percent. Detached cells which may originate from a number of different sites in the body ultimately make their way into circulation hence the name circulating endothelial cells (CECs). The abnormal occurrence of these cells in the circulation defines endothelial damage (Goon *et al.*, 2004) and may be suggestive of a disease condition in the host. This is particularly true when one considers that these cells are rarely seen in the healthy host. More significantly levels of CECs as a potential marker of endothelial damage have correlated with several other surrogate markers of endothelial damage particularly von willibrand factor (vWF) (Nadar *et al.*, 2005; Chong *et al.*, 2006). Conversely, CECs rather than being a consequence of disease may also be a cause of disease as have been suggested in other disease conditions. In cancer, CECs may not reflect endothelial damage but may rather indicate

angiogenesis, a process of new endothelium and blood vessel formation. It has also been shown that detached endothelial cells rather than being senescent and dead cells may be viable cells with the potential to proliferate. This has been demonstrated in sickle cell anemia where these cells have been cultured with varying degrees of success (Solovey *et al.*, 1997).

Several mechanisms have been proposed to explain the process of endothelial cell detachment. Despite the differences in these mechanisms, loss of endothelial cells to the circulation appears to result from the breakdown in cell to cell and/or cell to molecule communications or interactions which results in loss of signals mediating cell survival (Re *et al.*, 1994). The endothelium is held to the connective tissues by an underlying basement membrane rich in macromolecules which make up the extracellular matrix. Some of these molecules include integrins, collagen, laminin, fibronectin, vitronectin, vascular endothelium (VE) cadherin among others (Timpl, 1989; Paulsson, 2008). Loss of endothelial cells has been observed in viral infections and this has been linked to a break down in interactions between basement membrane proteins and endothelial cells (Visser *et al.*, 1989; Grefte *et al.*, 1993). The role of inflammatory cytokines particularly tumor necrosis factor and interferon gamma have also been suggested to result in the loss of endothelial cells (Rüegg *et al.*, 1998). High levels of CECs were demonstrated in blood of patients who have undergone medical procedures suggesting that cause of endothelial damage or vessel wall injury could be mechanical.

In fact, the detection or the levels of CECs have been associated with endothelial damage in disease with high levels suggested to be predictive of disease severity (Koc *et al.*, 2005; Lee *et al.*, 2005). These have been demonstrated in a wide range of diseases including cardiovascular diseases, immunological diseases and infectious diseases. High levels of CECs have been enumerated in patients with unstable angina and acute myocardial infections (Mutin *et al.*, 1999;

Makin *et al.*, 2004; Wang *et al.*, 2005). Other related studies have also shown the utility of these cells in predicting disease severity. In congenital heart disease, patients diagnosed with irreversible pulmonary arterial hypertension recorded high levels of CECs in their blood compared with patients with reversible pulmonary arterial hypertension. This appears to suggest that endothelial damage may be more pronounced in patients with irreversible pulmonary arterial hypertension (Smadja *et al.*, 2009). High levels have also been shown in acute phase of other cardiovascular diseases including stroke, atrial fibrillation (Nadar *et al.*, 2005) and diabetes mellitus (McClung *et al.*, 2005). In vasculitis, studies have shown increasing levels and decreasing levels of CECs in patients with acute vasculitis and patients recovering from the disease respectively (Nakatani *et al.*, 2003; Woywodt *et al.*, 2003). High levels of CECs have also been shown in autoimmune diseases like systemic lupus erythematosus and systemic sclerosis. In rickettsia conorii infections, severe disease was associated with high levels of CECs. Significant reductions in the levels of these cells were also demonstrated after treatment (George *et al.*, 1993).

Endothelial damage marked by the loss of endothelial cells from the endothelium is known to be repaired by resident cells at the site of damage. These cells proliferate, generating new endothelial cells with the capacity to migrate and incorporate into damaged sites (Hanahan *et al.*, 1996; Holash *et al.*, 1999). Repair of damage has also been shown to be augmented where necessary by bone marrow derived cells which are released into circulation and have the capacity for proliferation, migration, differentiation into endothelial cells and incorporation into sites of vascular damage (Asahara *et al.*, 1997). These cells are termed endothelial progenitor cells. They are different from CECs by virtue of being derived from the bone marrow and showing properties of hematopoietic stem cells. Both cells have been differentiated by the expression of a

combination of surface biomarkers (Duda *et al.*, 2007). The mobilization of EPCs from the bone marrow as well as a rapid rise in levels has been suggested to be induced by conditions that may cause endothelial damage (Gill *et al.*, 2001).

Levels of EPCs have also been associated with various disease conditions and in a few cases have been used as prognostic index for disease (Vasa *et al.*, 2001; Schmidt-Lucke *et al.*, 2005; Kung *et al.*, 2016). Reduced levels of EPCs have been observed in cerebral malaria patients compared with control groups which included uncomplicated malaria patients, severe malaria anemia patients and healthy controls (Gyan *et al.*, 2009; Oduro, 2015). Levels of EPCs in cerebral malaria have been shown to increase as patients recover. Levels of these cells in these patients were significantly high 7 days post recovery from coma (Oduro, 2015). Low levels of endothelial progenitor cells have also been demonstrated in patients with systemic sclerosis (Mok *et al.*, 2010). Reduced levels have also been established in patients with chronic lung disease (Fadini *et al.*, 2006).

2.7 Measurement of biomarkers: Anti-VSA antibody responses, circulating endothelial cells and endothelial progenitor cells

Antibody responses to variant surface antigens, circulating endothelial cells and endothelial progenitor cells were measured using fluorescence-activated cell sorting (FACS) technique (Ofori, 2002; Gyan *et al.*, 2009). FACS is a derivation of flow cytometry which combines cell sorting abilities with the simultaneous measurement and analysis of multiple cell parameters or characteristics. Thus, a basic flow cytometer is a machine that is able to simultaneously measure and analyze multiple parameters of a cell which may include the size and internal granularity. The ability to measure the physical properties of these cells is enhanced by a fluidics system of

the flow cytometer that ensures that cells to be measured flow in a single file while they are illuminated by a beam of laser light which may be produced from a single or multiple sources. As each cell encounters the laser, light is either reflected, refracted or diffracted onto optical filters which direct these signals onto detectors. The detectors in turn convert these optical signals to electronic signals which is collected and analyzed by a computer.

Basic flow cytometer relies on the light scattering properties of cells to collect information on physical properties of cells. Light which is diffracted from the surface of the cell is collected as forward scattered light, the amount of which reflects the size of the cell. Similarly, light reflected or refracted is collected as side scattered light which is proportional to the internal complexity of the cell. Data collected on these light scattering properties is used to distinguish different populations of cells in a heterogeneous mixture based on their size and internal complexity.

The ability of antibodies to be conjugated to fluorescent dyes and the property of these dyes to absorb light of a particular wavelength and emit one of a different wavelength have been utilized in flow cytometry to improve measurements and cell sorting abilities. Multiple properties of cells expressing varying antigens or cell surface markers are measured and analyzed using the levels of fluorescence emitted by these fluorescent molecules. Using these fluorescence and light scattering properties of these cells a FACS machine can simultaneously measure, sort and analyze different populations of cells.

Data generated from these analysis are usually presented as dot plots which can be further analyzed by flow cytometry software. Subpopulations of cells indicated on these plots could be further sorted out and analyzed by gating the subpopulations of these cells (Biosciences, 2000) as illustrated in figure 2.2.

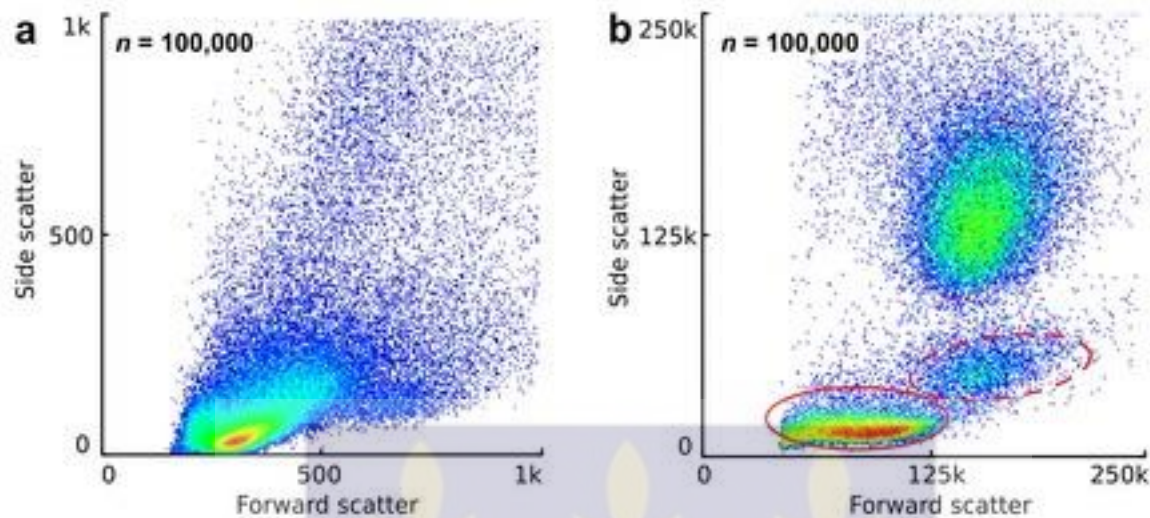


Figure 2.2: FACS analysis of whole blood

FACS of whole blood and of whole blood after red blood cell (RBC) depletion. **(a)** Forward vs. side scatter plot of whole blood showing a rather homogeneous distribution with no distinct peaks due to red blood cell scattering. **(b)** Depletion of red blood cells reveals three distinct subpopulations which can be identified as lymphocytes (47.6 %, red solid line), monocytes (3.7 %, dashed line) and granulocytes (38.7 %, dotted line) based on gating. Figure adapted from previous FACS analysis studies (Otto *et al.*, 2015).

2.8 Measurement of *Plasmodium falciparum* histidine rich protein 2 (PfHRP 2)

Plasmodium falciparum histidine rich protein 2 is a water soluble molecule produced by *P. falciparum* (Wellems *et al.*, 1986). This molecule is released by infected erythrocytes containing all stages of the parasite (Parra *et al.*, 1991). Thus, estimation of the concentration of this molecule in the blood may reflect the total parasite biomass in a patient including parasites which have been sequestered in the blood vessels and those found in circulation (Dondorp *et al.*, 2005). High concentrations of PfHRP 2 have been associated with severe malaria outcomes while healthy control samples have been shown to contain insignificant levels of the molecule.

More importantly, this molecule has been used to distinguish between severe outcomes of malaria particularly cerebral malaria (Seydel *et al.*, 2012). Using ELISA based techniques, the total parasite biomass (parasite density) could be estimated by measuring the concentration of *Pf*HRP 2 in plasma of parasite infected patients (Dondorp *et al.*, 2005).



CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study design

The study design is case control hence subjects were recruited based on the presence and absence of cerebral malaria. The cases included cerebral malaria patients while the controls comprised healthy children, uncomplicated malaria patients and severe malaria anemia patients. Uncomplicated malaria patients were recruited as part of the controls because of their potential risk of developing cerebral malaria following diagnosis with uncomplicated malaria. Severe malaria anemia which is also a complication of severe malaria was included as controls in order that any effect due to cerebral malaria may be clearly distinguished from severe malaria anemia.

3.2 Study sites

Patients diagnosed with the various categories of malaria were recruited from hospitals within Greater Accra region. These included Princess Marie Louise Hospital, La General Hospital, LEKMA Hospital, Ridge Hospital and Tema General Hospital. Healthy children were recruited from baby well/child welfare clinics and basic schools within and around the hospitals.

3.3 Duration of study/sampling time

Recruitment of patients into the study was carried out throughout the year primarily between May and October of 2014 and 2015 which coincided with the main malaria transmission season. Duration of study for individual subjects varied depending upon the nature of condition and time of patient recovery. The mean time for individual patient participation was 15 days. Samples were always obtained at the time of initial evaluation of the patient (day 0) regardless of the nature of condition, and on day 14 for cerebral malaria (CM) patients and uncomplicated malaria

(UM) patients. For cerebral malaria (CM) patients, a follow up sample was only obtained fourteen days after treatment initiation and post recovery from the coma.

3.4 Description of subject participation and methods for collecting specimens and data

Malaria patients between the ages of 1 and 12 years reporting for medical care were screened /recruited by rotating teams of study nurses and clinicians in study affiliated hospitals.

Two milliliters of venous blood was obtained from each study subject by the clinicians with appropriate consent from the patient/parent. Patients were screened for moderate or severe pallor on clinical examination. Children with hemoglobin levels of 7g/dL or less and who were hemodynamically stable were recruited into the study. Children not meeting this criteria were excluded. For children with severe malaria anemia, hemoglobin levels were invariably below 5 g/dL and therefore required transfusion. For these cohorts, a sample was only taken when the patient was stable and with the approval of the pediatrician, immediately before transfusion.

3.5 Primary outcomes measured

The primary outcome variables measured were plasma anti-VSA antibody responses and levels of circulating endothelial cells and endothelial progenitor cells. Figure 3.1 shows time points at which samples were taken.

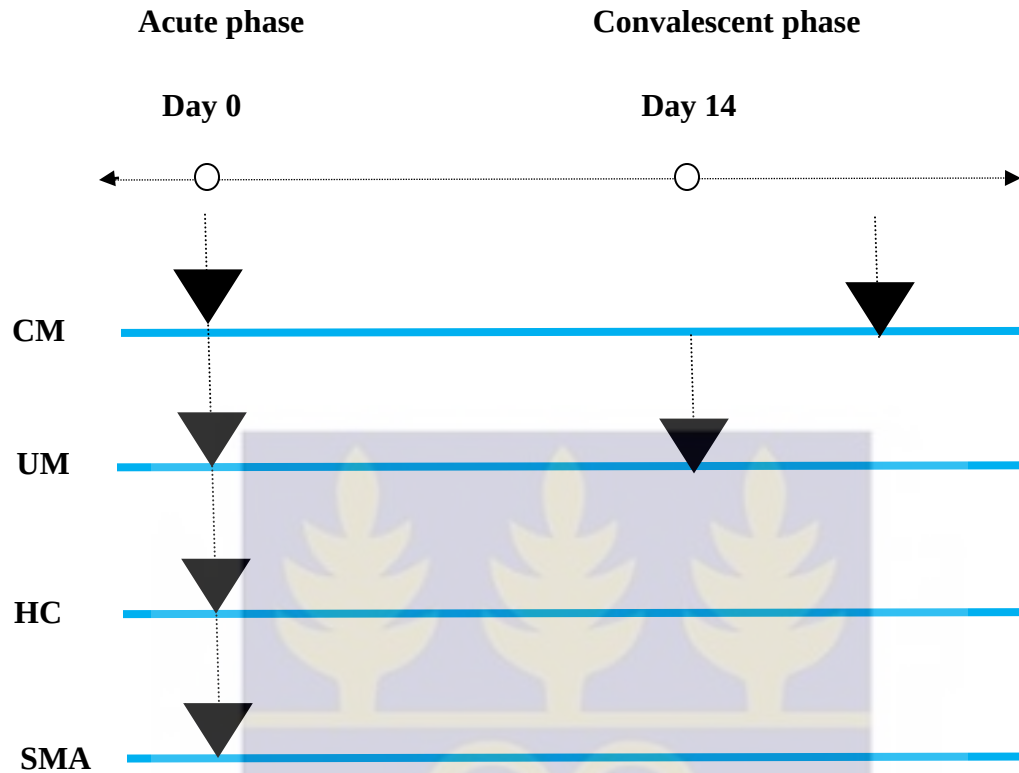


Figure 3.1: Time points for sample collection

Time points at which samples for each group were taken. Black arrows heads represent specific time points. For CM, samples were taken fourteen days following treatment initiation and post recovery from coma. For HC and SMA samples were only taken at one time point (day 0).

3.6 Inclusion/Exclusion Criteria

3.6.1 Specific Inclusion criteria

- **Clinical Malaria:** Presentation at a clinic with a history of fever or current fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) plus 5 or more *P. falciparum* parasites per high power field (HPF) (approx. 2500 /microliters). No other obvious cause found for the fever.
- **Cerebral Malaria:** Unconscious, with a Blantyre coma score (BCS) of ≤ 3 and duration of coma of at least 60 minutes, hemoglobin value $> 5\text{g/dL}$, no record of recent severe head trauma or other cause of coma or neurological diseases including meningitis/encephalitis (was assessed by culture of cerebrospinal fluid).
- **Uncomplicated Malaria:** Clinical malaria without any WHO criteria for severe malaria such as severe malaria anemia or respiratory distress. Patients were monitored briefly in the outpatient clinic at the discretion of the study physician prior to returning home; however, uncomplicated malaria patients were not hospitalized.
- **Severe Malaria Anemia:** Clinical malaria with hemoglobin $< 5\text{g/dl}$ without any WHO criteria for other severe malaria syndromes such as cerebral malaria or respiratory distress.
- **Healthy Controls:** No signs and symptoms of clinical malaria. Additionally, there should be no demonstration of parasites in the blood using microscopy.

3.6.2 Specific Exclusion Criteria

- Parent, guardian or proxy unable to give informed consent.
- Unwillingness to comply with requirements of the protocol.

- Evidence of concomitant infection at time of enrollment including meningitis/encephalitis and bacteremia.
- History of any underlying disease that compromised the diagnosis and outcome of the illness including chronic viral infection, chronic weight loss, diarrhea.
- Diseases/conditions known to alter levels of EPCs or induce endothelial damage: recent severe bleeding, sickle cell disease or trait, evidence of bacterial or viral infection, history of diabetes mellitus , cardiovascular disease, Hypertension or hypercholesterolemia, surgery within 1 month, bone fracture within 3 months, major trauma within 1 month (e.g. car accident), blood transfusion within 3 months.

3.7 Sample Size

The number of study participants required for this study was 185. This was determined by the formula below using the estimated prevalence of malaria in 2013 among children in sub-Saharan Africa. The prevalence of malaria was estimated to be 14 percent WHO (2014). The calculated sample size number was reduced to 80 as a result of the relatively low prevalence of severe malaria and the potential difficulty in recruiting these number of subjects particularly cerebral malaria patients within the period allotted for the study. In all, seventy two study participants were successfully recruited into the study.

$$N = \frac{t^2 \times p \times (1-p)}{m^2}$$

N - Sample size

t - Confidence level of 95% (standard value of 1.96)

m - Margin of error of 5% (standard value of 0.05)

p - Estimated prevalence of disease to be studied

3.8 Laboratory evaluations / assays

Patients with suspected malaria had laboratory tests done as required for routine care including: Full blood count, blood film for malaria parasites, sickle cell anemia test, blood cultures and cerebrospinal fluid cultures supplemented with biochemistry tests in patients with suspected cerebral malaria. In addition, two milliliters of peripheral blood was drawn for study related tests. Blood was collected into EDTA and heparin microtubes for further processing.

3.8.1 Processing of samples

Blood containing EDTA was spun at 1500 rpm for 10 minutes at 4°C but only after an aliquot has been taken for the estimation of levels of circulating endothelial cells and endothelial progenitor cells. The resulting plasma and RBC pellets were separated into cryovials and stored at -30°C. Blood collected into heparin containing microtube was spun at 1000 RCF for 15 minutes at 4°C. Plasma was stored at -30°C for later use. To the resulting RBC pellets, equal volume of freeze mix was added and stored in liquid nitrogen at – 196°C for later use. Processing of samples was carried out in Biosafety Cabinets (BSC) and aseptic protocol in handling these samples were followed diligently.

3.8.2 Estimation of levels of circulating endothelial cells and endothelial progenitor cells

Volumes of 2 microliters and 4 microliters each of blocking antibodies were added to two separate sets of falcon tubes comprising of four and two tubes respectively. Fifty microliters of blood samples collected in EDTA were added to each tube in the first set. To the second set of tubes, 100 microliters of blood was added. Tubes were vortexed gently at low speed for 10 seconds and then incubated at 4°C for 10 minutes. Three microliters of conjugated monoclonal

antibodies (BD biosciences, San Jose, California) were then added after incubation. The mixture was vortexed for 10 secs and incubated again at 4°C for 30 minutes. Pipetting and incubation were carried out in the dark to protect samples from light. Details of addition of fluorescent dye conjugated monoclonal antibodies are shown below in table 3-1. Following incubation, tubes were vortexed for 10 seconds and lysed with BD FACS lyse solution. Two milliliters of FACS lyse buffer was added to all tubes. Again, tubes were vortexed and incubated for 10 minutes at room temperature. Tubes were centrifuged at 1500 rpm for 7 minutes at 15°C. The resulting supernatant was discarded and the pellets re-suspended in 3 milliliters cold FACS flow at 4°C. The washing procedure was repeated and the resulting cells re-suspended in 250 microliters of cold FACS flow for acquisition of events. Using a Flow cytometer, 100000 events and 250000 events were acquired for the first and second sets of tubes respectively. Endothelial progenitor cells comprised percentage of cells among the population of leucocytes expressing these unique combination of cell surface biomarkers: CD45dimVEGFR2+CD34+CD133+ while circulating endothelial cells expressed on their surfaces CD11b, CD34+CD133+CD31bright.

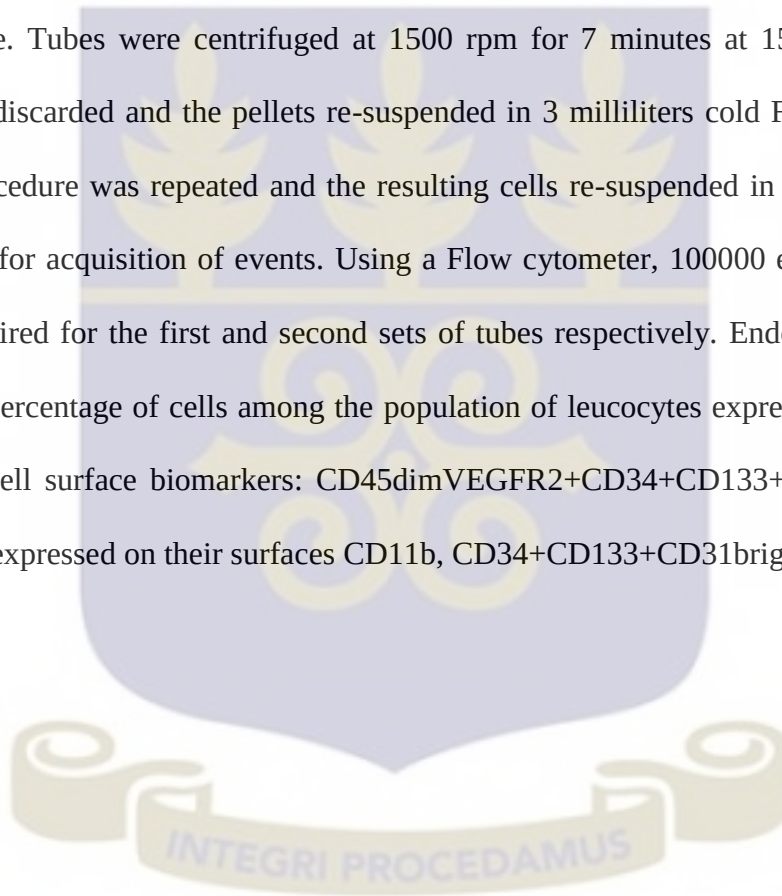


Table 3.1: Monoclonal antibodies for circulating endothelial cells and endothelial progenitor cell surface markers, and controls

Tube	Flourescein isothiocyanate conjugated monoclonal antibody (Vol. 3ul)	Phycoerythrin conjugated monoclonal antibody (Vol. 3ul)	Peridinin chlorophyll conjugated monoclonal antibody (Vol. 3ul)	Allophycocyanin conjugated monoclonal antibody (Vol. 3ul)
1	Unstained (Remains at 4°C)			
2	M-IgG1 k	M-IgG1	M-IgG1k	M-IgG
3	R-IgG 2b	M-IgG1	M-IgG1k	M-IgG
4	CD 15	CD 14	CD 4	CD 8
5	CD 11b	CD 133	CD 34	CD 31
6	CD 45	CD 133	CD 34	CD 309 (VEGFR-2)



3.8.3 Cultivation of parasites

Cryovials containing infected erythrocytes which had been preserved in freeze mix was removed from storage and placed in a 37°C water bath for 2 minutes to thaw. The thawed sample was then spun at 1500 rpm for 10 minutes. The resulting supernatant was collected and discarded. To the remaining RBC pellets, an equal volume of parasite thawing mix was added. The mixture was again spun at 1500 rpm for 10 minutes, and the resulting supernatant was collected and discarded as done before. 500 microliters of complete parasite medium (CPM) was then added, mixed gently and again spun at 1500 rpm for 10 minutes. The supernatant was collected and discarded again, after which 1 milliliter of CPM was finally added to complete the thawing procedure. Parasites were now ready for inoculation into culture flasks.

Five milliliters of complete parasite medium (CPM) was added to the sterile T-25 tissue culture flasks obtained from BD Biosciences. To this flask containing CPM, a volume of erythrocytes containing parasites and 200 microliters of packed erythrocytes were added. The mixture was then flushed gently with a special mix gas preparation for 30 seconds after which the flask was tightly capped and incubated at 37°C. Flask was labeled accordingly with date and specimen ID. CPM used was warmed to 37°C before use. Again, these procedures were carried out in a Biosafety Cabinet. All aseptic and safety protocols were properly followed.

Complete parasite medium was changed after 48 hours following inoculation of parasites and thereafter, changed on a daily basis to replenish used up nutrients in the media. Five milliliters of CPM was added following removal of used up media. A thin film slide was also prepared from the resulting erythrocyte pellets prior to addition of fresh media (CPM). Slide was stained accordingly, and the parasitemia and conditions of the parasite determined. Following addition of fresh media and flushing of mixture with a gentle flow of special mix gas, the flask was

incubated at 37°C. Flask was sub cultured when the parasitemia was determined to be more than two percent. All samples were aseptically processed in biosafety cabinets using recommended protocols. Details of the preparation of culture media and other reagents used is provided in appendix C.

3.8.3.1 Staining and determination of parasitemia

Properly prepared thin film slides from erythrocyte pellets of culture flasks were fixed in methanol for 30 seconds after adequately dried. The fixed smears were allowed to dry after which, they were stained with a 10 percent working solution of Giemsa stain for 10 minutes. Following staining, the slides were allowed to dry completely and parasitemia determined accordingly. Parasitemia, which denotes the percentage of infected erythrocytes equals the number of infected erythrocytes counted, divided by the total number of erythrocytes counted all multiplied by 100. A minimum of 500 cells were counted. Multiple infected cells as well as gametocyte infected cells were not included. Flasks that had more than 2 percent parasitemia were subcultured accordingly.

3.8.3.2 Subculturing of parasites

Parasitemia approximating 2 percent in a flask was reduced to 1 percent by inoculating a new flask with a volume of blood from the old flask. This was done to ensure that, rate of growth of parasites were not affected. The volume of blood required to achieve a parasitemia of 1 percent in both flasks were determined. Packed RBCs were added to both flasks to maintain the 4 percent hematocrit required. Five milliliters CPM was then added and the mixture gently flushed with a special gas mix for 30 seconds. Both flasks were then incubated at 37°C. Media were changed regularly on a daily basis.

3.8.4 Separation of late stage parasite infected erythrocytes

Parasite infected erythrocytes containing late trophozoites and schizonts were separated from uninfected cells and ring stages of the parasite by subjecting the cultures of appropriate parasitemia to Magnetic Activated Cell Sorting (MACS; Miltenyi BioTec, Bergisch Gladbach, Germany) technique (Radbruch *et al.*, 1994). MACS provide a high gradient magnetic field enabling malaria pigment producing parasites to be purified. Cultures approximating 4% parasitemia and containing sufficient late stage parasites (pigment producing parasites) were used. Cultures were centrifuged at 2000 rpm for 5 minutes. The supernatant was then discarded leaving behind the resulting erythrocyte pellets. The pellets were re-suspended in 2 milliliters of 2 percent FCS/PBS and loaded on top of the MACS column which had previously been incubated with 2 percent FCS/PBS for 10 minutes. The complete MACS set up comprised the iron filings containing Size C MACS column held in position between two magnets on the MACS equipment and a three way stopcock connected to the column. The three way stopcock was fitted with a 10 milliliters syringe and a 21 G pore size needle. Openings on the stopcock were regulated using a knob fitted on the device.

Loaded pellets containing infected erythrocytes were allowed to fully migrate into the matrix of the column. The matrix is the portion of the column containing the iron filings. The loaded pellets were incubated for 10 minutes in the column ensuring that pigment containing parasites were adequately attracted and held onto the iron filings within the magnetic field. Following incubation, the column was then washed with 50 milliliters of 2 percent FCS/PBS through the previously closed opening between the needle and the column.

To harvest pigment containing parasites, the column was completely removed from the MACS equipment and the needle disconnected from the three way stopcock. The column was then

inverted with the larger opening facing over a 50 milliliters falcon tube and using the syringe connected to the three way stopcock, the eluate was collected into the tube by flushing 50 milliliters of 2 percent FCS/PBS. The eluate was centrifuged at 1500 rpm for 10 minutes and the concentration of the harvested parasites determined.

3.8.5 Determination of concentration of parasites

Following centrifugation of the eluate, the resulting pellets were resuspended in 3 milliliters of 2 percent FCS/PBS. An aliquot of 20 microliters was reconstituted to 200 microliters. Parasites were counted using a calibrated counting chamber, counting all four large corner squares. The average number of cells/square as well as the dilution factor was calculated. From the resulting values, the concentration (cells/milliliters) was determined using the formula below. The concentration was then adjusted such that an aliquot of 100 microliters contained 2×10^5 cells.

$$\text{Concentration (cells/milliliters)} = \text{Average number of cells/square} \times \text{Dilution factor} \times 10^4$$

$$\text{Average number of cells/square} = \text{Number of cells} / \text{number of squares}$$

$$\text{Dilution factor} = \text{Final volume} / \text{initial volume}$$

3.8.6 Measurement of plasma anti-VSA antibody responses

Following adjustment of the concentration to 2×10^5 cells, a 100 microliters aliquot was added to 5 microliters of plasma from both patients and control samples. Control samples included plasma samples from donors who have not been exposed to any form of *Plasmodium* spp. infections and plasma from adults who live in malaria endemic areas. Five serial five-fold dilutions were prepared for the plasma pool of positive control. Plasma and parasite preparation was then incubated for 30 minutes at 5°C. Following incubation, the tubes were washed by adding 2

milliliters of 2 percent FCS/PBS, centrifuged at 2000 rpm for 5 minutes and the resulting supernatant discarded. Washing of tubes containing the parasites and plasma were repeated twice. Cells were re-suspended by gently agitating the tubes after each washing step. 100 microliters of Goat Anti-Human IgG-FITC conjugated (Dako, Glostrup, Denmark) and Ethidium bromide in the preparation 1:100 + 20 microliters/milliliters respectively was added thereafter and also incubated for 30 minutes at 5°C. Following incubation tubes were washed twice as described above. Cells were then re-suspended in 200 microliters of washing buffer and acquired using a Flow Cytometer (BD FACS Calibur, San Jose, California). Ten thousand cells were acquired with results expressed as median fluorescence intensity (MFI).

3.8.7 Measurement of *Plasmodium falciparum* histidine rich protein 2 (PfHRP 2)

Plasmodium falciparum histidine rich protein 2 was estimated using a quantitative sandwich enzyme immunoassay technique. Samples and reagents including but not limited to working standards and controls were brought to room temperature and prepared based on instructions by Kit manufacturers (R & D Systems Inc., Minneapolis, Minnesota). One hundred microliters of assay diluent was added to wells which had been pre-coated with antibodies specific for the analytes immediately before the addition of the samples, working standards and controls. For each well, 100 microliters was added. Following this step, the plate was covered with an adhesive strip provided. The position of reagents and samples were then recorded on a microplate template provided. The plate was then incubated for two hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. The contents of the well were aspirated following incubation and the plates washed with a washing buffer (0.05% Tween 20 in Phosphate Buffered Saline). The washing step was repeated three times making a

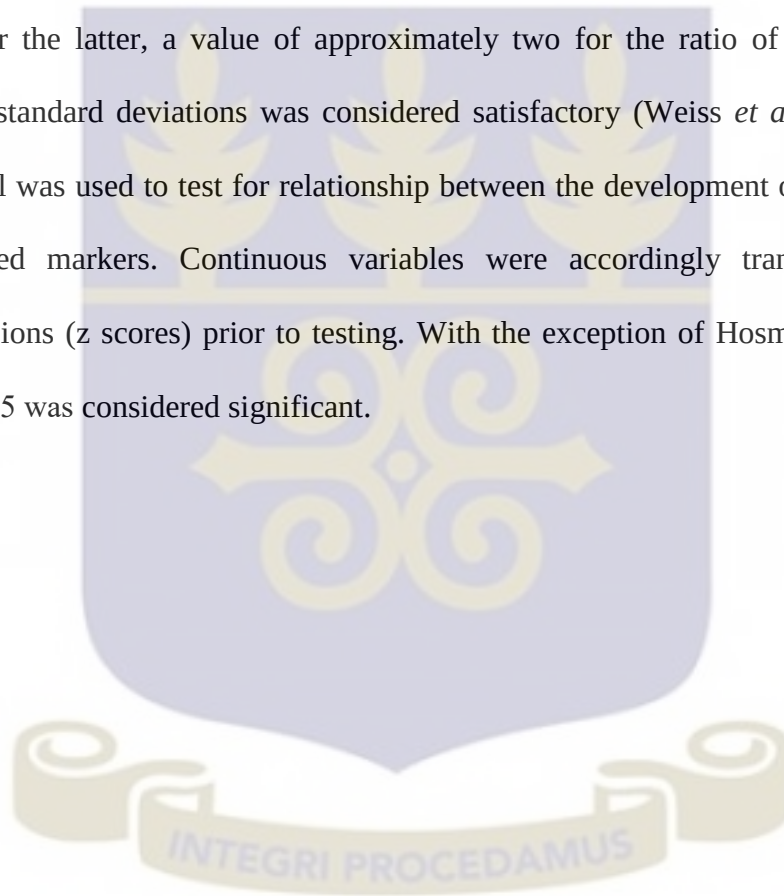
total of four washes. To ensure that there was no washing buffer left behind, the plates were inverted and blotted with a clean paper towel.

Two hundred microliters of enzyme linked antibody specific for the analytes were added to the wells, covered with an adhesive strip and then incubated as described above. Again following incubation, the plates were aspirated and washed four times as described above. The plates were then blotted using a clean paper towel. The aspiration, washing and blotting steps ensured that unbound substances were completely removed. Next, 200 microliters of substrate solution was added to each well, and incubated for 30 minutes in the dark. The reaction between the enzyme and the substrate was stopped by adding 50 microliters of stop solution to the wells. This resulted in a change in color from blue to yellow in the wells with varying intensities. The optical densities of the wells were then determined using a microplate reader (EL808 BioTek, Winooski, Vermont) set at 450nm. The levels of HRP 2 were determined from the optical densities recorded.

3.9 Statistical analysis

Flow cytometry data was analyzed using FlowJo Software Version 10 (FlowJo LLC, Ashland, Oregon). Results for levels of anti-VSA antibody responses were presented as median fluorescence intensity (MFI) while that of levels of circulating endothelial cells and endothelial progenitor cells were presented as percentage of cells analyzed. All subsequent analysis were carried out using GraphPad Prism Software Version 5.01 (GraphPad Software, Inc., San Diego, California) and SigmaStat Software Version 3.5 (Systat Software, Inc., San Jose, California). Graphs summarizing data were drawn using GraphPad Prism Software Version 5.01. For comparisons of means between two groups with matched samples, Wilcoxon sign ranked test

was used. Analysis of variance (ANOVA) was used for comparisons between three or more groups. When the differences between groups were found to be statistically significant, Tukey's multiple comparison test were carried out to determine differences between paired groups. Calculations of ANOVA were preceded by log transformation of data when there was evidence for violation of normality and equal standard deviation assumptions. The former was satisfied if the absolute values of skewness and kurtosis were less than two and nine respectively (Schmider *et al.*, 2010). For the latter, a value of approximately two for the ratio of the largest to the smallest sample standard deviations was considered satisfactory (Weiss *et al.*, 2008). Logistic Regression model was used to test for relationship between the development of cerebral malaria and the estimated markers. Continuous variables were accordingly transformed to their standardized versions (z scores) prior to testing. With the exception of Hosmer-Lemeshow test (H-L test) $p < 0.05$ was considered significant.



CHAPTER FOUR

4 RESULTS

4.1 Clinical characteristics

Clinical characteristics of approximately sixty four subjects for which there were available data have been summarized below. The number of subjects (sample size) for each group varied for each parameter or characteristic depending on available data.

Table 4.1: Summary of clinical data obtained from the study participants

Clinical Characteristics	Cerebral Malaria	Severe Malaria Anemia	Uncomplicated Malaria	Healthy Controls	<i>p</i> value
	n = 17	n = 14	n = 8	n = 25	
Age, mean $\pm$ SD	5.35 $\pm$ 3.20	2.62 $\pm$ 1.28	6.57 $\pm$ 2.99	8.16 $\pm$ 3.81	0.0002
Min age, max age	0.67, 12.00	1.00, 6.00	3.00, 11.00	1.5,12.0	
Male sex, n (%)	13 (76.47)	7 (50)	6 (75)	12 (48)	
Other findings, mean $\pm$ SD					
Hemoglobin, g/DL	8.53 $\pm$ 2.67	4.39 $\pm$ 0.62 ***	11.00 $\pm$ 1.16 **	12.18 $\pm$ 0.79 ***	< 0.0001
Platelets $\times 10^3/\mu\text{L}$	122.30 $\pm$ 147.80	115.20 $\pm$ 71.43	174.4 $\pm$ 113.90	329.90 $\pm$ 77.78 ***	< 0.0001
WBC $\times 10^3/\mu\text{L}$	10.79 $\pm$ 6.81	12.81 $\pm$ 7.64	7.25 $\pm$ 2.22	7.07 $\pm$ 3.46	0.0074
Parasite density	3002 $\pm$ 3021	1648 $\pm$ 2288	177.6 $\pm$ 331.7 **		0.0042

*SD, Standard deviation; sample size (n), For each variable differences between study groups were calculated using one way analysis of variance. Asterisks indicate post hoc test analysis between groups with cerebral malaria group as a comparison group. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.*

4.1.1 Age and gender

The study participants which included approximately forty one percent females were between the ages of 8 months and 12 years. There were statistically significant differences in ages between the groups (ANOVA, $F(3,57) = 7.74$, $p = 0.0002$). Post hoc tests indicated that, the mean age of SMA patients ($M = 2.62$, $SD = 1.28$) was significantly lower than both UM ($M = 6.57$, $SD = 2.99$) and HC ($M = 8.16$, $SD = 3.81$). There were no significant differences between the other groups. Overall, these results suggest that with the exception of SMA patients, all the other study groups were similar in age.

4.1.2 Hemoglobin levels

Comparison of the mean hemoglobin levels between the groups revealed that there was a significant difference between the groups. (ANOVA, $F(3,60) = 93.16$, $p < 0.0001$). Post hoc analysis revealed that the mean level of hemoglobin in the HC cohort ($M = 12.18$, $SD = 0.79$) was significantly higher than UM ($M = 11.00$, $SD = 1.16$), CM ($M = 8.54$, $SD = 2.67$) and the SMA group ($M = 4.39$, $SD = 0.62$). As expected the SMA group had the lowest level of hemoglobin and was significantly lower when compared to the other groups.

4.1.3 Platelets

Mean platelet levels when compared between the groups showed that the differences were statistically significant (ANOVA, $F(3,60) = 21.3$, $p < 0.0001$). Post-test analysis indicated that the mean levels of platelets were significantly higher in HC ($M = 329.9$, $SD = 77.78$) group than CM ($M = 122.3$, $SD = 147.8$), SMA ($M = 115.2$, $SD = 71.43$) and UM group ($M = 174.4$, $SD = 113.9$). There were no significant differences in the other groups.

4.1.4 Parasite density

Parasite density was estimated by measuring the levels of *Plasmodium falciparum* histidine rich protein 2 (*PfHRP 2*) in samples obtained from the study participants. The levels of *PfHRP2* were significantly different between the malaria groups (ANOVA, $F(2,32) = 6.53$, $p = 0.004$). Post hoc test analysis revealed that the levels of *PfHRP 2* were lower in the UM group ($M= 178$, $SD = 332$) compared with both CM ($M= 3002$, $SD = 3021$) and SMA groups ($M= 1648$, $SD= 2288$). There were no significant differences between CM and SMA groups.



4.2 Marked variation in anti-VSA_{CM} antibody responses between study groups

One of the core objectives of this study was to determine the levels of antibody responses to variant surface antigens (VSA) of parasite isolates obtained from cerebral malaria (CM) patients as well as other parasite isolates obtained from uncomplicated malaria (UM) patients and severe malaria anemia (SMA) patients. For all the VSAs of the three isolates tested, there were significant differences in the mean levels of plasma anti-VSA_{CM} antibody responses between the study groups (ANOVA, CM₁, $F(3, 66) = 2.78, p = 0.048$; CM₂, $F(3, 66) = 4.50, p = 0.006$; CM₃, $F(3, 68) = 2.88, p = 0.042$). Post hoc test revealed that, for the CM₃ parasite isolate, the mean level of anti-VSA_{CM} antibody responses was higher in the CM group than the HC. There were no significant differences in mean levels of anti-VSA antibody responses between the other paired groups. For CM₂ isolate, the mean level of anti-VSA_{CM} antibody responses among the SMA group was significantly higher when compared with the HC. There were no differences in the mean levels of anti-VSA antibody responses between the other paired groups. The pattern of anti-VSA_{CM} antibody responses for CM₁ isolate was different as there were no differences in the mean levels of anti-VSA antibody responses between the groups after pairwise comparisons. The variation in the anti-VSA_{CM} antibody responses between the groups for all three parasite isolates is illustrated in figure 4.1.

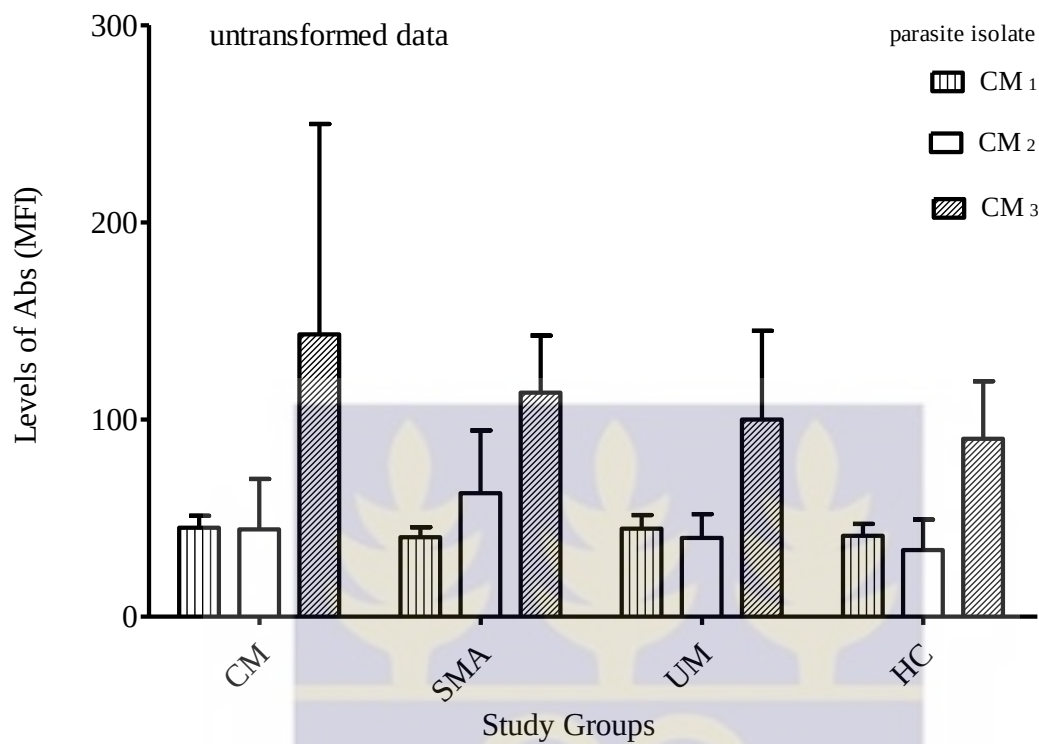
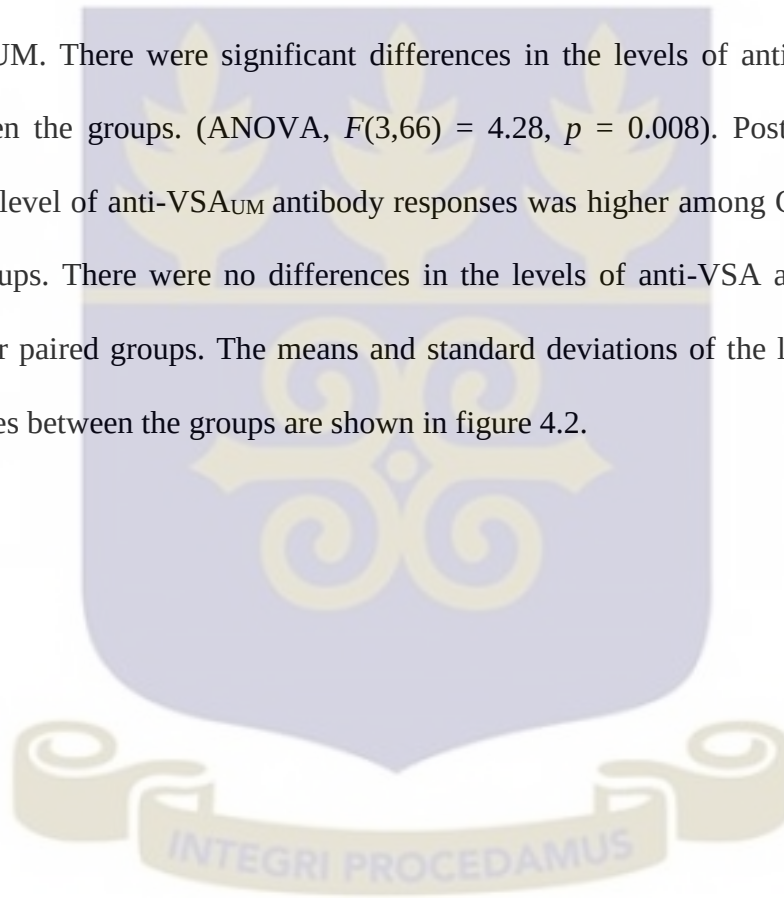


Figure 4.1: Levels of antibody responses to variant surface antigens of three parasite isolates obtained from cerebral malaria patients.

The column bars represent the mean levels of antibody responses to the variant surface antigens among the study groups while the error bars represent their corresponding standard deviations.

4.3 Levels of anti-VSA_{UM} responses is higher in cerebral malaria patients than uncomplicated malaria patients and healthy controls

Having evaluated the pattern of anti-VSA_{CM} antibody responses between the study groups, plasma from the study participants comprising 18 cerebral malaria (CM) patients, 15 severe malaria anemia (SMA) patients, 9 uncomplicated malaria (UM) patients and 28 healthy controls (HC) patients were tested against VSA of a parasite isolate obtained from a patient who had been diagnosed with UM. There were significant differences in the levels of anti-VSA_{UM} antibody responses between the groups. (ANOVA, $F(3,66) = 4.28$, $p = 0.008$). Post hoc test analysis revealed that the level of anti-VSA_{UM} antibody responses was higher among CM group than the UM and HC groups. There were no differences in the levels of anti-VSA antibody responses between the other paired groups. The means and standard deviations of the levels of anti-VSA antibody responses between the groups are shown in figure 4.2.



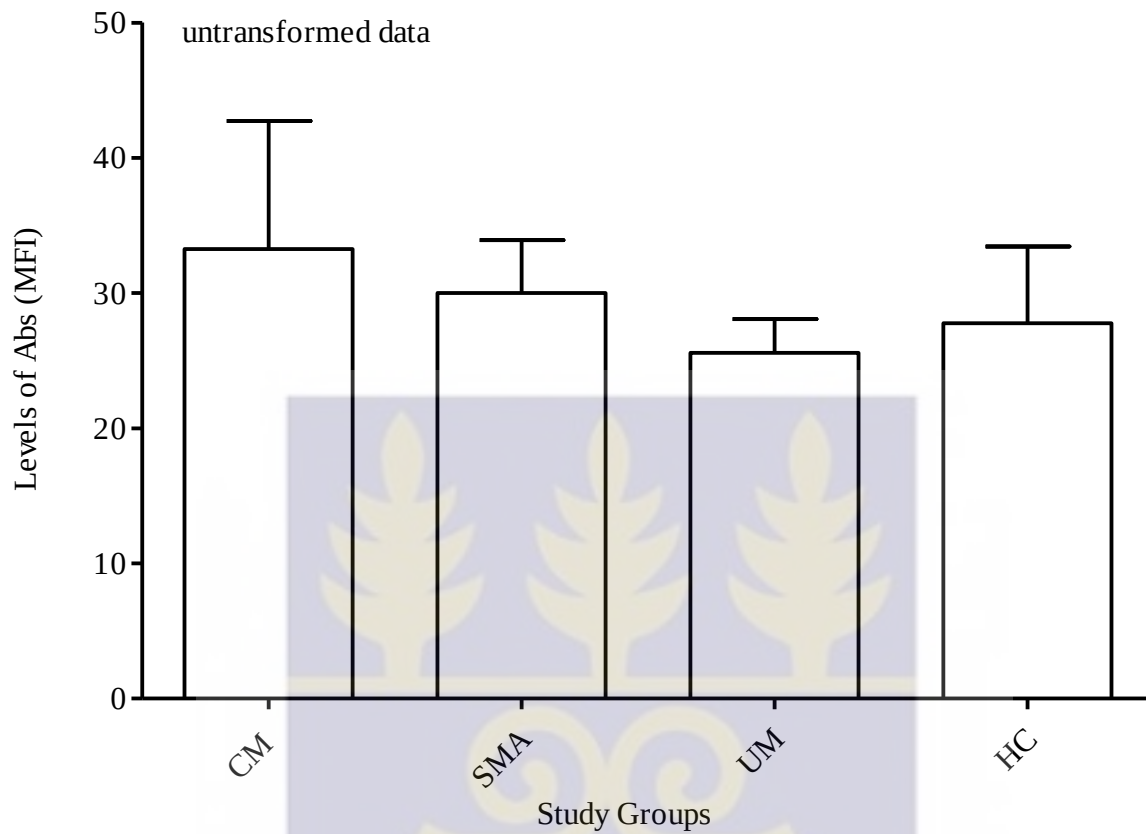
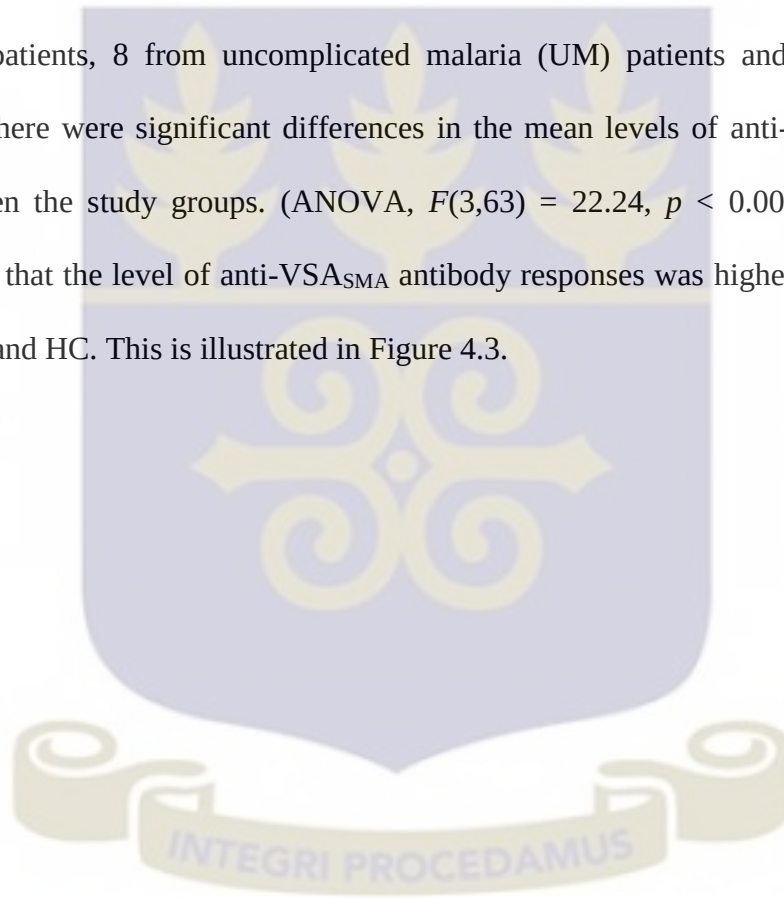


Figure 4.2: Levels of antibody responses to variant surface antigens of a parasite isolate obtained from an uncomplicated malaria patient.

The column bars represent the mean levels of antibody responses to variant surface antigens of the parasite isolate while the error bars represent their corresponding standard deviations.

4.4 Levels of anti-VSA_{SMA} antibody responses is higher in cerebral malaria patients than the other study groups

To determine the levels of anti-VSA_{SMA} antibody responses among the study groups, plasma obtained from the study participants were tested against VSA of a parasite isolate obtained from a patient who had been diagnosed with severe malaria anemia. Samples for which there was adequate plasma included 17 from cerebral malaria (CM) patients, 15 from severe malaria anemia (SMA) patients, 8 from uncomplicated malaria (UM) patients and 27 from healthy controls (HC). There were significant differences in the mean levels of anti-VSA_{SMA} antibody responses between the study groups. (ANOVA, $F(3,63) = 22.24$, $p < 0.0001$). Post hoc test analysis revealed that the level of anti-VSA_{SMA} antibody responses was higher in the CM group than SMA, UM, and HC. This is illustrated in Figure 4.3.



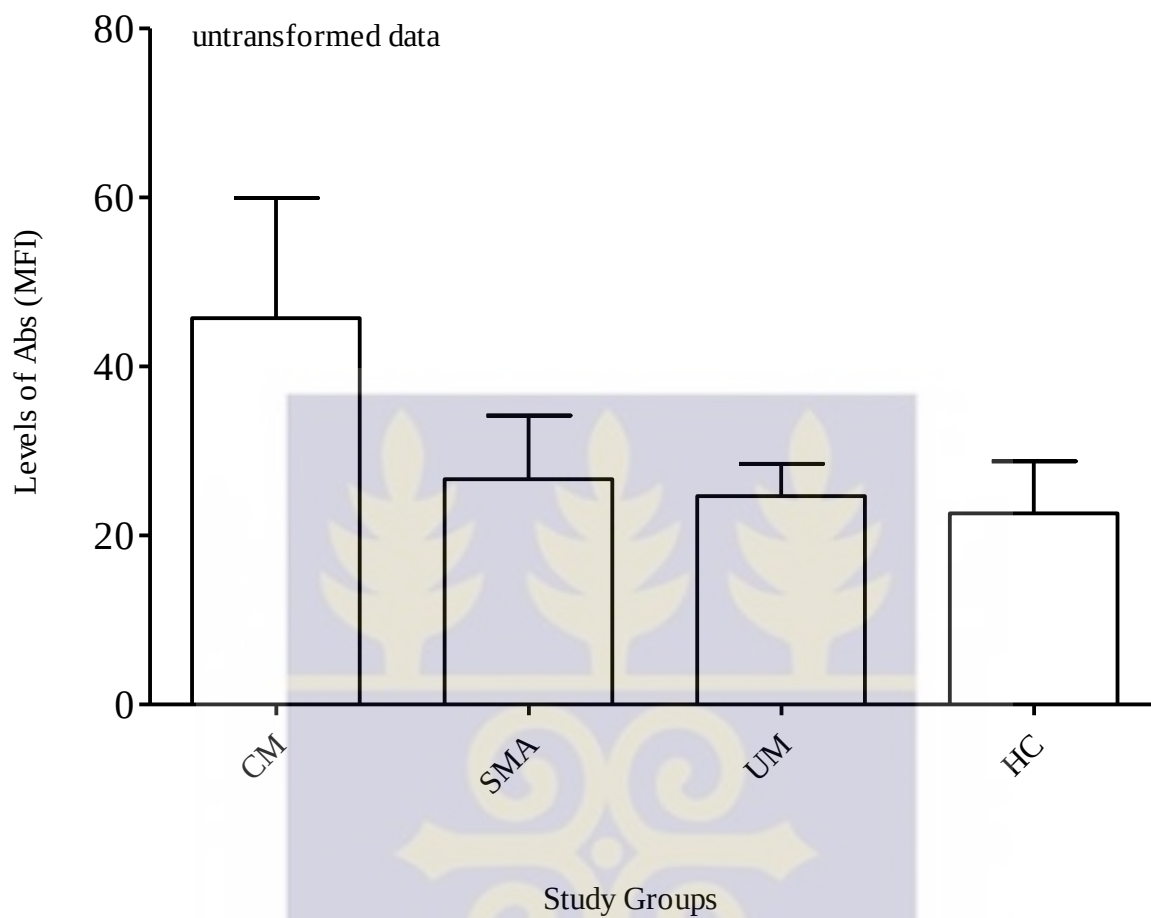
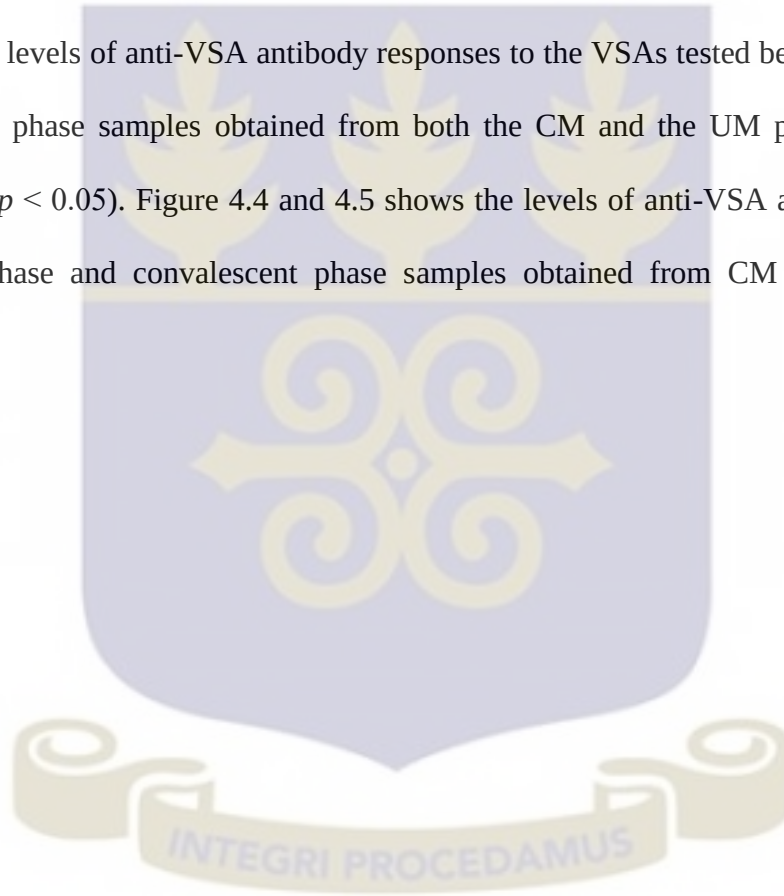


Figure 4.3: Levels of antibody responses to variant surface antigens of parasite isolates obtained from a severe malaria anemia patient.

The column bars represent the mean levels of antibody responses to the variant surface antigens among the study groups while the error bars represent their corresponding standard deviations.

4.5 There is no association between acute phase and convalescent phase plasma anti-VSA antibody responses

Two sets of six separate convalescent phase plasma samples were obtained from cerebral malaria patients and uncomplicated malaria patients who were successfully followed up till day 14. Convalescent phase samples from uncomplicated malaria patients (UM) were obtained fourteen days post initial evaluation of the patient and initiation of treatment. There were no significant differences in the levels of anti-VSA antibody responses to the VSAs tested between acute phase and convalescent phase samples obtained from both the CM and the UM patients (Wilcoxon signed rank test, $p < 0.05$). Figure 4.4 and 4.5 shows the levels of anti-VSA antibody responses between acute phase and convalescent phase samples obtained from CM and UM patients respectively.



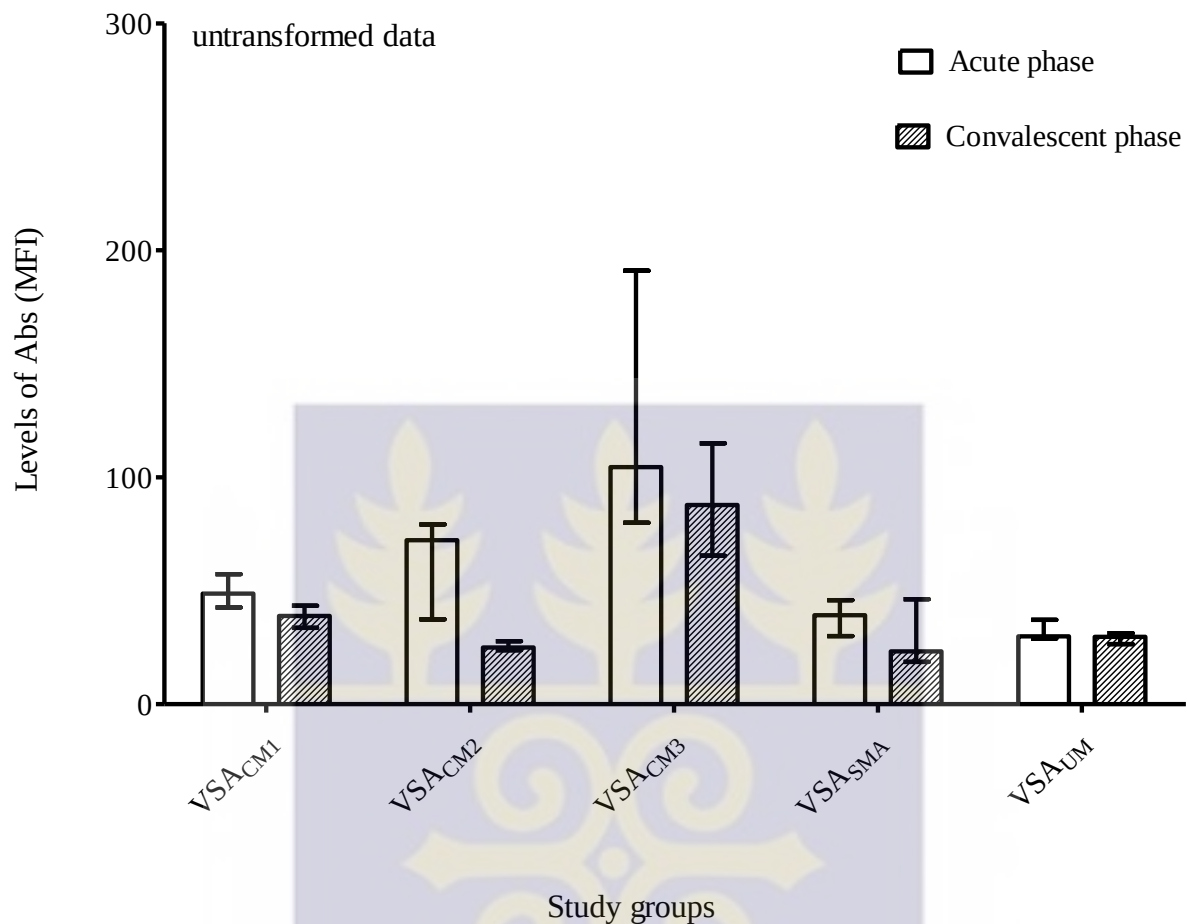


Figure 4.4 : Levels of acute phase and convalescent phase plasma anti-VSA antibody responses of cerebral malaria patients.

Variant surface antigens of the parasite isolates were obtained from patients who had been diagnosed with cerebral malaria, as well as patients diagnosed with severe malaria anemia and uncomplicated malaria. The column bars represent the median levels of anti-VSA antibody responses for the cerebral malaria patients while the error bars represent the interquartile range. The levels of anti-VSA antibody responses to the various VSAs are shown on the y-axis and the VSAs of the various parasite isolates are shown on the x-axis.

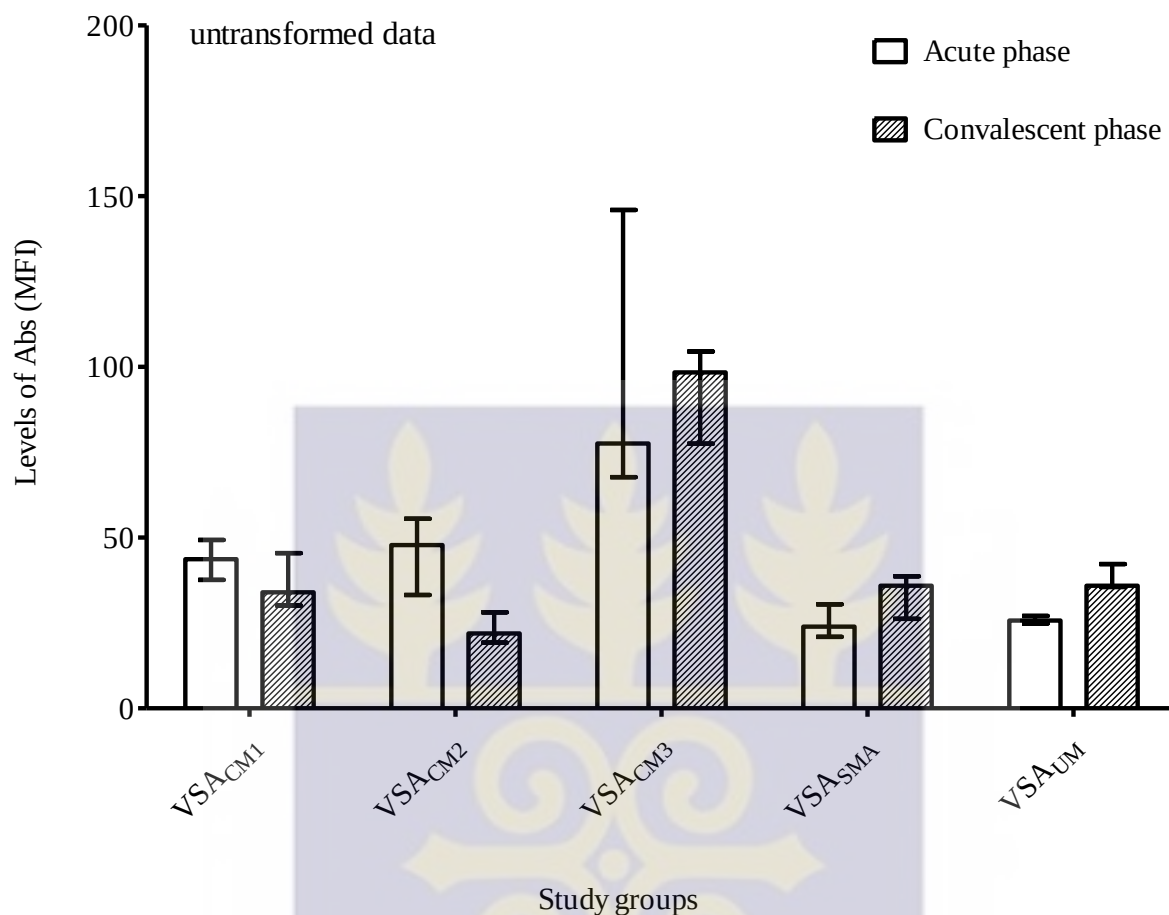
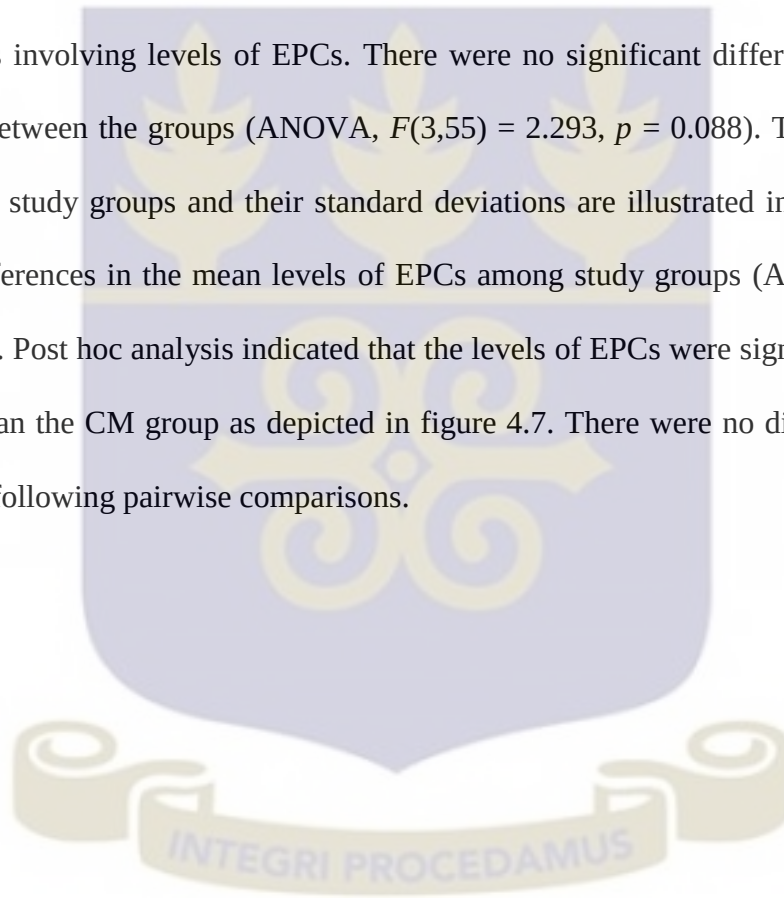


Figure 4.5: Levels of acute phase and convalescent phase plasma anti-VSA antibody responses of uncomplicated malaria patients.

Variant surface antigens of parasite isolates were obtained from patients who had been diagnosed with cerebral malaria, as well as patients diagnosed with severe malaria anemia and uncomplicated malaria. The column bars represent the median levels of anti-VSA antibody responses for the uncomplicated malaria patients while the error bars represent the interquartile range. The levels of anti-VSA antibody responses to the various VSAs are shown on the y-axis and the VSAs of the various parasite isolates are shown on the x-axis.

4.6 Levels of circulating endothelial cells and endothelial progenitor cells

To determine the level of endothelial damage and repair during cerebral malaria, circulating endothelial cells (CEC) as well as endothelial progenitor cells (EPC) were measured in seventy two study participants. But following log transformation of the data to satisfy assumptions of normality and equal standard deviations for ANOVA test, values for 13 samples were removed from the final analysis for the levels of CECs. Similarly, values for 6 samples were also removed from the analysis involving levels of EPCs. There were no significant differences in the mean levels of CECs between the groups (ANOVA, $F(3,55) = 2.293$, $p = 0.088$). The mean levels of CECs among the study groups and their standard deviations are illustrated in figure 4.6. There were marked differences in the mean levels of EPCs among study groups (ANOVA, $F(3,62) = 2.924$, $p = 0.041$). Post hoc analysis indicated that the levels of EPCs were significantly higher in the UM group than the CM group as depicted in figure 4.7. There were no differences between the other groups following pairwise comparisons.



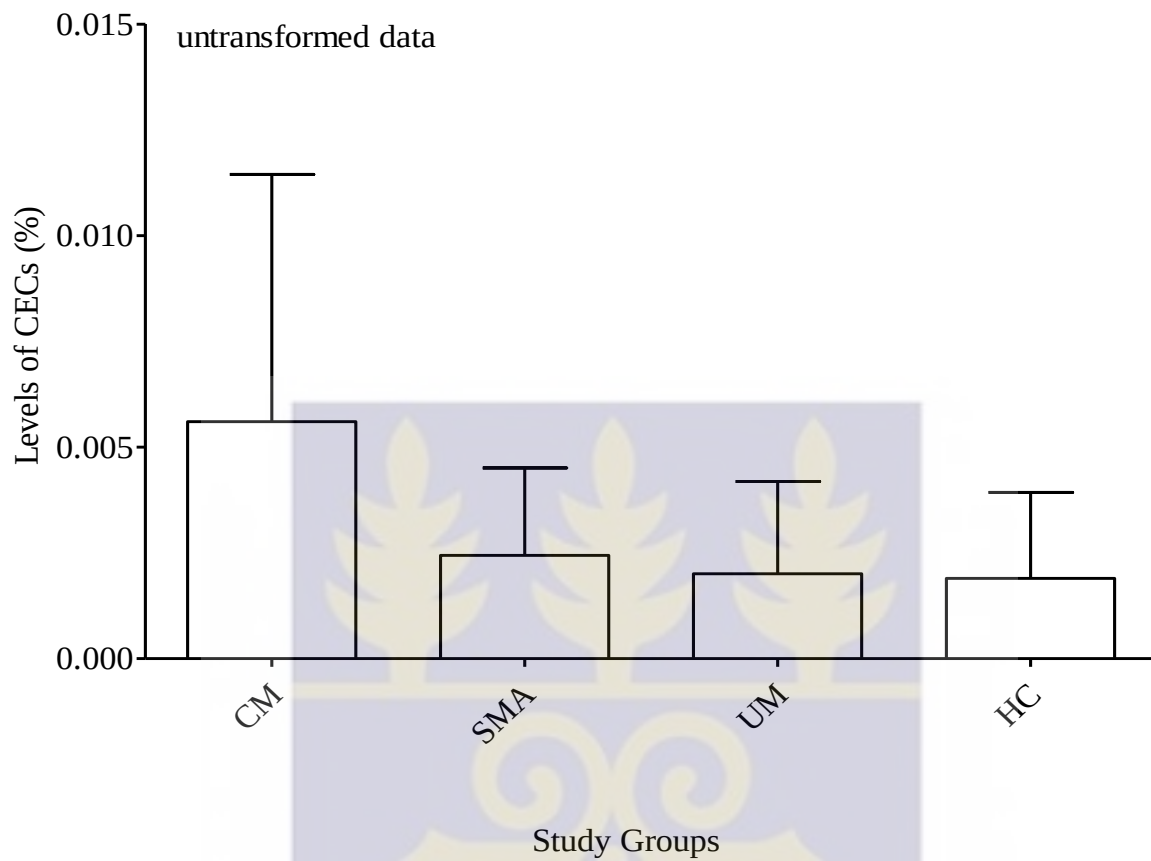


Figure 4.6: Levels of circulating endothelial cells among the study groups

The column bars represent the mean levels of CECs while the error bars represent their standard deviation



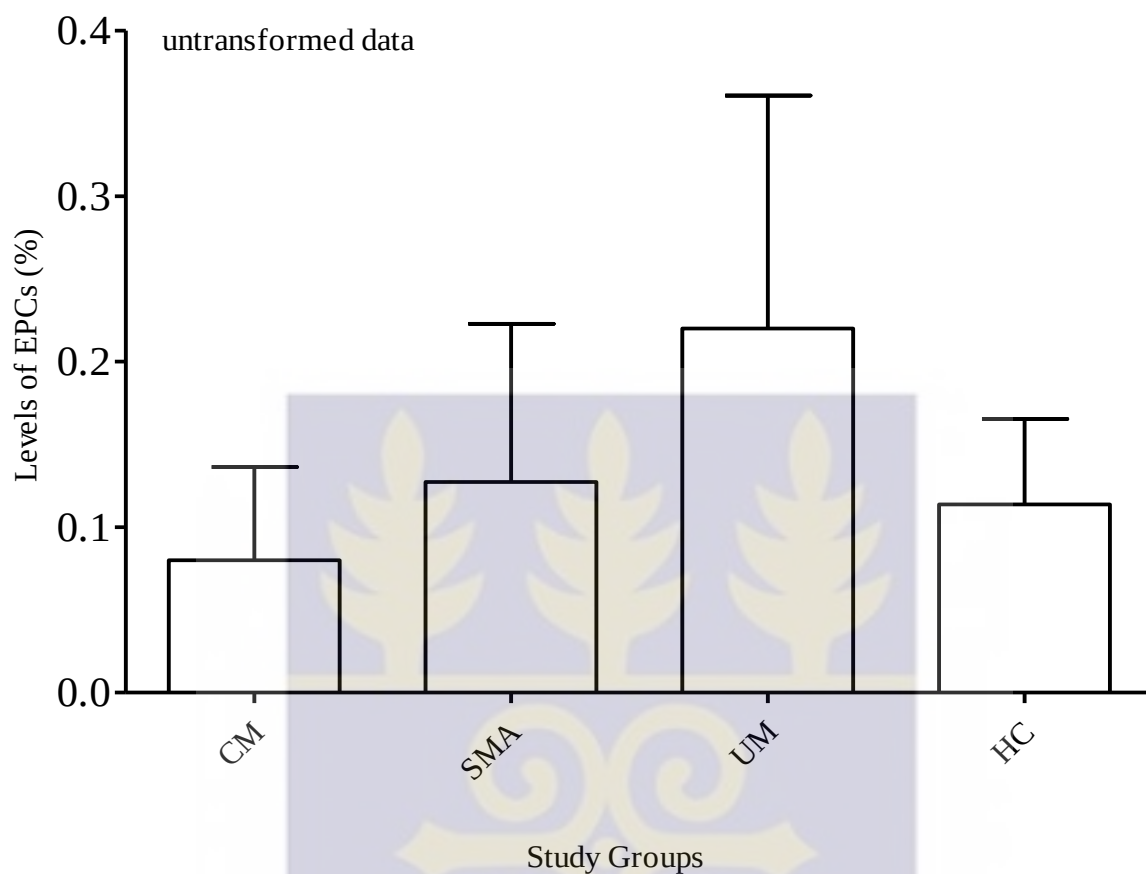


Figure 4.7: Levels of endothelial progenitor cells among the study groups

The column bars represent the mean levels of endothelial progenitor cells while the error bars represent their standard deviations

4.7 Development of cerebral malaria is negatively associated with the levels of endothelial progenitor cells

The aim of this study was to determine the relationship between the development of cerebral malaria and the levels of anti-VSA antibody responses, levels of circulating endothelial cells and endothelial progenitor cells. To this end, multiple logistic regression analysis was performed to model the impact of these biomarkers on the likelihood of a healthy child developing cerebral malaria and/or a child initially diagnosed with uncomplicated malaria progressing to cerebral malaria. A three-predictor logistic regression model indicated that the development of cerebral malaria by an uncomplicated malaria patient had a negative association with the levels of EPCs when the values for the other biomarkers were held constant. This result suggested that for every unit increase in the levels of EPCs the odds of an uncomplicated malaria patient developing cerebral malaria was lowered by 0.09. However, there were no associations between the development of cerebral malaria and the levels of circulating endothelial cells and levels of anti-VSA antibody responses. The detailed results of the logistic regression analysis for a UM patient and a healthy control is presented in Table 4.2 and 4.3.

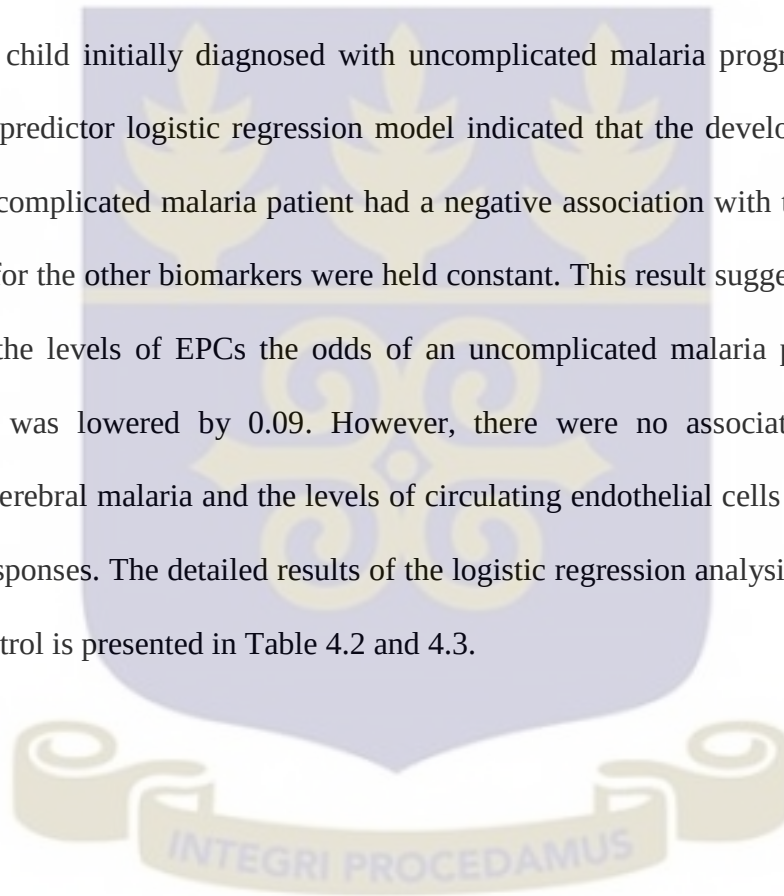


Table 4.2: Logistic regression analysis of the impact of the levels of biomarkers on the development of cerebral malaria by uncomplicated malaria patients

Predictor	Coefficient	Standard Error	Wald Statistic	<i>p</i> value	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	2.643	1.584	2.783	0.095	14.060	0.630	313.768
Anti-VSA	1.172	1.512	0.601	0.438	3.229	0.167	62.537
CEC	3.825	2.600	2.164	0.141	45.840	0.280	7491.951
EPC	-2.442	1.201	4.136	0.042	0.087	0.008	0.915
Test		χ^2		<i>p</i> value			
Overall model evaluation							
Likelihood ratio test		12.689		0.005			
Goodness-of-fit test							
Hosmer & Lemeshow test		12.172		0.144			
Reference response : UM							

Table 4.3: Logistic regression analysis of the impact of levels of the biomarkers on the development of cerebral malaria by healthy children

Predictor	Coefficient	Standard Error	Wald Statistic	<i>p</i> value	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	-0.324	0.401	0.652	0.419	0.723	0.329	1.588
Anti-VSA	1.041	0.684	2.313	0.128	2.831	0.740	10.823
CEC	0.847	0.646	1.718	0.190	2.332	0.657	8.274
EPC	-0.553	0.425	1.699	0.192	0.575	0.250	1.321
Test			χ^2	<i>p</i> value			
Overall model evaluation							
Likelihood ratio test			12.689	0.005			
Goodness-of-fit test							
Hosmer & Lemeshow test			12.172	0.144			
Reference response : HC							

CHAPTER FIVE

5 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Sequestration of parasite infected erythrocytes in the microvessels of the body is crucial to the pathogenesis of cerebral malaria (Moxon *et al.*, 2013; Milner Jr *et al.*, 2014). It is plausible that sequestration may be a stimulus which triggers a host of downstream responses in the host consequently resulting in endothelial damage (Moxon *et al.*, 2013) and repair of the damaged endothelium (Gyan *et al.*, 2009). Sequestration has been shown to be inhibited by the high levels of antibodies to variant surface antigens which are key facilitators of the process. Furthermore, high levels of these antibodies have also been associated with protection from malaria (Ricke *et al.*, 2000; Dodoo *et al.*, 2001). Hence, the hypothesis that children who develop cerebral malaria have low levels of anti-VSA antibodies which may enhance sequestration and ultimately result in high levels of circulating endothelial cells and low levels of endothelial progenitor cells.

In this study, high levels of anti-VSA antibody responses were observed among children who were diagnosed with cerebral malaria compared with the other study groups. Also in this study, high levels of anti-VSA antibody responses were seen among patients diagnosed with severe malaria anemia but unlike the cerebral malaria group, the levels of anti-VSA antibody responses were not significant when compared with the other groups. Furthermore, levels of anti-VSA antibodies at the time of diagnosis and when the patients had recovered were not different for both cerebral malaria patients and uncomplicated malaria patients. In previous studies involving Gambian children, low levels of anti-VSA antibody responses were observed among severe malaria patients while high levels were seen among patients diagnosed with uncomplicated

malaria. Similar results were also observed in the same patients following one month of treatment and successful recovery which suggested that severe malaria was associated with low levels of anti-VSA antibody responses and the conclusion that these levels of antibodies were associated with high susceptibility to infections (Tebo *et al.*, 2002). While this finding contrasts with that of the present study, a more recent study conducted in Sudan had reported high levels of anti-VSA antibody responses among severe malaria patients with higher levels observed in cerebral malaria patients, followed by severe malaria anemia patients and uncomplicated malaria patients (Theander *et al.*, 2006).

Findings from the present study suggest that high levels of anti-VSA antibodies observed among the cerebral malaria patients may be explained by a unique feature of the disease in inducing the production of cross reactive antibodies with broad range of specificity to the variant surface antigens tested. High levels of anti-VSA antibodies have been associated with disease protection suggesting that these patients may be protected from future infections mediated by parasites which express similar variant surface antigens. Given that these high levels of antibodies were observed at the time of diagnosis, it may not be erroneous to suggest that high levels of these antibodies may contribute to disease pathogenesis possibly through tissue damage. These findings may also support the rejection of the hypothesis that children develop cerebral malaria because of the low levels of antibodies to variant surface antigens mediating the disease. The inability to separately analyze the levels of antibodies in plasma from the same patients from which the parasites were isolated (homologous samples) made interpretations of the results supporting the hypothesis difficult. This limitation was due to the low numbers of cerebral malaria parasite isolates used in the study which in turn can be partly explained by the difficulty in adapting these parasites to culture. The limited quantity of available plasma also proved to be

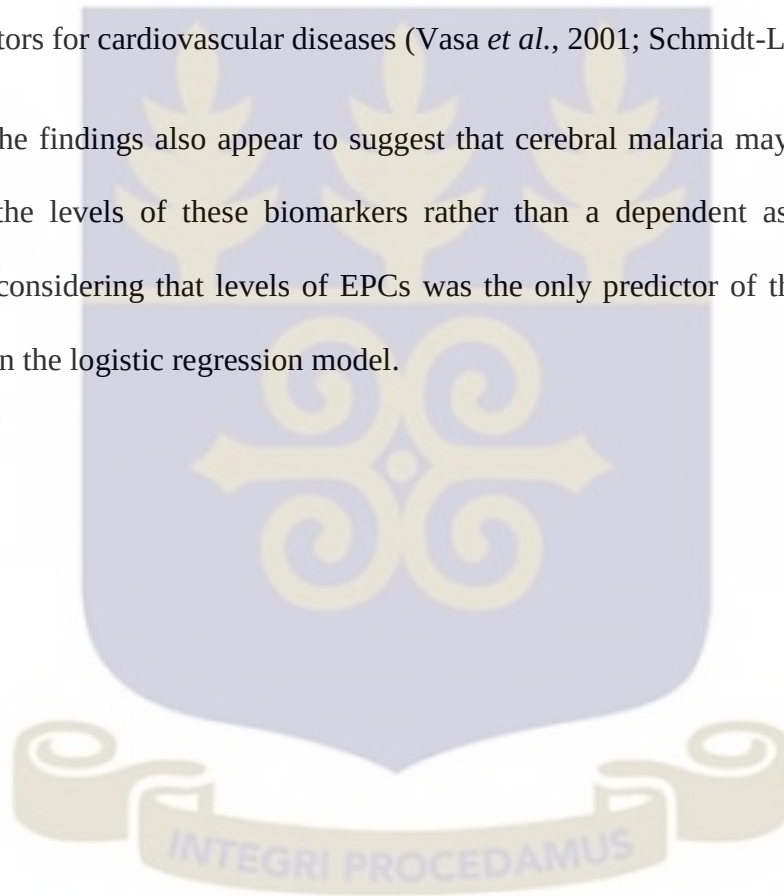
a limitation for this particular assay. One other possible limitation that may alter the inferences made from the above results is the inability to type parasite isolates used for the assay because of limited funding. It may well be that development of the disease was indeed associated with low levels of antibodies to variant surface antigens mediating the disease as have been suggested by earlier studies (Ricke *et al.*, 2000; Dodoo *et al.*, 2001; Tebo *et al.*, 2002). Despite, the higher levels of anti-VSA antibody responses among the cerebral malaria patients, logistic regression analysis revealed that there was no association between the development of the disease and the levels of anti-VSA antibody responses.

It was also hypothesized that, development of cerebral malaria was associated with high levels of circulating endothelial cells (CECs) and low levels of endothelial progenitor cells (EPCs). In this study, higher levels of CECs were observed among cerebral malaria patients than the other study groups but the results were not statistically significant. This finding is in contrast with that of unpublished data from earlier studies (Oduro, 2015) which reported significantly higher levels in cerebral malaria patients within the same laboratory and using the same combination of cell surface markers but a significantly larger sample size than was used in this study. While the present finding suggest that endothelial damage within the study subjects were the same and not peculiar to cerebral malaria, the same finding also failed to rule out entirely the possibility of high magnitude of endothelial damage in cerebral malaria pathogenesis.

Also in this study, levels of EPCs were significantly lower than the uncomplicated malaria group suggesting that the ability/or mechanism of repairing endothelial damage augmented by EPCs may be impaired in cerebral malaria patients, thus explaining why these patients developed cerebral malaria. Previous studies had demonstrated lower levels of EPCs among cerebral malaria patients (Gyan *et al.*, 2009). Indeed, multiple logistic regression analysis revealed that

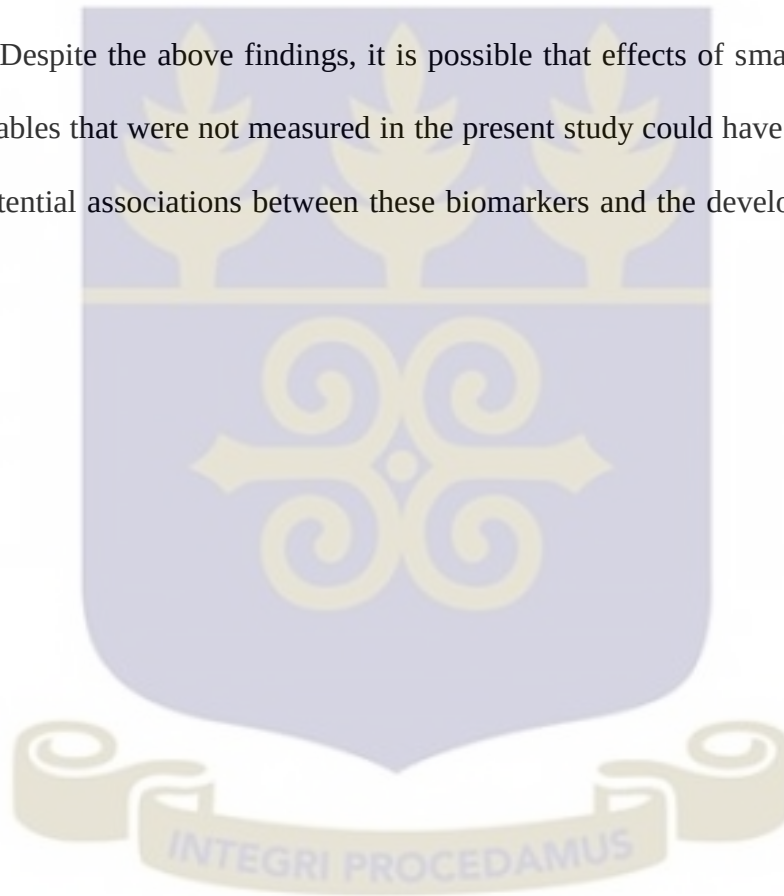
levels of EPCs were negatively associated with development of cerebral malaria. The results suggested that for every unit increase in the levels of EPCs, the odds of developing cerebral malaria particularly for an uncomplicated malaria patient was lowered by 0.09 when the levels of CECs and anti-VSA antibodies was adjusted for or held constant. The results also suggested a plausible use of levels of EPCs given proper validation as a biomarker for assessing risk/or prognosis of cerebral malaria. Indeed, levels of EPCs have been evaluated as potential risk and prognostic indicators for cardiovascular diseases (Vasa *et al.*, 2001; Schmidt-Lucke *et al.*, 2005).

Taken together, the findings also appear to suggest that cerebral malaria may be independently associated with the levels of these biomarkers rather than a dependent association. This is particularly true considering that levels of EPCs was the only predictor of the development of cerebral malaria in the logistic regression model.



5.2 Conclusions

This study has shown that there is an association between the development of cerebral malaria and the levels of EPCs. Using multiple logistic regression analysis, it was shown that for every unit increase in the levels of EPCs, the odds of developing cerebral malaria was lowered by 0.09 when the levels of CECs and anti-VSAs were held constant. This study has also shown that levels of anti-VSA antibodies among cerebral malaria patients were higher than non cerebral malaria patients. Despite the above findings, it is possible that effects of small sample size and confounding variables that were not measured in the present study could have underestimated or overestimated potential associations between these biomarkers and the development of cerebral malaria.



5.3 Recommendations

Given the limitation of small sample size which may have underestimated or overestimated potential associations that may have existed between the development of cerebral malaria and the levels of biomarkers evaluated, this study recommends further investigations with larger sample sizes for both patient plasma and number of parasite isolates. This may improve the statistical efficiency and make conclusions regarding the relationship between these biomarkers and the development of cerebral malaria definite. The study also recommends the evaluation of other biomarkers associated with pathogenetic features of the disease.



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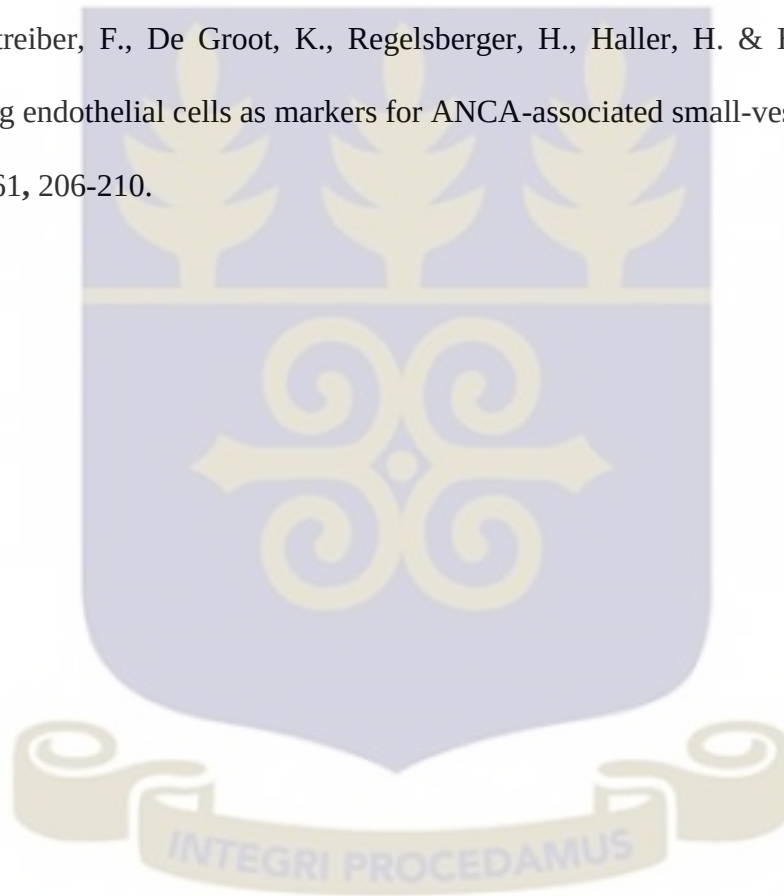
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APPENDICES

Appendix A: Consent form

A. Consent for children with malaria

Adapted from the study entitled: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben A. 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 Dr. Mame Yaa Nyarko at Prince Marie Louise Hospital (Tel: 0244 018888) or Dr. 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. Samuel Ayete-Nyampong, Chairman, Institutional Review Board, NMIMR, University of Ghana (021 501178/9) or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 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. Samuel Ayete-Nyampong, Tel 21-501-178/179 or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 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 Person who obtained Consent

B. Consent for healthy controls

Adapted from the study entitled: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben A. 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)

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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 Dr. Mame Yaa Nyarko at Prince Marie Louise Hospital (Tel: 0244 018888) or Dr. 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. Samuel Ayete-Nyampong, Chairman, Institutional Review Board, NMIMR, University of Ghana (021 501178/9) or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 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. Samuel Ayete-Nyampong, Tel 21-501-178/179 or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 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 Person who obtained Consent



Appendix B: Ethical Clearance



UNIVERSITY OF GHANA SCHOOL OF MEDICINE AND DENTISTRY

Ref. No.: **MS-AA/C.2/Vol.18^A**.....

12th February 2015

Mr. Gideon Obeng
Mphil, Department of Microbiology
SBAHS

ETHICAL CLEARANCE

Protocol Identification Number: MS-Et/M.5 – P 3.2/2014-2015

The Ethical and Protocol Review Committee of the School of Medicine and Dentistry on 11th February 2015 unanimously approved your research proposal.

TITLE OF PROTOCOL: "Microvascular Damage in Cerebral Malaria: The Role of Plasmodium Faciparum Erythrocyte Membrane Protein 1 (PfEMP 1)"

PRINCIPAL INVESTIGATOR: Mr. Gideon Obeng

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till November, 2015.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:.....

PROFESSOR ANDREW A. ADJEI

(FOR: CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Dean, SMD
Head of Department
Research Office

COLLEGE OF HEALTH SCIENCES

• P. O. Box GP4236, Accra, Ghana. • Telephone: +233 (0) 302 666 987/8 • Fax: +233 (0) 302 663 062
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Appendix C: Preparation of reagents

Parasite Freeze Mix

100 ml preparation

Sorbitol solution (4.2 % sorbitol in 0.9 % NaCl)..... 72 ml

Glycerol..... 28 ml

Procedure

Sorbitol solution and glycerol were measured separately using a calibrated measuring cylinder and the mixture constituted in a flask. The mixture was aliquoted into 50 ml falcon tubes and stored at 4°C.

Parasite Thawing Mix

100 ml preparation

Sodium Chloride..... 3.5g

Deionized water.....100 ml

Procedure

Salt was weighed with a balance and dissolved in water. The mixture was sterilized by filtering through 0.22 um pore filter. The sterilized product was aliquoted into 50 ml falcon tubes and stored at 4°C.

Parasite Washing Medium

500 ml preparation

RPMI 1640..... 493.5 ml

Gentamicin 10 mg/ml..... 2.5 ml

L-Glutamine.....4.0 ml

Procedure

From the 500 ml commercially constituted RPMI 1640, 6.5 ml was taken and stored in a 15ml falcon tube. Gentamicin and L-Glutamine was added to make the mixture up to 500 ml. The preparation was stored at 4°C and used within 30 days.

Albumax

Hypoxanthine..... 0.2 g

RPMI 1640..... 1.0 L

Albumax..... 50 g

Procedure

Hypoxanthine was dissolved in the RPMI 1640. This was followed by the addition of albumax. The mixture was filtered using a 0.22 um filter after which it was aliquoted into 50 ml falcon tubes and stored at -20°C

Complete Parasite Medium (CPM)

500 ml preparation

RPMI 1640.....	433.5 ml
Gentamicin 10 mg/ml.....	2.5 ml
L-Glutamine.....	4.0 ml
Albumax.....	50 ml
Normal Human Serum (O positive).....	10 ml

Procedure

From 500 ml commercially prepared RPMI 1640, 66.5 ml was aliquoted into a separate tube. Gentamicin, L-Glutamin, and Albumax were added sequentially to the 433.5 ml RPMI 1640. Normal human serum was filtered with a 0.8 um filter, after which the product was added to the initial medium to obtain a complete parasite medium. The medium was stored at 4°C and was stable for 7 days.

Giemsa Stain (Working solution)

Working Giemsa buffer.....	45 ml
Stock Giemsa (filtered).....	5 ml

Washing of human RBCs (O Rhesus D positive)

Blood was collected in a vacutainer containing citrate phosphate dextrose (CPD) and stored for 48 hours before further processing. Following 48 hours of storage, blood was transferred into a 50 ml falcon tube and centrifuged for 10 minutes at 2000 rpm. The resulting supernatant was aspirated and discarded. Parasite washing medium (PWM) was then added to make the mixture up to 40 ml. The mixture was thoroughly mixed and centrifuged for 10 minutes at 2000rpm. The resulting supernatant was aspirated and discarded as done previously. Again, PWM was added, the mixture centrifuged and the supernatant aspirated and discarded. An equal volume of PWM was added to the RBC pellets thereafter. Washed blood was stored at 4°C and used within two weeks.

Special mix gas

Balanced Nitrogen.....	92.5 %
Oxygen.....	2 %
Carbon dioxide.....	5.5%



Appendix D: Details of Logistic Regression Analysis

Logistic regression analysis of healthy control subjects likelihood of developing CM

Overall model evaluation

Pearson Chi-square Statistic: 39.808 (P = 0.304)

Likelihood Ratio Test Statistic: 10.889 (P = 0.012)

-2*Log (Likelihood) = 44.748

Goodness-of-fit test

Hosmer-Lemeshow Statistic: 5.195 (P = 0.736)

Threshold probability for positive classification: 0.500

Classification Table:

	Predicted Reference	Predicted Positive	Totals
Actual Reference Responses	21	3	24
Actual Positive Responses	8	9	17
Totals	29	12	41

Details of the Logistic Regression Equation

Predictor	Coefficient	Standard Error	Wald Statistic	P value
Constant	-0.220	0.398	0.304	0.581
Anti-VSA _{CM1}	0.592	0.409	2.094	0.148
CEC	1.274	0.725	3.090	0.079
EPC	-0.606	0.396	2.339	0.126

Predictor	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	0.803	0.368	1.752
Anti-VSA _{CM1}	1.808	0.811	4.031
CEC	3.574	0.864	14.785
EPC	0.546	0.251	1.186

Positive response (1): CM

Reference response (0): HC

Logistic Regression analysis of healthy control subject's likelihood of developing CM**Overall model evaluation****Pearson Chi-square Statistic:** 34.940 (P = 0.423)**Likelihood Ratio Test Statistic:** 11.251 (P = 0.010)**-2*Log (Likelihood) =** 40.719**Goodness-of-fit test****Hosmer-Lemeshow Statistic:** 7.845 (P = 0.449)**Threshold probability for positive classification:** 0.500**Classification Table:**

	Predicted Reference	Predicted Positive	Totals
Actual Reference Responses	21	3	24
Actual Positive Responses	7	8	15
Totals	28	11	39

Details of the Logistic Regression Equation

Predictor	Coefficient	Standard Error	Wald Statistic	P value
Constant	-0.234	0.412	0.322	0.570
Anti-VSA _{CM2}	0.998	0.564	3.130	0.077
CEC	1.256	0.709	3.133	0.077
EPC	-0.679	0.407	2.786	0.095

Predictor	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	0.791	0.353	1.775
Anti-VSA _{CM2}	2.713	0.898	8.198
CEC	3.510	0.874	14.095
EPC	0.507	0.228	1.126

Positive response (1): CM

Reference response (0): HC

Logistic Regression analysis of healthy control subjects likelihood of developing CM**Overall model evaluation****Pearson Chi-square Statistic:** 36.204 (P = 0.412)**Likelihood Ratio Test Statistic:** 12.689 (P = 0.005)**-2*Log (Likelihood)** = 41.152**Goodness-of-fit test****Hosmer-Lemeshow Statistic:** 12.172 (P = 0.144)**Threshold probability for positive classification:** 0.500**Classification Table:**

	Predicted Reference	Predicted Positive	Totals
Actual Reference Responses	22	2	24
Actual Positive Responses	9	7	16
Totals	31	9	40

Details of the Logistic Regression Equation

Predictor	Coefficient	Standard Error	Wald Statistic	P value
Constant	-0.324	0.401	0.652	0.419
Anti-VSA _{CM3}	1.041	0.684	2.313	0.128
CEC	0.847	0.646	1.718	0.190
EPC	-0.553	0.425	1.699	0.192

Predictor	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	0.723	0.329	1.588
Anti-VSA _{CM3}	2.831	0.740	10.823
CEC	2.332	0.657	8.274
EPC	0.575	0.250	1.321

Positive response (1): CM

Reference response (0): HC

Logistic Regression analysis of uncomplicated malaria patient's likelihood of developing CM

Overall model evaluation

Pearson Chi-square Statistic: 16.439 (P = 0.562)

Likelihood Ratio Test Statistic: 12.597 (P = 0.006)

-2*Log (Likelihood) = 15.670

Goodness-of-fit test

Hosmer-Lemeshow Statistic: 6.896 (P = 0.548)

Threshold probability for positive classification: 0.500

Classification Table:

	Predicted Reference	Predicted Positive	Totals
Actual Reference Responses	4	3	7
Actual Positive Responses	1	15	16
Totals	5	18	23

Details of the Logistic Regression Equation

Predictor	Coefficient	Standard Error	Wald Statistic	P value
Constant	3.120	1.606	3.774	0.052
Anti-VSA _{CM1}	0.752	0.688	1.197	0.274
CEC	5.023	3.224	2.427	0.119
EPC	-2.783	1.311	4.509	0.034

Predictor	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	22.637	0.972	526.941
Anti-VSA _{CM1}	2.122	0.551	8.170
CEC	151.924	0.274	84342.534
EPC	0.0618	0.00474	0.807

Positive response (1): CM

Reference response (0): UM

Logistic Regression analysis of uncomplicated malaria patient's likelihood of developing CM

Overall model evaluation

Pearson Chi-square Statistic: 14.878 (P = 0.460)

Likelihood Ratio Test Statistic: 8.243 (P = 0.041)

-2*Log (Likelihood) = 16.192

Goodness-of-fit test

Hosmer-Lemeshow Statistic: 9.762 (P = 0.282)

Threshold probability for positive classification: 0.500

Classification Table:

	Predicted Reference	Predicted Positive	Totals
Actual Reference Responses	3	3	6
Actual Positive Responses	1	13	14
Totals	4	16	20

Details of the Logistic Regression Equation

Ind. Variable	Coefficient	Standard Error	Wald Statistic	P value
Constant	2.557	1.558	2.692	0.101
Anti-VSA _{CM2}	0.695	1.009	0.474	0.491
CEC	3.883	2.952	1.730	0.188
EPC	-2.248	1.165	3.723	0.054

Ind. Variable	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	12.898	0.608	273.610
Anti-VSA _{CM2}	2.003	0.277	14.487
CEC	48.560	0.149	15801.417
EPC	0.106	0.0108	1.036

Positive response (1): CM

Reference response (0): UM

Logistic Regression analysis of uncomplicated malaria patient's likelihood of developing CM

Overall model evaluation

Pearson Chi-square Statistic: 14.408 (P = 0.638)

Likelihood Ratio Test Statistic: 11.592 (P = 0.009)

-2*Log (Likelihood) = 15.930

Goodness-of-fit test

Hosmer-Lemeshow Statistic: 9.246 (P = 0.322)

Threshold probability for positive classification: 0.500

Classification Table:

	Predicted Reference	Predicted Positive	Totals
Actual Reference Responses	4	3	7
Actual Positive Responses	2	13	15
Totals	6	16	22

Details of the Logistic Regression Equation

Predictor	Coefficient	Standard Error	Wald Statistic	P value
Constant	2.643	1.584	2.783	0.095
Anti-VSA _{CM3}	1.172	1.512	0.601	0.438
CEC	3.825	2.600	2.164	0.141
EPC	-2.442	1.201	4.136	0.042

Predictor	Odds Ratio	5% Conf. Lower	95% Conf. Upper
Constant	14.060	0.630	313.768
Anti-VSA _{CM3}	3.229	0.167	62.537
CEC	45.840	0.280	7491.951
EPC	0.0870	0.00827	0.915

Positive response (1): CM

Reference response (0): UM