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ENDOTHELIAL BIOMARKERS AND THE PATHOGENESIS OF CEREBRAL MALARIA

BY

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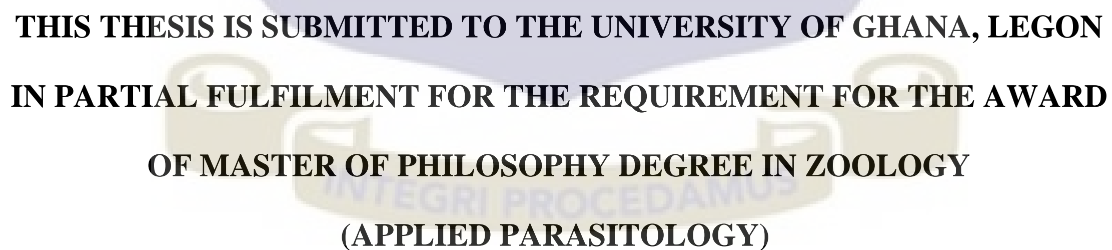

(APPLIED PARASITOLOGY)

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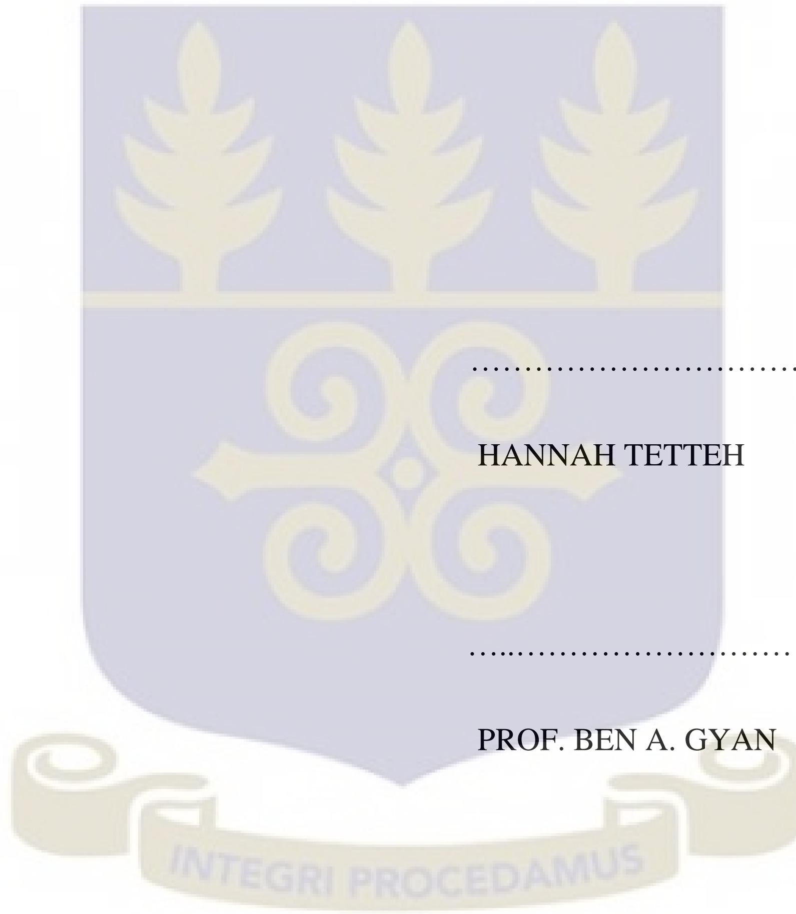
DECLARATION

The laboratory experiments described in the thesis was performed by me at Noguchi Memorial Institute for Medical Research of the College of Health Sciences, University of Ghana. The work was done under the supervision of Prof. Ben A. Gyan and Dr. Bethel Kwansa-Bentum All references cited in this thesis have been fully acknowledged in the reference section.



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DEDICATION

To my father, Mr. Emmanuel Tetteh, my mother Mrs. Deborah Tetteh, my dear aunt, Janet, all my siblings, my niece and nephew.

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My utmost appreciation goes to Jehovah God for giving me the strength and wisdom to carry out this project.

My appreciation goes to my father, Mr. Emmanuel Tetteh and mother Mrs. Deborah Tetteh for taking me through my education up to this level and giving me advise and care which has brought me this far. To all my siblings for their support and encouragement towards my education.

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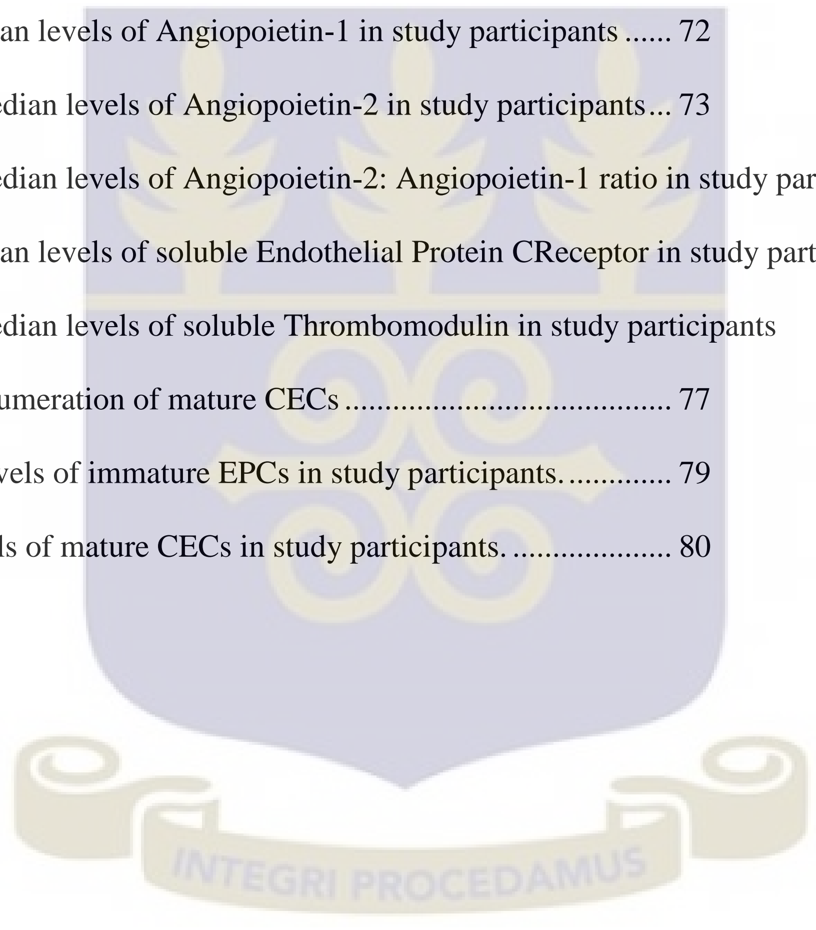

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ABBREVIATIONS

ACT	Artemesinin-Combination Therapy
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APC	Activated Protein C
APC	Allophycocyanin
BCS	Blantyre Coma Score
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CECs	Circulating Endothelial Cells
CEPCs	Circulating Endothelial Progenitor cells
CM	Cerebral Malaria
EDTA	Ethylene diaminetetra acetic acid
ELAM-1	Endothelial Leukocyte Adhesion Molecule
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
EPCR	Endothelial Protein C Receptor
FACS	Fluorescence –Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
G6PD	Glucose-6-Phosphate Dehydrogenase
Hb	Haemoglobin
HC	Healthy Control

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HIV/AIDS	Human Immuno Virus/ Acquired Immune Deficiency Syndrome
ICAM-1	Intercellular Adhesion Molecule-1
IFN-ɣ	Interferon-ɣ
Ig	Immunoglobulin
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
KDR	Kinase-insert Domain Receptor
LLINs	Long-Lasting Insecticidal Nets
LT- α	Lymphotoxin- α
MAb	Monoclonal Antibody
MMP-9	Matrix Metalloproteinase-9
NGS	Normal Goat Serum
NMS	Normal Mouse Serum
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein Complex
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
SDF-1	Stromal Cell Derived Factor-1
sEPCR	soluble Endothelial Protein C Receptor
SMA	Severe Malaria Anaemia
sTM	soluble Thrombomodulin
Tek	Tyrosine Kinase

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TM	Thrombomodulin
TNF- α	Tumor Necrosis Factor-α
UM	Uncomplicated Malaria
UNICEF	United Nations International Children's Emergency Fund
USAID	United States Agency for International Development
VCAM-1	Vascular Cell Adhesion Molecule
VEGF2	Vascular Endothelial Growth Factor 2
WHO	World Health Organization

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ABSTRACT

Cerebral malaria occurs due to abnormality in the function of the brain endothelium. This signifies that the brain endothelium plays a crucial role in the outcome of malaria in children. Regulated balance between angiopoietin-1 and angiopoeitin-2 is responsible for the control of normal endothelial cell function. The angiopoietin-Tie-2 system has been shown to regulate endothelial cell function and vascular integrity.

Endothelial Protein C Receptor (EPCR) and Thrombomodulin (TM) are receptors on the endothelium and are required for the activated protein C cellular pathway which is known to have some microvascular protective effects. Elevated serum or plasma TM and EPCR levels suggested to be due to endothelial damage have been shown to be found in diseases associated with systemic or locally increased levels of inflammatory cytokines such as malaria.

The study aimed to investigate the relationship between Ang-1, Ang-2, sEPCR and sTM and the pathogenesis of *P. falciparum* malaria infection in children with circulating endothelial progenitor cells and circulating endothelial cells which are known to be responsible for endothelial repair when damage occurs. Children between the ages of 2 to 12 years presenting with CM or UM were recruited from 5 hospitals in Accra and healthy children within the same age range were also recruited as controls. In addition to clinical and haematological determinations, levels of Ang-1, Ang-2, sEPCR and sTM in the plasma of all subjects were measured using ELISA. It was found

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that Ang-1 levels were higher in the healthy children compared to children who had uncomplicated or cerebral malaria and was associated with immature EPC levels ($P < 0.05$). It was also found that soluble TM was highest in children in the cerebral malaria group compared to uncomplicated malaria and healthy control groups ($P < 0.05$). The study suggests that Ang-1 but not Ang-2 is a predictor of endothelial damage and EPC recruitment. Also sTM was found to be a predictor of endothelial damage.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is an important parasitic disease and the leading cause of mortality in the world today. Malaria is an infectious disease caused by the parasitic protozoan called *Plasmodium*. It is endemic in over 100 developing countries in the tropical regions with extensions into sub-tropical regions with altitudes below 1,500m (Zofou *et al.,* 2014). The main vector responsible for transmitting malaria is the female *Anopheles* mosquito. *Plasmodium falciparum* malaria is the most virulent of all the species that cause malaria and accounts for 90% of malaria cases worldwide. Of these 86% of malaria related mortality occurs in sub-Saharan Africa due to *Plasmodium falciparum* infections (Perkins *et al*., 2011). Worldwide an estimated 5% of the population is infected with malaria though the burden is currently decreasing slightly (White, 2009). The decrease in malaria burden however, is not evenly distributed worldwide because out of 35 countries among which 5 are in Asia, the other 30 are in sub-Saharan Africa and these countries account for 98% of global malaria deaths. (Roll Back Malaria Partnership, 2011; World Malaria Report, 2012).

Malaria affects people of all ages and sex but the most vulnerable people are young children, pregnant women, HIV/AIDS patients, international travelers from non-endemic areas. Among the risk groups, young children, in endemic regions of Africa where nearly a quarter of all childhood deaths are due to malaria, are the most susceptible to severe malaria and contribute the bulk of malaria deaths worldwide (Miller *et al.,* 2013; WHO, 2011a). In Sub-Saharan Africa where large numbers of deaths also occur due to malnutrition, HIV/AIDS, tuberculosis and other diseases, malaria not only adds to the disease burden but also social and economic burden by slowing down

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development in diverse ways (Sachs & Malaney, 2002). Worldwide efforts are being put in place by different organizations such as WHO, USAID, Bill and Melinda Gates foundation and others to curb malaria with an aim of eradication through research, training and education.

Although the ACT is effective in the treatment of uncomplicated malaria, it is difficult to use it to treat cerebral malaria since the parasites are mostly sequestered and are not found in the peripheral blood where there is maximum drug action. There is therefore the need to develop new therapies in order to reduce the high mortality of severe malaria (Miller *et al.*, 2013). In Ghana about 3.5 million people contract malaria each year out of which 25% of the deaths occur among children under the age of five. Most children who survive cerebral malaria often have some neurological damage leading to lifelong defects (UNICEF, 2007). In Ghana, malaria is responsible for up to 60% of out-patient clinical cases. This implies that the malaria burden in Ghana is quite high and hence is a major challenge to development due to the mortality and morbidity it causes in children and especially adults of the working class. Even though the devastating effect of malaria is high, people overlook its devastating effect by accepting it as a normal everyday sickness in the country. Due to this, over the years the promotion of a malaria-free environment has not yet been fully appreciated in Ghana. Currently though efforts are being put in place to reach a goal of eradication (Asante & Asenso-Okyere, 2003).

Symptoms of *falciparum* malaria ranges from asymptomatic infections to severe malaria. Clinical features of malaria includes fever, chills, sweating, headaches, vomiting, muscle aches, in the severe form of malaria there are clinical features such as renal failure, hyperparasitemia, hypoglycemia, severe anemia, respiratory distress and cerebral malaria (Marsh *et al.*, 1995; WHO, 2000). Cerebral malaria which occurs only in *Plasmodium falciparum* malaria is one of the most

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severe forms of malaria and accounts for 80% of deaths. It causes shock, unrousable coma and eventually death (Goldman, 1999). The major feature of cerebral malaria which is the unrousable coma has no known direct cause. There have been extensive studies on the pathogenesis of malaria, there is still no actual information on why some children develop complications like cerebral malaria resulting in death. Though there have been several proposed hypotheses and work done using both in vivo and in vitro models, the full mechanism of pathogenesis of cerebral malaria is yet to be fully known.

A compromised microcirculation, with sequestration of parasitized red blood cells is known so far to be one of the main reasons for the pathogenesis of cerebral malaria (Dondorp *et al*., 2005a). Studies conducted within the past two decades reveal that the severity of the cerebral malaria is dependent on the damage that occurs when infected red blood cells adhere to the microvessels of the brain endothelium and also cause occlusion by forming rosettes with uninfected red blood cells (Goldman, 1999). The outcome to malaria infection however is mainly determined by the parasite mass and the response of the host to the infection. A range of protective immunological responses are involved that determine disease severity and survival. There are therefore several experiments being conducted to investigate this phenomenon. Several studies have shown that damaged endothelial cells are replaced by the replication of own cells and also by the migration and integration of bone marrow –derived circulating endothelial progenitor cells (CEPCs) to sites of damage in the microvasculature (Asahara *et al.,* 1997). Work done by Gyan *et al*. (2009) showed that children who presented with cerebral malaria had low levels of endothelial progenitor cells compared to those who presented with mild malaria and healthy controls and they were able to confirm the mobilization of endothelial progenitor cells from bone to sites of micro vascular damage by measuring levels of stromal derivative factor-1(SDF-1) which correlated positively

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with disease severity.

Other factors aside the circulating endothelial progenitor cells that are stipulated to be responsible for the production and repair of microvessels and also play roles in the pathogenesis of cerebral malaria include inflammatory cytokines, platelets, endothelial receptors, intercellular adhesion molecule-1 (ICAM-1), thrombomodulins and several others. Regulated balance between angiopoietin-1 and angiopoeitin-2 is responsible for the control of normal endothelial cell function. The angiopoietin-Tie-2 system has been shown to regulate endothelial cell function and vascular integrity. Angiopoietin-1 (Ang-1) engages endothelial receptor Tie-2, promoting endothelial cell quiescence and survival, whereas these effects are generally inhibited by Angiopoietin-2 (Ang-2) (Brindle *et al*., 2006). Previous reports have demonstrated that there are low Ang-1 levels and elevated Ang-2 levels in severe malaria compared to uncomplicated malaria (Lovegrove *et al*., 2009). According to Conroy *et al*. (2010), increased ratio of serum or plasma Ang-1/ Ang-2 ratio is a very good predictor of adverse outcomes in certain disease conditions such as diabetes, sepsis and malaria. Since the endothelium may have a crucial role in mediating malaria pathogenesis it is therefore necessary to evaluate the relationship between angiopoietin-1 and angiopoeitin-2.

Endothelial Protein C Receptor (EPCR) is a receptor on the endothelium and is required for the activated protein c cellular pathway which is known to have some cytoprotective effects (Thiyagarajan *et al*., 2007). Some studies done on baboons suffering from *E. coli* sepsis have shown that EPCR signalling decreased inflammation and thus prevented fatality of the disease on the animals (Feistritzer *et al.,* 2006). Another studies also showed that EPCR signaling played a cardioprotective role in lipopolyscharide-induced endotoxemia in mice (Iwaki *et al.,* 2005). These findings and several others give an indication that EPCR signalling has protection on the

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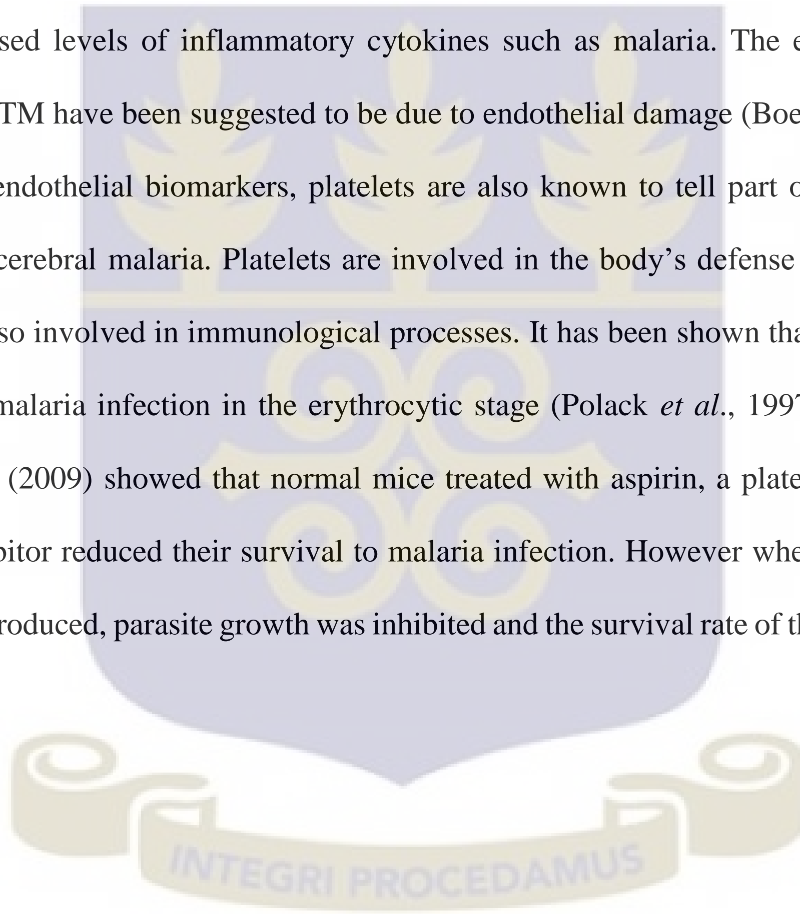
endothelium, with the brain endothelium not being an exception.

Thrombomodulins (TM) are mainly expressed on vascular endothelial cells of arteries, veins and capillaries, lymphatic endothelial cells, mesothelial cells, malignant cells and sometimes on activated smooth muscle cells. Its soluble form is found in serum, plasma and urine. Elevated serum or plasma TM levels have been shown to be found in diseases associated with systemical or locally increased levels of inflammatory cytokines such as malaria. The elevated serum or plasma levels of TM have been suggested to be due to endothelial damage (Boehme *et al*., 1996). Aside from the endothelial biomarkers, platelets are also known to tell part of the story in the pathogenesis of cerebral malaria. Platelets are involved in the body's defense against microbial pathogens and also involved in immunological processes. It has been shown that platelets protect the host during malaria infection in the erythrocytic stage (Polack *et al*., 1997). Work done by McMorran *et al*. (2009) showed that normal mice treated with aspirin, a platelet activation and aggregation inhibitor reduced their survival to malaria infection. However when purified human platelets were introduced, parasite growth was inhibited and the survival rate of the mice increased.



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1.2 Justification

Elimination of the parasite does not completely resolve clinical consequences of infection. Unique protective effects of certain vascular proteins make them attractive for exploitation to provide novel therapeutic options (Brindle *et al*., 2006). To ascertain the effect of these proteins or biomarkers, it is necessary to evaluate levels of these protein growth factors when one is infected with malaria. Differentiating cerebral malaria (CM) from other causes of serious illness in African children is problematic, owing to the non-specific nature of the clinical presentation and the high prevalence of incidental parasitaemia. Rapid diagnosis and early intervention can potentially therefore improve the clinical outcomes in individuals affected by malaria since those who present with mild forms of malaria might be harboring sub-clinical endothelial cell activation that will ultimately drive progression to a severe form of the disease. Peripheral blood biomarkers of endothelial cell activation/dysfunction may therefore be clinically useful to identify those individuals who, from among all patients with mild malaria, will progress to severe disease.

1.3 Hypothesis

Endothelial biomarkers, Ang-1, Ang-2, sEPCR and sTM are associated with severity of *Plasmodium falciparum* malaria infection and may help identify children with cerebral malaria.

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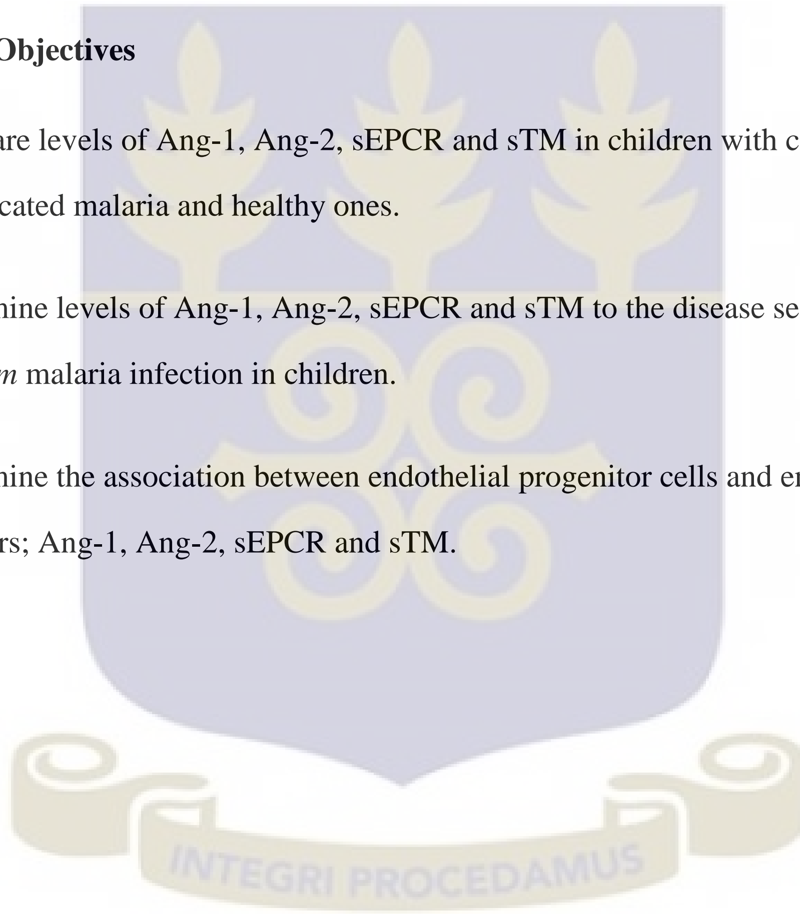
1.4 General Objective

The aim of this study was to investigate the relationship between Ang-1, Ang-2, sEPCR and sTM and the pathogenesis of *P. falciparum* malaria infection in children with circulating endothelial progenitor cells and circulating endothelial cells.

1.5 Specific Objectives



- To compare levels of Ang-1, Ang-2, sEPCR and sTM in children with cerebral malaria, uncomplicated malaria and healthy ones.
- To determine levels of Ang-1, Ang-2, sEPCR and sTM to the disease severity of *P. falciparum* malaria infection in children.
- To determine the association between endothelial progenitor cells and endothelial biomarkers; Ang-1, Ang-2, sEPCR and sTM.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is an infectious disease that is transmitted through the bite of an infected female *Anopheles* mosquito, it can also be gotten though rarely through the transfusion of infected blood and the use of needles or syringes contaminated with the blood of an infected person (Malaria, 2011). The disease in humans is caused by the parasitic protozoans: *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax* and in Southeast Asia, *Plasmodium knowlesi* (Perkins *et al*., 2011). Malaria is generally characterized by periodic attacks of chills and fever, nausea, anemia, spleen enlargement. In a severe or complicated form it is characterized by algid malaria, cerebral malaria, severe malaria anemia and respiratory distress. The most fatal of all the malaria is the one caused by *Plasmodium falciparum* because it is the only species that causes cerebral malaria which is responsible for most malaria death in children under five years old.

INTEGRI PROCEDAMUS

2.2 History of the Malaria

Malaria is a term that was taken from the Italian word "mal" and "aria" which means bad air (*Plasmodium vivax*, 2014). This was so because malaria occurred mostly in areas that were marshy and had foul smell therefore it was concluded that the disease was being caused by the foul air emanating from such environments. The term malaria was coined in 1827 by Macculoch. (*Plasmodium vivax*, 2014). By the year 1880, it was discovered that the disease was not caused by

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foul air but rather by a parasite when Charles Laveran, a French military doctor first observed the parasite in the blood of a patient. Six years later, the parasite was observed in the red blood cells of patients by Camilo Golgi. He was able to establish that there were at least two forms of the disease in terms of their periodicity. In 1890, Grassi and Filetti first introduced the species *Plasmodium vivax* and *Plasmodium malariae*. William Welch reviewed and named the malignant tertian malaria parasite *Plasmodium falciparum* in 1897. In 1922, *Plasmodium ovale* was described by John William Watson. The parasite which infect monkeys, *Plasmodium knowlesi* was first described by Knowles and Gupta in 1931 however its first human infection was documented in 1965.The mode of transmission was suggested in the year 1894 by Richard Pfiffer as through blood sucking insects. In the year 1902 a British army doctor, Sir Ronald Ross, won a Nobel Prize for the discovery of the oocysts of Plasmodium in the crop wall of female anopheles mosquito on 20th August 1897. He also demonstrated that malaria parasites could be transmitted from infected humans to mosquitoes. Due to the significance of his discovery, till date, 20th August of every year has been marked as the world malaria day. Currently Malaria has been largely eliminated from North America and Europe but still persists in tropical and Subtropical regions of the world (*Plasmodium vivax*, 2014; CDC, 2012).

INTEGRI PROCEDAMUS

2.3 Malaria, the global burden

According to WHO, (2010), malaria still causes significant morbidity and mortality worldwide despite the decline in number of cases and deaths over the last few years with the bulk of the burden observed in children under five years of age in Sub-Saharan Africa. According to Roll Back Malaria, (2011), malaria is the fifth and second cause of death from infectious diseases

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worldwide and in Africa respectively. Malaria caused up to 500 million clinical episodes annually with a resulting one million deaths in children under 5 years old in Sub-Saharan Africa in the year 2000 alone. Recent estimates show that as many as 3.3 billion people live in areas at risk of malaria in 109 countries or territories (Roll Back Malaria, 2011). Malaria not only puts a burden on the health of individuals, it also puts a heavy economic burden on endemic countries and contributes to the cycle of poverty people face in many countries. The economic burden of malaria is felt in household of individuals and health systems in endemic countries (Sicuri *et al*., 2013). In a talk given at a conference by the World Bank in Washington in September, 2013, it was indicated that disease burden is now defined better by disability instead of premature mortality. Also according to The World Bank group, (2013), in Sub-Saharan Africa, while premature death and disability from some communicable diseases are on the decrease, malaria and HIV/AIDS still continue to account for more health losses. Also in most of Sub-Saharan African countries, a larger percentage of healthy years were lost due to disability in 2010 compared to 1990. HIV/AIDS and malaria accounted for a larger proportion of disability in Sub-Saharan Africa than the world as a whole (The World Bank group, 2013).

Due to the burden of malaria, some malaria interventions have been put in place. Currently, malaria treatment and prevention strategies recommended by the WHO include Indoor Residual Spraying (IRS), Long-Lasting Insecticidal Nets (LLINs), Artemisinin-Combination Therapies (ACTs) and Intermittent Preventive Treatment (IPT). These strategies are designed to kill mosquitoes that enter houses or other sleeping areas, repel and kill mosquitoes, treat against malaria and also provide antenatal malaria treatment respectively. Worldwide, efforts are being put in place by different organizations such as WHO, USAID, Bill and Melinda Gates foundation and others to curb malaria with an aim of eradication through training and education. Also several initiatives have been put

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in place to train and equip scientists from endemic regions to make a difference in the treatment, diagnosis, management and eradication of malaria in their countries. Some of the initiatives are the Roll Back Malaria program launched in 1998, the Multilateral Initiative on Malaria (MIM) which had its 6th conference in 2013 and the West African Regional Workshop on Protozoan Pathogens which had its 2nd training in 2014. Also there are courses which certify participants and make them competent for the management of malaria. One such course is the International Diploma Course on Malaria Program Planning and Management which run its course from 1996 to 2012 in Iran. The course provided scientists, medical personnels and health workers with the skills for decision making, how to win the war against malaria in their respective countries and was also similar to the policy of malaria control for capacity building in malaria endemic areas of the world (Mesdaghinia *et al*., 2013).

Other strategies currently put in place to manage malaria include vaccine development, drug development and vector control (Zofou *et al*., 2014). Each of these strategies fall short in adequacy to some extent. A vaccine for malaria is yet to be developed but the one that currently looks promising is the RTS, S vaccine which targets the blood stage of the plasmodium parasite (Agnandji *et al.*, 2012). Also drug and insecticide resistance is a challenge for drug development and vector control respectively. For vector control, destruction of vectors nests including larvae, indoor residual spraying, intermittent preventive therapy, insecticide treated bed nets and biological control have been employed (Morel *et al*., 2005). The challenge facing vector control now however is insecticide resistance to almost all classes of insecticides (Zofou *et al*., 2014; WHO, 2012). For drug development, monotherapies are no longer used due to increasing drug resistance by the plasmodium parasites. Combination therapy drugs are now used in the treatment

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of malaria in order to prevent or delay resistance by the parasites thus the drug now recommended by WHO is Artemisinin based Combination Therapies (ACT) and quinolines as the preferred partner drug to artemisinins (WHO, 2012). Because there is a likelihood of resistance being developed to the artemisinin-based combination therapy due to possible weak adherence of patients, there is the need to develop novel antimalarial drugs with improved efficiency and efficacy (Banek *et al*., 2014).

Although malaria prevention and treatment tools have been put in place in several endemic countries, malaria predominantly affects rural and poor populations that have little or no access to these tools. Apart from the inaccessibility of these strategies to most rural communities, some challenges that might hinder the success of these strategies include drug resistance due to improper use of malaria medicines, insecticide resistance for the vectors and also the improvement of access to the latest anti-malaria tools.

2.3.1 Malaria, the situation in Ghana

Ghana is an endemic malaria country and a high transmission area with approximately more than 1 case occurring per 1000 population. The major plasmodium species causing malaria is the *Plasmodium falciparum* which accounts for 80%-90% of all cases. *Plasmodium malariae* accounts for 20%-36% and *Plasmodium ovale* accounts for 0.15% of malaria cases in the country (World Malaria Report, 2013). The main vectors of the disease are the *Anopheles gambiae, Anopheles funestus and Anopheles arabiensis* (World Malaria Report, 2013). In Ghana, about 3.5 million people contract malaria each year with 25% of the deaths occurring within this number being that

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of children under the age of five. Malaria has a negative effect on children and most children who survive severe malaria often have some neurological damage that might hinder their development (UNICEF, 2007). In children, malaria have been linked to absenteeism from school among school-age children. (Chuma *et a*l., 2010). In a study conducted by Fernando & Wickremasinghe (2003), they found that even one bout of malaria was negatively associated with student performance on the language and mathematics tests. Zuilkowski & Jukes, (2014) therefore suggested based on their work findings that preventing early childhood malaria may reduce dropout at a relatively low cost.

With up to 60% of out-patient clinical cases being malaria in Ghana, the disease burden is quite high and therefore is a major concern for health workers and scientists within the country. Efforts are thus being put in place to help curb the disease (Asante & Asenso-Okyere, 2003). As at now all efforts and programs put in place with regards to malaria in Ghana is targeted towards control. (World Malaria Report, 2013). The policies and strategies adopted on a national level includes; nationwide education, free distribution and promotion of the use of insecticide treated nets (ITNs) or long lasting insecticide nets (LLINs) for all age groups, indoor residual spraying (IRS), Intermittent Preventive Treatment (IPT) for pregnant women, Intermittent Preventive Treatment in Infants (IPTi), strengthening of health services and the science community thru the organization and hosting of workshops in the field of malaria and several others. (Felix & Asenso-Okyere, 2003; World Malaria Report, 2013).

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Also aside from the national programs that are ongoing, smaller communities are also participating in the fight against malaria. One example is that in 2005, Anglogold Ashanti implemented an integrated malaria programme in Obuasi, a mining town located in the middle belt of the country. The programme covered a large area, including mine housing and infrastructure, private housing and buildings in Obuasi town and in villages within the Obuasi Municipal district. The programme chalked success by reducing the burden of malaria in the community and also reduced absenteeism due to malaria morbidity (Anglogold Ashanti, 2009). Currently the drugs used to treat unconfirmed and *Plasmodium falciparum* malaria in Ghana are quinolone and Artemeter Lumifatherine combination therapy. The available method of diagnosis are the rapid diagnostic test (RDT) and also microscopy. The RDT available in the country however is able to diagnose *Plasmodium falciparum* malaria only. There is no surveillance system put in place yet with regards to malaria. Also currently the source of funding with regards to malaria treatment, control, elimination is obtained from Globalfund, USAID/PMI, WHO/UNICEF and the Government on a small scale. Bill and Melinda Gates foundation also gives funds that are directed mainly towards research (World Malaria Report, 2013).

2.4 The distribution of Malaria

Malaria is endemic in over 100 developing countries in the tropical regions with extensions into sub-tropical regions with altitudes below 1,500m (Zofou *et al.*, 2014; Roll Back Malaria Partnership, 2011). The location and severity of malaria are mostly determined by climate and ecology (Gallup & Sachs, 2001). The area of potential transmission is controlled by climatic factors such as temperature, humidity and rainfall, climate change, immigration and emigration

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as well as the socio-economic conditions of the population. These factors influence the development of both the vector and the parasite. With regard to distribution (see figure 1), *Plasmodium falciparum* and *Plasmodium malariae* have been reported to be found within the same geographical area whilst *Plasmodium vivax* and *Plasmodium ovale* are also reported to occupy complementary niches (Perkins *et al*., 2011). *P. falciparum*, which is responsible for most severe malaria cases and deaths, is generally found in tropical regions and *P. malariae* is also mainly found in tropical regions of the world. *P. vivax* generally is common in Asia and the Eastern Mediterranean and not in Sub-Saharan Africa due to the lack of the duffy antigen by Africans. *P. ovale* is found mainly in Africa and occasionally in Southeast Asia and the Western Pacific (Perkins *et al*., 2011).

Ghana can broadly be divided into the Coastal, Forest and Savannah agro-ecological zones. According to the Ministry of Health (MOH), (1991), each of these zones exhibit different characteristics in relation to the vector and the parasite due to differences in temperature, rainfall and humidity patterns as well as the ecology (Felix & Asenso-Okyere, 2003). In the Coastal zone, malaria transmission is intense and perennial however during the dry season, transmission reduces markedly. The coastal zone falls into the coastal savannah and the coastal lagoons and mangrove swamps. The coastal savannah stretches from the lower Volta Region through the Accra Plains to the lower Central Region with the main parasite species being *Plasmodium falciparum*. The coastal lagoons and mangrove swamps occurring along the shores has *Anopheles melas* as its main vector. Malaria transmission is intense, perennial and quite stable in the rain forest zone. Environmental conditions throughout the year in the forest zone of Ghana is favourable for malaria transmission thus the forest zone has the highest endemicity in the country. Peak transmission is usually soon

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after the major rainy season. The principal vector is the *Anopheles gambiae* complex while the predominant parasite species is the *Plasmodium falciparum*. The Northern Savannah zone covers the three Northern Regions of Ghana. The rainfall pattern in this zone is quite inconsistent. Malaria transmission is intense and perennial however it reduces drastically during the long dry seasons which occur from October to April. The principal vector is the *Anopheles arabiensis* while the principal parasite is the *Plasmodium falciparum*.

Figure 1: A map showing global malaria transmission by country.
(blogs.scientificamerican.com/guest-blog/2013/06/11/breaking-bad-with-breakbone-fever/)

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2.5 Taxonomy of the *Plasmodium* species

Plasmodium falciparum is classified as follows; kingdom Protista, phylum Apicomplexa, class Sporozea, subclass Coccidia, order Eucoccidiorida, suborder Haemosporina and genus *Plasmodium* (Karapelou, 1987). There are over one-hundred named species of Plasmodium infecting vertebrates of all kinds. Four out of the over hundred species of Plasmodium parasites; *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale* and *Plasmodium vivax* are known to cause malaria in humans. Studies in Southeast Asia have shown that a fifth species, *Plasmodium knowlesi*, known to infect monkeys can also infect humans (Perkins *et al.,* 2011).

2.5.1 *Plasmodium vivax*

Plamodium vivax (P. vivax) is the most common and widely distributed malaria parasite. It occurs in Asia and Eastern Mediterranean and accounts for 43% of malaria cases worldwide. *P. vivax* malaria is rarely reported in Sub-Saharan Africa mainly because Africans lack the duffy gene which makes them naturally resistant to the parasite (*Plasmodium vivax*, 2014). The parasite is known to cause benign tertian uncomplicated malaria in humans. Enlargement of the liver is the most common complication caused by *P. vivax* malaria. *P. vivax* malaria can relapse several years after initial infection due to the formation of hypnozoites in the liver. White (2003) indicated that the longest incubation period reported for a relapse of *P. vivax* malaria is 30 years.

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2.5.2 *Plasmodium ovale*

Plasmodium ovale (P. ovale) is the rarest malaria parasite that infects humans. It occurs mainly in the tropical regions of the world however it is not very common in Central and Eastern Africa. It is known to cause mild tertian uncomplicated malaria in humans (*Plasmodium vivax*, 2014).

2.5.3 *Plasmodium malariae*

Plasmodium malariae (P. malariae) accounts for 7% of malaria cases worldwide and has a cosmopolitan distribution. It has been reported to cause malaria in humans as well as in wild animals. It is known to cause quartan malaria in humans. This species of plasmodium can become active again in the blood after several years of dormancy (Garnham, 1966).

2.5.4 *Plasmodium falciparum*

Plasmodium falciparum malaria is the most virulent of all the Plasmodium species and is predominant in Sub-Saharan Africa (Perkins *et al*., 2011). It causes malignant tertian malaria or cerebral malaria in humans. The known symptoms of P. *falciparum* malaria are headache, high fever with chills, sweats, nausea, vomiting, cough, abdominal and back pain. Other serious complicated symptoms include severe anemia, jaundice, thrombocytopenia, pallor and the most severe form of malaria which is cerebral malaria (Barnwell, 1989).

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2.6 The life cycle of Plasmodium falciparum

The malaria parasite has a life cycle which occurs in two different hosts; the invertebrate host (vector) mosquitoes and the vertebrate hosts. The parasite passes through several stages of development in each of the host. In the invertebrate host it passes through the stages of fertilization, meiosis, ookinete formation, oocyst formation and sporogony. In the vertebrate host it passes through the stages of pre-erythrocytic schizogony, erythrocytic schizogony and erythrocytic gametogony (Greenwood *et al*., 2008). All of these stages have their own unique shapes and structures and also mechanisms that help the parasite to evade the immune system (Floren *et al*., 2002)

2.6.1 Pre-erythrocytic Schizogony (asexual liver stage)

This stage starts when a vertebrate host is bitten by an infected female anopheles mosquito during feeding. With the mosquito bite, tens to a few hundred invasive sporozoites are inoculated into the skin first which then moves into the blood stream. The sporozoites circulate in the blood stream and then reach the liver within few hours where they enter into few hepatocytes, multiply and grow within parasitophorous vacuoles (Yamauchi *et al*., 2007). Each sporozoite develops into a schizont that reproduces asexually to generate over 20,000 merozoites (exoerythrocytic merozoite) (Jones & Good, 2006). It takes approximately between 5-16 days depending on the parasite species for the merozoites to rupture the liver cells and infect red blood cells in the peripheral blood at the lung capillaries (Good & Doolan., 2007; Silvie *et al*., 2008). This begins the erythrocytic schizogony. The pre-erythrocytic phase has little pathology and no symptoms, as only a few hepatocytes are affected.

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2.6.2 Erythrocytic Schizogony Phase (asexual blood stage)

Asexual development of the malaria parasite occurs mainly in the red blood cells. The merozoites released from the liver recognize, attach, and enter the red blood cells (RBCs) within a minute of their release. This minimizes the exposure time of the parasite surface antigens to host immune cells therefore the parasite is protected from host immune response (Cowman & Crabb, 2006). After entering the red blood cell, the merozoite undergoes a trophic period and then replicate asexually. The young trophozoite which is ring shaped increases in size, loses its ring shape and becomes a trophozoite. The parasite at this stage ingests the host cell cytoplasm and digests haemoglobin which gives hemozoin as a by-product. Red blood cells containing matured trophozoites ruptures to release newly formed merozoites into peripheral blood. Within the red cells, repeated cycles of parasitic development occur with precise periodicity, and at the end of each cycle, hundreds of fresh daughter parasites are released that invade more number of red cells (Miller *et al*., 2002). It has been widely studied and proven that the release of the antigens and waste products after the rupture of infected red blood cell accounts for the intermittent fever paroxysms associated with malaria.

2.6.3 Erythrocytic Gametogony (sexual blood stage)

After the rupture of the red blood cells to release merozoites, some of the parasites develop into gametocytes which are the sexual forms of the parasite. The male and female gametocyte (micro

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and macro gametocytes respectively), are ingested by a female *Anopheles* mosquito during a blood meal. The parasite then continues its sexual life cycle in the mosquito because mosquitoes are the definitive hosts for the malaria parasites.

2.6.4 Stages in the mosquito (sporogonic stages)

When the female *Anopheles* draws a blood meal from an individual infected with malaria, the male and female gametocytes of the parasite find their way into the gut of the mosquito. The male gametocyte undergoes exflagellation and produces 6-8 motile male gametes and the female gametocyte produces one gamete. The male and the female gametocyte fuse in the mosquito midgut to form an ookinete. The actively moving ookinetes then burrow into the mosquito midgut wall to develop into oocyst. The oocyst has several sporozoites developing in it. After the sporogonic phase of 8–15 days, the oocyst bursts and releases sporozoites into the body cavity of the mosquito, from where they travel to and invade the mosquito salivary glands (Ferguson & Read, 2004). When an infected mosquito bites a susceptible vertebrate host, it injects the sporozoites into the host and the life cycle begins again.

2.7 General signs and symptoms of malaria (Malaria pathogenesis)

Symptoms of falciparum malaria range from asymptomatic infections to severe malaria. Clinical features of malaria include fever, chills, sweating, headaches, vomiting, muscle aches, mild

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diarrhea, dizziness, dry cough. Progression from some of these general symptoms may lead to complications of the disease such as renal failure, hyperparasitemia, hypoglycemia, severe anemia, respiratory distress and cerebral malaria (Marsh *et al*., 1995; WHO, 2000).

2.7.1 Complications of Plasmodium falciparum malaria

Several complications arise due to *P. falciparum* malaria progession when left untreated. Some complication of falciparum malaria are severe anemia (1% mortality), Marsh *et al*. (1995), acidosis (15% mortality) or coma (18% mortality), severe liver failure, circulatory collapse, hypoglycaemia, hyperpyrexia, acute pulmonary, oedema and renal failure (Dondorp, 2010). The placenta is also one preferred site of sequestration for infected red blood cells, thus *falciparum* malaria is a major cause of maternal death, abortion, stillbirth, premature delivery and low birth weight in endemic areas (Ramasamy, 1998).

2.7.2 Severe malaria anemia

Blood stage infection of P. *falciparum* malaria induces anemia. Severe malarial anemia caused by P. *falciparum* is responsible for approximately a third of the deaths associated with disease (Haldar & Mohandas, 2009). In holoendemic transmission areas, severe malaria anemia mortality can exceed 30% in pediatric populations. Development of severe malaria anemia seems to be multi-factorial. It involves lysis of infected red blood cells coupled with inefficient and/or decreased production of erythrocytes in the bone marrow erythrocytes which leads to failure in the ability to replenish the reduced pool of erythrocytes (Haldar & Mohandas, 2009; Abdalla *et al*., 1980;

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Phillips *et al.,* 1986). Also destruction of parasitized and non-parasitized circulating red blood cells by the spleen may be a contributory factor to severe malaria anemia (Buffet *et al*., 2009).

An imbalance in the range of pro- and anti-inflammatory cytokines, chemokines, growth factors, and effector molecules released in an attempt to control the parasitemia can induce damage to the host, including suppression of the erythropoietic response leading to anemia development (Helleberg *et al.,* 2005). Tumor necrosis factor-alpha (TNF-α) and Interleukin-10 (IL-10) have been and reported to be involved in the development of severe malaria anemia (Akanmori *et al.,* 2000; Casals-Pascual *et al*., 2006). Also haemozoin in the bone marrow have been implicated to play a role in the pathogenesis of malarial anemia through ineffective erythropoiesis (Aguilar *et al*., 2014).

2.7.3 Respiratory distress and metabolic acidosis

Metabolic acidosis is present in 40–60% of cases of severe malaria, often presenting as respiratory distress. It occurs when there is a buildup of lactic acid concentration in the blood as a result of products of anaerobic glycolysis from inadequately perfused host tissues (Miller *et al*., 2013). The liver is responsible for removing lactate from the blood, however its inability to compensate for the high rate at which malaria parasites produces lactate leads to metabolic acidosis in malaria. Also direct effect of cytokines such as TNF-α and IL-1 have been implicated in pathogenesis of metabolic acidosis (Krishna *et al*., 1994). Some symptoms that characterize respiratory distress are the presence of pulmonary oedema, intercostal muscle retraction, deep acidotic breathing and involvement of abdominal muscles when breathing (WHO, 2000).

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2.7.4 Cerebral malaria

Cerebral malaria which occurs only in falciparum malaria is one of the most severe forms of malaria and accounts for 80% of deaths. It causes shock, unrousable coma and eventually death (Goldman, 1999).The major feature of cerebral malaria which is the unrousable coma has no direct known cause. Other symptoms of cerebral malaria are stiffness of the neck and convulsions or partial motor seizures which occurs in both non-immune adults and children. Seizures may be subtle, such as repetitive eye or hand movements. The level of consciousness after a seizure is usually lower than that preceding it (Snow *et al*., 2001). A compromised microcirculation, with sequestration of parasitized red blood cells in capillary brain endothelium is known so far to be the main feature in the pathogenesis of cerebral malaria (Dondorp, *et al.*, 2005b).

Immunohistochemical staining suggest that there are endothelial activation, inflammation and disruption of the blood-brain barrier involved (Turner *et al*., 1994). Inflammatory cytokines such as interferon-ɣ (IFN-ɣ), tumor necrosis factor-α (TNF- α) and lymphotoxin- α (LT- α) have been shown in some studies to influence the biochemical pathways in the brain during falciparum malaria infection thereby participating in the pathogenesis of CM (Chen *et al*., 2000a; Engwerda *et al*., 2002; Hunt & Grau, 2003; Lou *et al*., 2001; Weiser *et al.*, 2007). Also Linares *et al*., (2012) found out in a cerebral malaria experiment conducted using mice that there is upregulation of ICAM-1, VCAM-1, e- selectin and p-selectin expression in all cerebral regions before infected red blood cells accumulation. This implicates inflammatory cytokines as contributing factors in the pathogenesis of cerebral malaria.

Cerebral malaria in children is associated with long-term neurocognitive sequellae in some

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children after recovery from coma (Casals-Pascual *et al*., 2008). It has been reported that, about 14–25% of children who recover from CM develop cognitive impairment such as speech and language impairment, epilepsy, behavior and neuropsychiatric disorders such as inattentiveness, impulsiveness and hyperactivity (Carter *et al*., 2005). The causes of sequelae are largely unknown thus have been postulated to be multifactorial with risk factors being hypoglycemia, seizures, depth and duration of coma, and hyporeflexia (John *et al*., 2008). In clinical settings, cerebral malaria is defined based on the Blantyre coma scale in order to assess young children with severe malaria (Warrell *et al.,* 1990). Impaired consciousness, measured by the Blantyre coma score, is consistently associated with increased risk of lasting neurological injury and death (Beare *et al*., 2004).

2.8 Pathogenesis of cerebral malaria

Despite treatment, CM mortality can be as high as 30%, while 10% of survivors of the disease may experience short- and long-term neurological complications. The pathogenesis of CM is mediated by alterations in cytokine and chemokine homeostasis, inflammation as well as vascular injury and repair processes (Wiser, 2000).

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2.8.1 Sequestration of Plasmodium falciparum in cerebral malaria

Several factors contribute to the pathogenesis of cerebral malaria however sequestration of parasitized red blood cells is thought to be the main factor in the pathogenesis of cerebral malaria (MacPherson *et al*., 1985). Sequestration occurs when red blood cells infected with mature forms of *Plasmodium falciparum* in the peripheral blood are withdrawn and bind to the endothelial lining

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(cytoadherence, figure 2) of organs such as brain, kidney, eye, liver, placenta, heart in the body with varying degree (White *et al*., 1992; Newbold *et al*., 1999; Rogerson *et al*., 2004). Post mortem studies done in some studies have shown that sequestration of infected red blood cells is greater in the brain compared with other organs (MacPherson *et al*., 1985; Oo *et al*., 1987).

Cytoadherence has been shown to be facilitated by the electron-dense protuberances called knobs, on the surface of the infected erythrocyte which serves as specific points of contact between infected red blood cells and the endothelial lining (Dondorp *et al*., 2005a). Endothelial surface molecules such as thrombospondin, ICAM-1, CD36, E-selectin, ELAM-1 and VCAM-1 have been reported to also mediate cytoadherence (Grau & Lou, 1994). The infected red blood cells also bind to other uninfected red blood cells (rosetting) which eventually leads to microvascular occlusion, a contributing factor to the resulting coma in cerebral malaria (Dondorp *et al.,* 2004). It has been suggested that infected red blood cells begin cytoadherence during the trophozoite and schizont stages since that is when the expression of knobs occurs (Nash *et al.,*1992). Cytoadherence has been suggested to be an immune evasion strategy and also a means to evade spleen destruction by the parasite. It has also been postulated that sequestration of infected red blood cells enables the *Plasmodium falciparum* to be in an oxygen-depleted environment which favours their optimal growth (Chen *et al*., 2000b).

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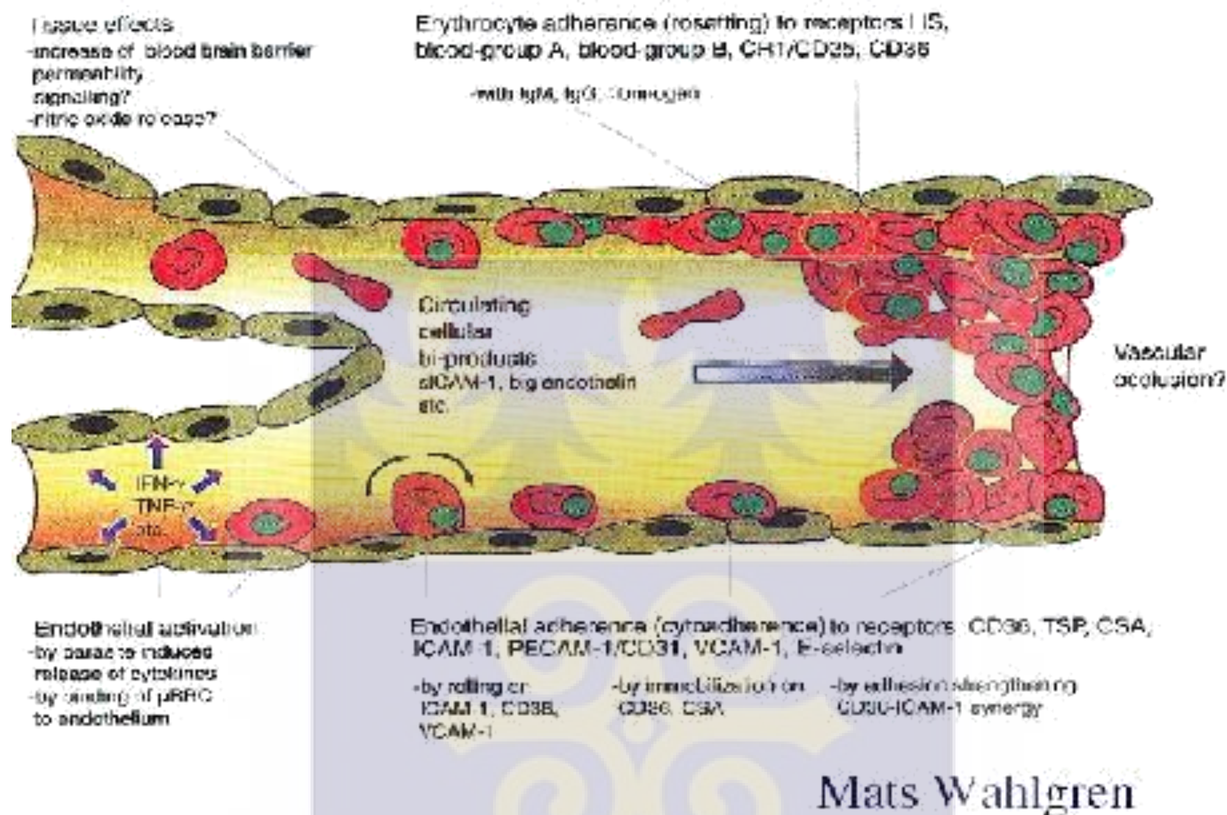

Figure 2: Obstruction of microvascular blood flow by cytoadherence
(Adapted from Chen *et al*., 2000 a)

INTEGRI PROCEDAMUS

2.9 Host endothelial cell

The vascular endothelium is a complex organ that can interact with and respond to its environment, shifting from quiescent to activated and back again. The endothelium is present in every organ in humans and is responsible for physiological functions, such as regulation of oxygen and nutrients transport, blood pressure, coagulation, inflammatory processes and angiogenesis. It is made up of monolayer of endothelial cells (ECs) which serves as a margin between circulating blood and the

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surrounding tissue (Hristov *et al*., 2003). Endothelial cells are known to come from various lineages such as the endothelial lineage and hematopoietic lineage each with similar function. CECs and EPCs are known as non-hematopoietic cells. CECs are believed to be derived from mature endothelium while EPCs are derived from the bone marrow mediated by eNOS-derived nitric oxide (NO) however it is believed that immature EPCs can differentiate into CECs when they mature (Hristov *et al*., 2003; Yoder, 2012). It has been suggested that signals from the bone regeneration site mobilize EPCs from the bone marrow into the peripheral circulation Lee *et al*. (2008) however certain amount of bone marrow-derived EPCs have been observed to be present in peripheral blood where they increase in response to certain cytokines (Iwaguru *et al*., 2002). They are used in the formation of new vessels and in repairing existing vascular endothelium when there is injury by migrating and spreading into the site of injury (Schwartz *et al.,* 1980). The bone marrow is made up of different types of stem/progenitor cells such as the multi-potent adult progenitor cells, able to regenerate several tissue layers, mesenchymal stem cells, and haemangioblasts, the common putative precursors of haematopoietic and the endothelial lineages (Orlic *et al*., 2002).

The rate of endothelial layer renewal is low about 0–1% per day in healthy individuals. Human endothelial progenitor cells (EPCs) have been generally defined as circulating cells that express a variety of cell surface markers similar to those expressed by vascular endothelial cells, adhere to endothelium at sites of hypoxia/ischemia, and participate in new vessel formation (Yoder, 2012). Circulating endothelial cells (CECs) were first described over 30 years ago in smears of peripheral blood (Erdbruegger *et al*., 2006) however Asahara *et al*. (1997) were the pioneers in the identification of EPCs and showing that they are derived from the bone marrow with an endothelial lineage. EPCs are generally characterized by the expression of three markers, CD133, CD34, and

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the vascular endothelial growth factor receptor-2 but during differentiation, they lose CD133 and start to express CD31. They are likely to proliferate and to differentiate into mature CECs (Hristov *et al*., 2003).

When there is injury to the endothelium, it gets activated, loses its normal function and hence becomes pro-coagulant and pro-adhesive at the surface (Aird, 2003). CECs replicate in order to replace damaged endothelial cells but they have a limited ability in migrating and proliferating to sites of endothelial damage thus EPCs support them by effectively migrating and proliferating from the bone marrow to endothelial injury sites in order to repair it (Urbich & Dimmeler, 2004). It has been reported that insufficient or dysfunctional EPCs and levels of associated factors which effect EPC mobilization and function such as the chemokines, stromal cell derived growth factor 1 (SDF-1) and matrix metalloproteinase-9 (MMP-9), have been shown to project poor outcomes in several diseases associated with microvascular damage which supposes that bone marrow-derived circulating endothelial progenitor cells (cEPCs) mediates microvascular repair by incorporating into damaged sites (Gill, 2001). Microvascular damage is therefore implicated in the pathogenesis and disease progression for a range of infectious syndromes such as sepsis, acute lung injury, dengue hemorrhagic fever and shock syndrome and malaria (Darwish & Liles, 2013).

One common feature of malaria infection is endothelial cell injury and increased numbers of circulating endothelial microparticles could serve as indicators of endothelial activation in patients with malaria complicated by coma (Combes *et al*., 2004). For example in *Plasmodium falciparum* malaria, damage to the microvasculature have been shown to occur by the sequestration of infected red blood cells in the microvasculature together with vascular occlusion and/or a cytokine-mediated sepsis like syndrome which has been proposed as a cause of cerebral malaria (Weatherall

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et al., 2002; Adams *et al*., 2002; Taylor *et al.,* 2004). In line with this Gyan *et al*. (2009) revealed that children with cerebral malaria (CM) had significantly lower levels of EPCs compared to uncomplicated malaria (UM), asymptomatic parasitemia (AP) or healthy controls (HC) and found that mean levels of SDF-1 was significantly increased in children with CM and UM as compared to asymptomatic parasitemic children or healthy controls. High CEC levels have been found in severe acute myocardial infarction disease (Mutin *et al*., 1999). Freestone *et al*. (2005) also showed that patients with atrial fibrillation and an acute cardiovascular or cerebrovascular event also had significant elevated CECs compared to patients with uncomplicated chronic atrial fibrillation.

Despite the restorative ability of EPCs, their number in circulation and function are negatively affected by age-associated changes. As one ages, there is a decline in the expression of pro-angiogenic factors such as growth factors, cytokines, and hormones. This is likely to diminish the generation, mobilization, migration, and survival of EPCs. Chang *et al*. (2007), demonstrated that decreased SDF-1 and VEGF expression in aged tissues impaired EPC trafficking to sites of ischemia. Also it is likely that there will be enhanced vascular disease risk with increasing age as oxidative cellular damage might accumulate in EPCs thereby waning its survival capacity and protective function. He *et al*. (2009), demonstrated that early EPCs derived from old subjects had significantly reduced levels and activity of the antioxidant enzyme glutathione peroxidase-1 (GPx-1) and were more sensitive to oxidative stress-induced apoptosis as compared to EPCs of younger subjects. Age can therefore be said to be a significant risk factor for the development of vascular diseases.

Endothelial cells express a variety of proteins in serum or plasma which plays significant roles as indicators of endothelial activation which may be useful clinically as biomarkers of disease

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severity or diagnosis in systemic infectious diseases. Some of these are von Willebrand factor (vWF), thrombomodulin, soluble E-selectin, angiopoietins and many others (Ortiz *et al*., 2003; Rojas *et al*., 2005).

2.9.0 Host endothelial cell markers

Circulating endothelial cells (CECs) and circulating endothelial progenitor cells (CEPCs) have been identified as two cell populations that play crucial functions in the development and maintenance of blood vessels (Duda *et al*., 2007). Due to their importance in determining the outcome of several diseases, they have generated a lot of interest in the biomedical research field. Specific surface markers for CECs and EPCs identified by hematopoietic functional assays have increased the understanding of these cells. There is no clear cut marker for these cells therefore slight variations in the markers used for the phenotypic identification of CECs and EPCs exists in different studies. One way of phenotypically identifying and counting cell expressing surface markers for EPCs and CECs is by means of the flow cytometry using whole blood or separated mononuclear cells (Timmermans *et al*., 2008). Timmermans *et al*. (2008) used the cell surface markers CD34, VEGF2 (KDR), CD133, and CD45 to identify endothelial cells and indicated that CD34þ KDRþ (VEGFR-2) cells not expressing the CD45 antigen are the actual circulating EPCs. Duda *et al*. (2007) suggested that to identify viable CEC and EPCs phenotypically, it was best to use the cell surface markers; CD31-FITC, CD34- APC, CD133-PE, CD45-PerCP and VEGFR2 (KDR)-PE. Gyan *et al.* (2009) also used CD34 + /VEGFR2 + and CD34 + /CD133 + to identify and differentiate between the levels of CEC and EPCs respectively in children presenting with cerebral malaria (CM), uncomplicated malaria (UM) and asymptomatic parasitaemia. CD11b has

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also been suggested as a cell surface marker that identifies endothelial cells in addition to the above mentioned ones. Hristov *et al.* (2003) indicated that though EPCs are characterized by the expression of the surface markers, CD133, CD34, and the vascular endothelial growth factor receptor-2 (VEGFR-2), during differentiation they lose CD133 and start to express CD31, vascular endothelial cadherin, and von Willebrand factor.

CD34 is known to be expressed on hematopoietic precursors and endothelial cells (Duda *et al,* 2007; Leone *et al*., 2009). They play critical role in angiogenesis and neovasculogenesis during both tumour growth and wound healing. Stellos & Gawaz, (2007) indicated the recruitment and proliferation of CD34 by the cytokine-mediated SDF-1 after endothelial injury in a mouse model experiment.

CD 133 is known to be highly expressed on hematopoietic stem cells and lack the ability to give rise to endothelial cells during in vitro culture, in vitro or in vivo from blood vessels (Timmermans *et al.,* 2007; Duda *et al*., 2007). In addition to hematopoietic cells, it has been reported that CD 133 coexpression with CD34 and VEGFR-2 aids in recognizing early circulating EPCs (Reviewed in Ribatti, 2004; Timmermans *et al.,* 2007). In human haematopoietic lineages, CD133 antigen expression has been said to be restricted to CD34+ cells (Yin *et al.,* 1997), CD133 has also been reported to be a marker of tumour-initiating cells in a number of human cancers (Mizrak *et al*., 2008).

VEGF2 (KDR) is known to express the CD45 antigen. VEGF2 (KDR) is present on endothelial cells, certain monocytes and hematopoietic precursors whilst CD45 is a pan-hematopoietic marker. They have both been shown to be absent in mature endothelial cells (Timmerman, 2008; Duda *et*

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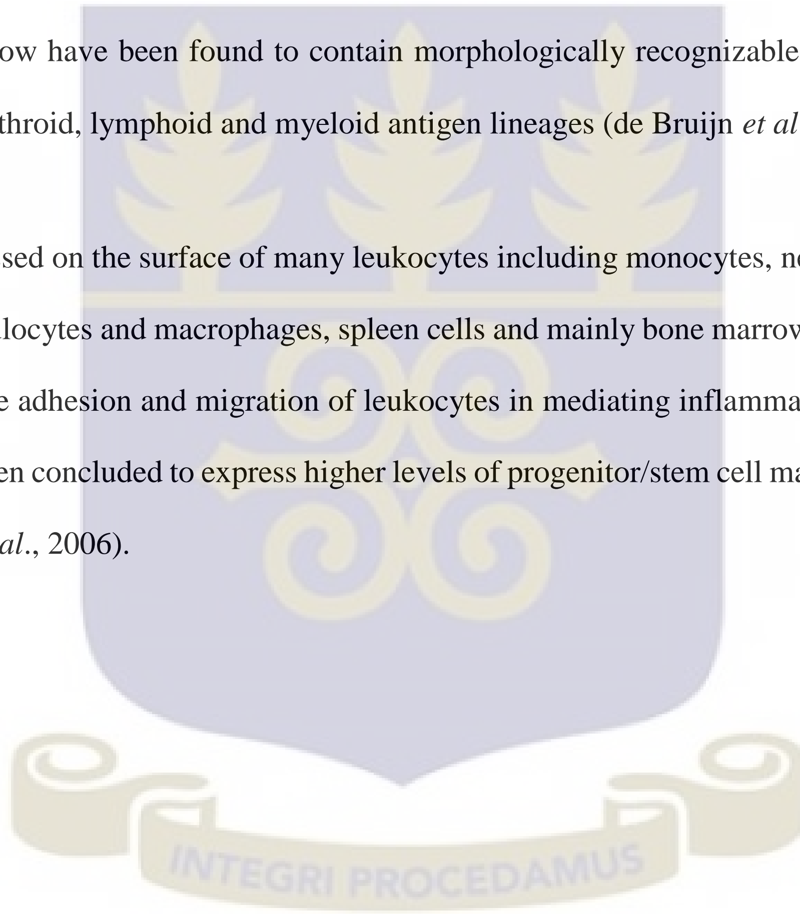
al., 2007).

CD 31 (PECAM-1) is known to be expressed on a series of cell populations in the blood including platelets, myeloid cells, B lymphocytes, certain T lymphocyte cell subsets, bone marrow precursor cells and NK cells. It has been shown to demonstrate angiogenic properties (Loomans *et al*., 2006; Ge *et al*., 2012). Loomans *et al*. (2006) demonstrated that a majority of EPCs are derived from immature CD31 and that they are the main source of bone marrow derived EPC. The CD31 cells in the bone marrow have been found to contain morphologically recognizable precursors of the granulocytic, erythroid, lymphoid and myeloid antigen lineages (de Bruijn *et al*., 1994).



CD 11b is expressed on the surface of many leukocytes including monocytes, neutrophils, natural killer cells, granulocytes and macrophages, spleen cells and mainly bone marrow cells. It has been shown to regulate adhesion and migration of leukocytes in mediating inflammatory responses. In all EPCs have been concluded to express higher levels of progenitor/stem cell markers than mature EC (Loomans *et al*., 2006).

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2.9.1 The role of Angiopoietin-1 in cerebral malaria

Angiopoietin-1 (Angpt1) is a secreted 70-kDa glycoprotein and a member of the angiopoietin family of growth factors (Jeansson *et al.,* 2011). Ang-1 is produced primarily by vasculature support cells, smooth muscle cells that surround the endothelial cell monolayer and specialized pericytes such as podocytes in the kidney and ITO cells in the liver (Page & Liles, 2013; Jeansson *et al.,* 2011). Ang-1 therefore acts in a paracrine manner (Felcht *et al*., 2012). Ang-1 is a ligand of the Tie-2 receptor, which belongs to a family of vascular tyrosine kinase (Tek) receptors expressed primarily in endothelial cells (Hansen *et al*., 2009). In endothelial cells, Ang1 activates Tie2 and becomes an effective mediator of angiogenesis and functions post-development to prevent vascular leakage and promote endothelial vascular quiescence by strengthening endothelial cell junctions and down-regulating surface adhesion molecules, such as VCAM-1 and E-selectin (Thurston *et al*., 2000; Gamble *et al*., 2000). Ang-1 is detected in soluble form in the plasma or serum. Concentrations of Ang-1 is known to exceed that of Ang-2 which is its antagonist (Lukasz *et al.,* 2008).

In a study conducted by Jeansson *et al*. (2011), they demonstrated that Ang-1 has a powerful ability to modulate the vascular response after injury when Ang-1 deficiency coupled with microvascular stress or injury in their mice model resulted in organ damage, accelerated angiogenesis, and fibrosis. Also Suri *et al*. (1996) found that mice deficient in Ang1 had disrupted interactions between ECs and peri-ECs that severely impaired vascular function, leading to embryonic death of the mice. This shows that Ang-1 is needed for the proper function of the vascular organs. Endothelial Ang-1 is expressed widely in normal adult tissues and is usually constitutively expressed in brain endothelium (Chittiboina *et al.,* 2013). Due to the protective effect of Ang-1, a

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number of Ang-1/Tie2 targeted strategies are being investigated in pathology of several diseases such as acute sepsis, stroke, arteriosclerosis, lung injury and acute kidney injury (Mei *et al*., 2007).

Activation of the endothelial cells causes the dysregulation of Ang-1 and Ang-2 balance which can lead to several disease complications. Altered Ang-1 and Ang-2 levels have been implicated in tumor-associated angiogenesis, cancer and hypertension. For example one feature of *P. falciparum* malaria includes the adhesion of mature parasitized erythrocytes to the microvasculature of vital organs and acute endothelial activation. This leads to the release of Weibel-Palade body products such as von Willebrand factor (VWF), propeptide (VWFpp) and angiopoietin-2 (Ang-2) into the systemic circulation. And these biomarkers have been identified as indicators of malarial disease severity (Fiedler & Augustin, 2006; Larkin *et al*., 2009).

Angiopoietins are one of the few factors that are being investigated in the pathogenesis of cerebral malaria since are regulators of the endothelium. A study was done by Lovegrove *et al.* (2009) to analyze the predictive value of angiopoietins for CM diagnosis in Thailand and Uganda patients. They found that the levels of Ang-1 were significantly reduced in CM patients compared to healthy controls and mild malaria patients whilst Ang-2 levels were significantly elevated in CM patients compared to control groups. Ang-1, Ang-2 and the ratio of Ang-2/Ang-1 were all shown to accurately discriminate between CM and mild malaria patients. This study proves that there is an endothelial dysregulation involved in the pathogenesis of cerebral malaria.

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2.9.2 The role of Angiopoietin-2 in cerebral malaria

Angiopoietin-2 (Ang-2) is produced in low levels primarily by endothelial cells and stored in Weibel–Palade bodies for rapid release upon exposure to various inflammatory stimuli. They have a half-life of more than 16 hours and act as autocrine regulator of endothelial cell functions. The overexpression of Ang2 in ECs destabilizes quiescent ECs through an internal autocrine loop and leads to EC detachment and a vessel regression (Hu & Cheng, 2009). The effect of Ang-2 is dependent on VEGF. In the presence of VEGF, Ang-2 has been shown to be involved in the promotion of rapid increase in capillary diameter, remodeling of the basal lamina, stimulating sprouting of new blood vessels, proliferation and migration of endothelial cells however in the absence of VEGF, Ang-2 is involved in the promotion of endothelial cell death and vessel regression (Lobov *et al.,* 2002).

Ang2 has been reported to act as a circumstance dependent agonist or antagonist for Tie2 (Augustin *et al.,* 2009). Ang2 has been shown to liaise with Ang1 in modulating endothelial development from circulating cord blood CD34+ progenitors. Also whiles Ang-1 regulates initial EC commitment of the CD34+ progenitor cells, Ang-2 contributes to it's the proliferation (Sato *et al.*, 1995). Also the synergic action of Ang-2 and Ang-1 directly activates ECs and neutrophils to promote a pro inflammatory response through Tie2- and β2 integrin–mediated signaling (Suri *et al.*, 1996). Also elevated Ang-2 levels were reported in septic patients and correlated with disease severity and predicted subsequent development of shock or death (Parikh *et al.,* 2006; Kümpers *et al.,* 2008). Contrastingly, elevated Ang-2 levels increased the survival of mice who were induced with *Escherichia coli* or *Pseudomonas aeruginosa* experimental sepsis when recombinant Ang-2 was administered to them few hours prior to the experimental induction (Tzepi *et al.*, 2012).

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Ang-2 antagonizes Ang-1 function resulting in endothelial activation and increased vascular Permeability. Ang-2 has been reported to serves as a marker of secondary injury, modulator of blood brain barrier breakdown and endothelial apoptosis (Chittiboina *et al*., 2013). Angiopoietin dysregulation indicates a perturbation of the normally low Ang-2: Ang-1 ratio, whether by decreased Ang-1, increased Ang-2 levels or both. The ratio of Ang-2/Ang-1 in serum serves as a good predictor of disease severity thereby implicating them in various disease pathogenesis (Page & Liles, 2013). Chittiboina *et al*. (2013) suggested that the early phase of injury in the endothelium is associated with a decrease in levels of Ang-1 and an up regulation of Ang-2.

Elevated Ang-2 levels have been shown to correlate with injury severity, organ failure, acute physiology and chronic health (Chittiboina *et al*., 2013)**.** In *P. falciparum* malaria, Ang-2 has been shown to sensitize the endothelium to high levels of tumour necrosis factor which leads to an increase in the expression of adhesion molecules such as ICAM-1 to which parasitized red blood cells bind (Fiedler *et al*., 2006). Also (Yeo *et al*., 2008)) demonstrated that increased plasma Ang-2 concentrations were associated with increased venous lactate, plasma intercellular cell adhesion molecule-1 concentrations, parasite biomass, and mortality in severe malaria patients. They also found the Ang-2 concentrations were good predictors of death in the patients.

2.9.3 The role of Thrombomodulin in cerebral malaria

Thrombomodulin (TM) is an endothelial cell transmembrane glycoprotein which serves as an important anti-coagulant receptor for thrombin (Dittman *et al*., 1990). TM is present in large quantities on the surface of the endothelium of arteries, veins and capillaries, lymphatic endothelial cells (Page & Liles, 2013). TM is expressed at a lower constitutive levels in the brain compared

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with other organs. TM forms a complex with thrombin and this complex catalyzes the generation of the anticoagulant molecule activated protein C (figure 3) which prevents thrombin from converting fibrinogen to fibrin and also from exerting other procoagulant effects (Moxon *et al*., 2013).

TM is known to also exhibit an anti-inflammatory effect, regulate cell adhesion and proliferation through its lectin-binding domain and possibly have unique functions during development (Nawroth & Stearn, 1986; Takano *et al*., 1990; Kodama et al., 1990). When the endothelium gets activated perhaps due to inflammatory cytokines such as TNF, it becomes susceptible to coagulation hence cell-surface thrombomodulin expression reduces, possibly due to shedding of the molecule into a soluble form, soluble thrombomodulin (sTM) (Page & Liles, 2013). sTM is found in serum, plasma and urine. Esmon, (1981) demonstrated using a mouse model that increased serum TM level is associated with a decreased expression of TM on the endothelial surface in vivo. Nevertheless, elevated serum TM levels are found in diseases associated with systemical or locally increased levels of inflammatory cytokines such as malaria, dengue fever, sepsis and other diseases and syndromes such as cardiovascular diseases, acute coronary syndrome, pulmonary thromboembolism, and severe hemorrhage (Jansson *et al*., 1997; Salomaa, 1999).

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In a study conducted by Ohnishi, (1999) using Malian children with uncomplicated and non-cerebral severe malaria, they showed that sTM levels was elevated during infection and declined with recovery. They also showed that the levels of sTM correlated with both parasitemia and disease severity, and were higher in children with severe malaria than in those with uncomplicated malaria. In another malaria study by Matondo *et al.* (2008), where patients presenting with uncomplicated *P. falciparum* malaria and uninfected individuals as controls was used, it was demonstrated that sTM levels was higher in patients than uninfected control subjects, and the sTM levels positively correlated with levels of pro-inflammatory cytokines and anemia which is a marker of disease severity. The elevated serum levels of TM in both studies suggest that endothelial cell damage occurs in the *P. falciparum* malaria. sTM has been proposed as both a diagnostic and prognostic tool of endothelial activation or dysfunction (Faust *et al*., 2001).

2.9.4 The role of Endothelial Protein C Receptor in cerebral malaria.

Endothelial cell protein C receptor (EPCR) is a type 1 transmembrane glycoprotein 1 and is mainly expressed on endothelial cells of large vessels (Fukudome *et al.,* 1994). EPCR is expressed on aortic endothelial cell, surface of monocytes, natural killer cells, neutrophils, eosinophils, immature hematopoietic stem cells, brain capillary endothelial cells and embryonic giant trophoblast (Balazs *et al*., 2006; Stephenson *et al*., 2006). EPCR is expressed at a lower constitutive levels in the brain compared with other organs (Moxon *et al*., 2013). The generation of activated protein C is needed by the TM-thrombin complex to prevent procoagulation effects in the endothelium which is augmented by the endothelial protein C receptor (see figure 3). Activated protein C have been shown to down regulate the production of inflammatory cytokines from

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monocytes12-14 and suppress leukocyte adhesion molecule expression in endothelial cells. EPCR is known to bind to circulating protein C and present it to the TM-thrombin complex where it is converted rapidly to activated protein C. Therefore both EPCR and TM receptors are required to generate APC in response to elevations in thrombin levels (Moxon *et al*., 2013). EPCR signaling has been shown to decrease inflammation. Some study found that APC binding to EPCR rescued baboons from *E. coli* sepsis and also another study found that EPCR plays a cardioprotective role in lipopolyscharide-induced endotoxemia in mice (Iwaki *et al.,* 2005).

Downregulation of EPCR causes the EPCR to be shed from the endothelium and become soluble form called soluble EPCR. sEPCR is found in plasma or serum in humans and has been shown to bind PC and APC with the same affinity as the membrane bound EPCR (Liaw *et al*., 2000). Both EPCR and sEPCR when bound to protein C enables activation by the thrombin–thrombomodulin complex for anticoagulant activity however when bound to activated protein C inhibits the anticoagulant activity of activated protein C (Kurosawa *et al*., 1997). The down regulation of these by inflammatory cytokines leading to pathologic conditions have been hypothesized to impair the ability to generate activated protein C despite high thrombin levels. A dysfunction in the pathway of anticoagulant protein C pathway has been implicated in several disease complications such as bacterial sepsis, dengue hemorrhagic fever, pediatric stroke and severe malaria (Faust et al., 2001; Herak et al., 2009; Cabello-Guti´errez et al., 2009).

During *P. falciparum* severe malaria, the malaria parasite protein PfEMP-1 has been shown to bind to EPCR. Turner *et al*. (2013) demonstrated using autopsies from Malawian children with CM that loss of EPCR occurs in cerebral malaria, localized to sites of sequestered parasitized red blood cells. Moxon *et al*. (2014) showed that in cerebral malaria there is activation of coagulation

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which signifies a dysregulation in endothelial anticoagulant receptors. It was thereby suggested that defect in the endothelial homoeostatic pathway mainly affects the brain, where constitutive expression of thrombomodulin and EPCR is low.

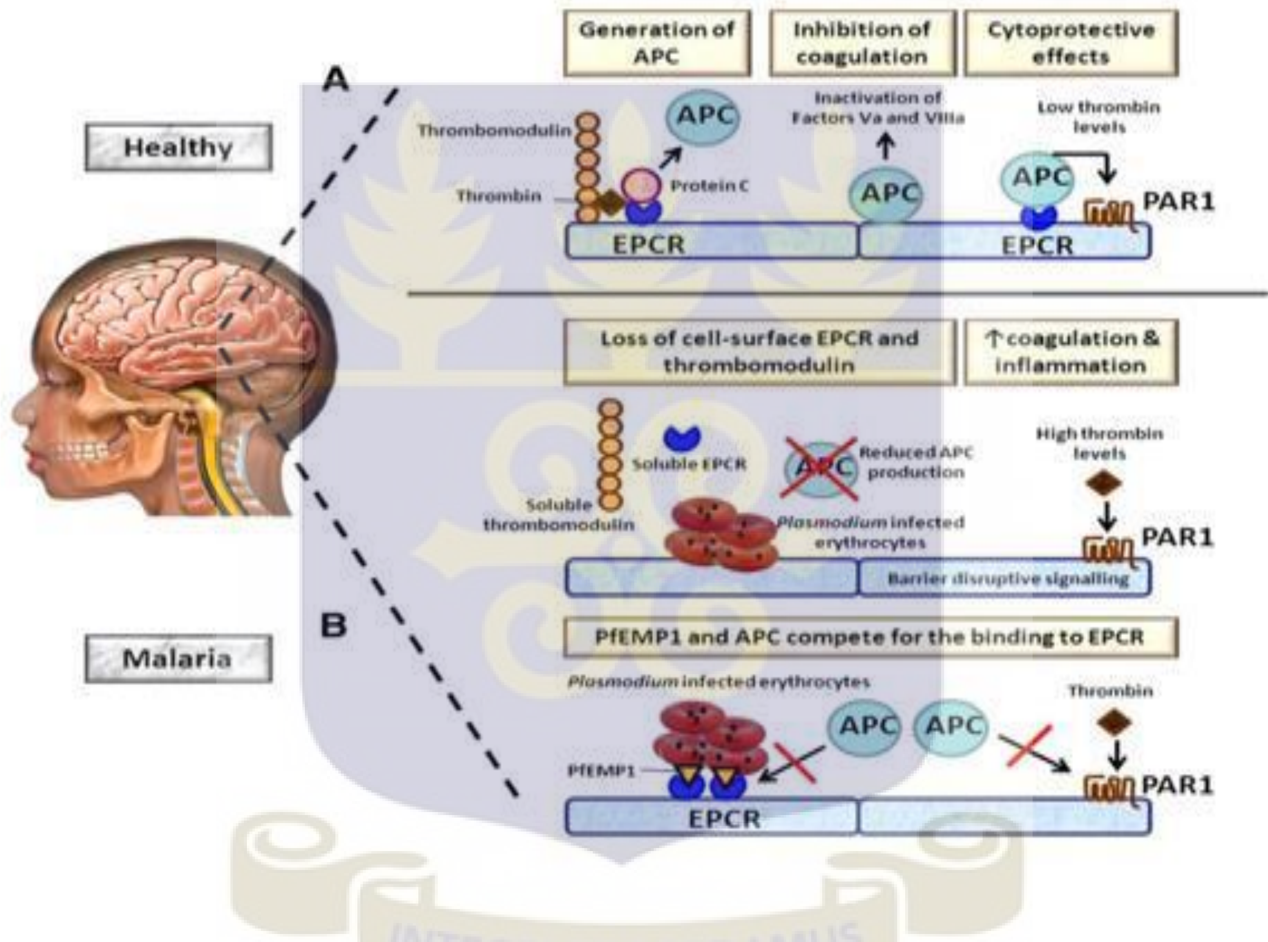

Figure 3: The role of thrombomodulin and endothelial protein C receptor in cerebral malaria (Adapted from Moxon *et al*., 2014)

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2.10.1 Various hypothesis underlying the pathogenesis of cerebral malaria.

Plasmodium falciparum is the main cause of severe clinical malaria and death. Several complications that arise due to severe malaria is believed to come about as a result of coagulopathy, microvascular changes and microvascular occlusion (WHO, 2000). The development of cerebral malaria has been attributed to several factors such as parasite-specific factors, endothelial sequestration, release of bioactive molecules, together with host inflammatory responses. There are a number of hypotheses proposed to be the underlying factors in the pathogenesis of cerebral malaria.

2.10.2 The Mechanical hypothesis

Sequestration of infected red blood cells and occlusion in the brain microvasculature has been implicated as a feature of cerebral malaria (Heyde *et al.,* 2006). The mechanical hypothesis was first proposed in 1894 by Marchiafava & Bignami, (1894) and it postulates that *P. falciparum* infected red blood cells bind to the post capillary venules, causing considerable obstruction of blood flow, decreased tissue perfusion and decreased removal of waste products (lactic acid). Finding of mature *Plasmodium falciparum* parasites within deep vascular endothelium confirmed the mechanical hypothesis (Marchiafava & Bignami, 1894). Dondorp *et al*. (2004) too have confirmed this hypothesis by autopsy studies as well as *in vivo* observations of the microcirculation which showed variable obstruction of the microcirculation in severe malaria.

Infected red blood cells may adhere to endothelial cells as well as uninfected red blood cells leading to both host pathogenesis and parasite survival (Sherman *et al.,* 2003).

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2.10.3 Microvascular Damage

Damage to the microvasculature is one feature of cerebral malaria (Dondorp *et al.,* 2008). Damage to the endothelium due to the sequestration of infected red blood cells leads to a decreased expression of the anticoagulant thrombomodulin and endothelial protein C receptor in the brain which makes it vunerable to destruction. Disruption of microvascular blood flow has been directly observed in human clinical studies of severe malaria (Dondorp *et al.,* 2008). The damage of blood vessels in the brain caused by parasite sequestration induces the production of the chemokine, Stromal Derived Factor-1 (SDF-1) in the bone marrow for the mobilization of endothelial progenitor cells to sites of endothelial damage to initiate repair (Shirozu *et al*., 1995; Hattori *et al*., 2001). Also infected red blood cells adherence to the endothelium have been shown to cause endothelial activation signals that promote adhesion, coagulation and inflammation (Francischetti *et al*., 2007; Cabrales *et al*., 2010)

2.10.4 The Cytokine hypothesis

The cytokine (inflammation) hypothesis was proposed by Maegraith in 1948 and states that malaria parasites and other infectious agents prompt a systemic inflammatory response that causes multi-organ failure and death. Also some cerebral malaria patients still die despite being given treatment that clears the peripheral blood parasitaemia (Horstmann *et al.,* 1985). Some studies have showed that not all cerebral malaria patients have parasites sequestered and therefore confirms the cytokine hypothesis (Hunt & Grau, 2003; Kwiawtowski *et al*., 1997). *Plasmodium falciparum* malaria may lead to upregulation of inflammatory cytokines serum or plasma. Some inflammatory cytokines that are increased are IL-1, IL-6, tumor necrosis factor (TNF) and NO

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(Nebl *et al*., 2005). The role of some inflammatory cytokines in the pathogenesis of cerebral malaria was demonstrated using knockout mice and inhibitory monoclonal antibodies and it was found that inflammatory cytokines were in despensable in the development of cerebral malaria (Schofield & Grau., 2005).

2.10.5 The Humoral hypothesis

The humoral hypothesis proposes that malaria toxins released from *Plasmodium falciparum* parasite in humans stimulates release of pro-inflammatory cytokines from macrophages. These cytokines may not be harmful to the host but induce additional and uncontrolled production of nitric oxide which may cause damage to the blood brain barrier eventually leading to reduced consciousness as occurs in cerebral malaria (Clark & Rockett, 1994). TNF- α and IL-1 (endogenous pyrogens) are known to induce fever in malaria in order to protect the host against peripheral blood parasites however high concentrations of these cytokines are correlated with cerebral malaria (Clark & Schofield, 2000).

2.11 Clinical Immunity to cerebral malaria

The clinical manifestations of malaria have been attributed to the asexual erythrocytic stage plasmodium parasites (Wiser, 2000). There are a number of clinical manifestations of cerebral malaria yet three symptoms are primarily common to patients: (1) unrousable coma that lasts for 24-72 hours; (2) impaired consciousness with non-specific fever; and (3) generalized convulsions and neurological sequellae.

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Cerebral malaria affects mainly children though adults from non-endemic countries develop it. However very young children who are less than one year old are passively immuned to malaria through maternal antibodies after which they gradually develop acquired immunity to malaria. They become susceptible to severe malaria with high mortality after 2 years (White, 1998). The liver stage of infection is termed as clinically silence because it is presumably does not induce any known immunological response. Protective immune response is therefore directed to the pre-erythrocytic parasite stage to merozoites or the antigens induced on the surface of infected red blood cells (Marsh *et al*., 2006; Engwerda *et al*., 2005). Immune response is acquired in distinct stages under right conditions of transmission intensity. The initial stage reduces the likelihood of severe diseases; the second phase limits the clinical impact of uncomplicated malaria; and the third phase provides partial protection against parasite burden.

The patho-physiology of malaria is multifactorial and requires both innate and adaptive immunity participation. Adaptive immunity plays an essential role in memory immunity to malaria and also the B cells and /or their products plays essential roles in parasite clearance during chronic malaria (Abdalla & Pasvol, 2004; Grun & Weidanz, 1981). Clinical immunity to malaria in general is characterized by host and parasite interactions. The ability of the host to control peripheral parasite replication and regulate inflammatory cytokines mediates the pathology associated with malaria infection to an extent (Abdalla *et al*., 2004).

2.11.1 Innate immunity/ Natural immunity

Innate immunity plays an important role in the response to pathogens and influences the

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development of adaptive immunity (Kadowaki *et al*., 2000). Innate immune response is needed in the clearance of *P. falciparum* from peripheral blood of the host especially during acute infection (Mohan & Stevenson, 1998). The spleen is responsible for the removal of peripheral parasites by destroying infected red blood cells. Macrophages, monocytes and neutrophils are some cells that seem to play a role in innate immunity by clearing parasites and reducing falciparum malaria infection associated clinical symptoms (Zhou *et al*., 2012).

2.11.2 Acquired immunity/ Adaptive immunity

Adaptive or acquired immunity refers to antigen-specific immunity that develops over a longer period (Shils & Shike, 2006). It involves humoral and cell mediated immunity produced by B and T lymphocytes respectively. The lymphocytes are categorized into three based on their function and cell-membrane components; B cells, T cells, and natural killer cells (Goldsby, *et al.*,2003).

Adaptive immunity to *P. falciparum* infection is obtained slowly despite repeated exposure to the parasite. Due to adaptive immunity an individual may clear peripheral parasites by controlling pro-inflammatory cytokines but will not trigger pathology (Ramasamyr, 1998). It has been shown in one study that adults may acquire immunity to malaria faster than children (Baird, 1995).

2.11.3 Humoral immune mechanisms

The erythrocytic stage of malaria is responsible for clinical symptoms and it is also the diagnostic stage. Immune protection against blood stage malaria is humoral (Chandele *et al.*,

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2011). Humoral immunity is mediated by antibodies such as Immunoglobulin-G (IgG) and Immuniglobulin-M (IgM). Malaria infection induces both polyclonal and specific immunoglobulin production (Troye-Blomberg & Perlmann, 2002). IgG is the most important antibody in the clinical immunity to malaria even though antibodies of different isotypes may have protective functions.

Antibodies may protect against malaria by a variety of mechanisms which could lead to the inhibition of merozoite invasion of erythrocytes (Wahlin *et al.,* 1984), inhibition of intra-erythrocytic growth or enhance clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration in small vessels and promoting elimination by the spleen (Udeinya *et al.,* 1981; Treutiger *et al.,* 1992).

2.11.4 Cellular immune mechanisms

Over the years, some studies have shown that $CD4^+$ T cells could transfer protection for blood-stage immunity in the *Plasmodium yoelii* infection. Also Parasite-specific memory $CD4^+$ T cells were found in the blood of human volunteers deliberately exposed to very low doses of malaria parasites with very few or no parasite-specific antibodies (Good & Enqwerda, 2011). This supports the claim that in the development of a malaria vaccine, it should be based on the generation of parasite-specific $CD4^+$ T cells.

$CD4^+$ T cells have been demonstrated to be associated with protection against malaria through the control of growth of blood parasite stages (Pierrot *et al.,* 2007). $CD4^+$ T cells are known to play a significant role in the generation of $CD8^+$T cells during a parasitic infection in order to effectively fight against it.

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Studies have shown that $CD4^+$ T cells are crucial to the development of $CD8^+$ T cell responses against hepatocytes infected with malaria parasites (Carvalho *et al.,* 2002). Another studies conducted by Weiss *et al*. (1993) using mice after immunization with *Plasmodium yoelii* sporozoites indicated that the mice were not protected by sporozoite immunization after depletion of CD4+ cells during blood stage malaria. This therefore indicates that $CD4^+$ T cells are essential in protection against malaria and thus serves as an important model for malaria vaccine development in humans in which $CD4^+$ T cells must be put into consideration.

Studies have shown that in murine malaria models, liver resident memory $CD8^+$ T cells are important for protective immunity as they efficiently eliminate malaria infected hepatocytes in vivo (Tse e*t al*., 2010). This therefore indicates that $CD8^+$ T cells are the first immune cells that aid in fighting malaria parasites in the body since the sporozoites attacks the liver first when they enter into the body.

Weiss *et al.* (1988) also found out that $CD8^+$ T cells were required for protection in mice immunized with malaria sporozoites. The results they obtained indicated that cytotoxic T cells are critical for immunity to large numbers of sporozoites. It was found out that $CD8^+$ T cells were responsible for liver stage immunity to *Plasmodium* parasites.

Also studies using the rodent parasites *Plasmodium berghei* and *Plasmodium yoelli* have shown that $CD8^+$ T cells are critical for protective immunity specifically against liver stages of infection. Immunization with radiation-attenuated sporozoites induced long-lasting protective immunity against challenge with sporozoites but not with infected erythrocytes. This therefore shows that $CD8^+$ T cells are effective in fighting against Plasmodium parasites during the liver stage than the

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erythrocyte stage. (Schofield *et al*., 1987; Weiss *et al*., 1988). In contrast with what others have done regarding $CD8^+$ T cells being effective to only liver stage Plasmodium parasite, (Good & Enqwerda, 2011) showed that $CD8^+$ T cells can transfer immunity in blood-stage malaria in mice by *P. yoelii*. However immunity was developed only after there was repeated exposure to different strains of *P. yoelii.* This therefore shows that $CD8^+$ T cells are not very effective in blood stage malaria.

In conclusion, both $CD4^+$and $CD8^+$ T cells are needed for protection against some parasites including *P. falciparum*. These T cells protect against different phases of *P. falciparum* infection (Shils & Shike, 2006). A study conducted by Kitchen *et al.* (2005) showed that CD4 expression on CD8 T lymphocytes modulates cytotoxic T lymphocyte function and is critical in vivo for optimal cell-mediated immunity to viral and allo antigens. The study shows that T lymphocytes specifically $CD4^+$ and $CD8^+$ T cells are responsible for protection against parasitic infections which includes infection caused by the *Plasmodium falciparum*. Studies have also shown that both $CD4^+$ and $CD8^+$ T cells are involved in acquired immunity to blood-stage *Plasmodium chabaudi* AS (Podoba & Stevenson, 1991).

Malaria has two stages in human which is the liver stage and the blood stage malaria. Different components of the T cells are expected to act on each stage and it has widely accepted that it is antibodies and $CD4^+$ T cells that play critical roles in the immune response during the blood stage of malaria (Milner, 2011). However Chandele *et al.* (2011) showed that blood-stage malaria infection results in a striking T-cell response and that activated CD8 and CD4 T cells have phenotypic and functional characteristics that are consistent with conventional antigen-specific effector and memory T cells during an infection with *Plasmodium yoelii*.

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2.11.5 Genetic susceptibility to Malaria

Some individuals are infected by malaria several times but they do not develop any clinical complications whiles others may develop complications and even die from recurrent infections.

It has been found that totally different genetic factors may determine an individual's initial resistance to infection, development of severe complications after infection or the prospect of acquiring effective long term immunity. Certain genetic factors such as sickle cell anemia, G6PD, β-thalasaemia, platelet factor 4 and Duffy-antigen determines such outcomes.

For example it has been demonstrated that Platelet factor 4 and Duffy-antigen are significant molecules in platelet-mediated killing of *P. falciparum* (McMorran *et al.,* 2012).

2.12 The Enzyme-Linked Immunosorbent Assay (ELISA) Technique

ELISA uses a primary binding assay technique which is a sensitive immunoassay that involves binding either an antigen or antibody unto a solid phase and adding the other. It uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein, especially an antigen or antibody in various body fluids such as serum/plasma, urine, cerebrospinal fluids, sperm and culture supernatant (Savige *et al*., 1998). It is designed for the detecting and quantifying substances such as peptides, proteins, antibodies and hormones. Its basic principle is using an enzyme (tag) to detect the binding of antigen and antibody. The enzyme functions by converting a substrate which is colourless into a coloured product when an antigen/antibody reaction or binding occurs. ELISA has several applications in our everyday life. It is used for detection of antibodies in blood sample for past exposure to disease e.g. Lyme disease, trichinosis, HIV, bird flu and Ebola. It can be used to track the spread of disease outbreaks such as cholera. It is also used

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in detecting potential food allergens in milk, peanuts, walnuts, almonds, banana and eggs (Parasuraman, 2013).

2.12.1 The different types of ELISA

There are various types of ELISA including the direct, indirect, and sandwich (or capture) ELISA. The direct ELISA is suitable for the detection of proteinaceous antigens and may require pre-purification of sample. Direct ELISA can be performed when microwell plates are coated with a sample containing the target antigen, and the binding of labelled antibody is quantitated by a colorimetric, enzymatic, or fluorometric end-point. After the test sample has been attached a solid phase (microwell plate), an antibody conjugated with a label is incubated with a captured antibody or antigen. The excess conjugate is washed off and incubated with a chromogen, after which a colour change indicates a specific antibody-antigen/ antibody reaction. Direct ELISA is fast to perform and eliminates possible non-specific binding of secondary antibody. However with direct ELISA, reactivity of primary antibody may reduce conjugation and also there is little signal amplification.

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The indirect ELISA utilizes an unlabeled primary antibody in conjunction with a labelled secondary antibody. The indirect ELISA is required in which a conjugated secondary antibody (e.g mouse IgG1, goat IgM, rabbit IgG1, chicken IgY), is targeted to the isotype of the primary antibody. With the indirect ELISA, the immunoreactivity of the primary antibody is not compromised and multiple binding of the secondary antibody affords some signal amplification increasing the overall sensitivity of the assay.

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Sandwich ELISA (Capture ELISA) is a sensitive assay that measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigens to be measured must contain at least two antigenic sites, capable of binding to antibody, since at least two antibodies act in the sandwich. This type of ELISA design for the quantitation of antigens are essential when the concentration of antigens is low and/or are contained in high concentrations of contaminating protein. It is capable of determining absolute amount of antigen in an unknown sample if a purified antigen standard is available. It is fast and accurate when used.

2.12.2 Fluorescent Activated Cell Sorter (FACS) Technique (Flow cytometry)

Flow cytometry is a technique for counting, sorting and examining microscopic particles, such as cells, bacteria and even chromosomes, by suspending them in a stream of fluid and passing them through an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. Flow cytometry involves the analysis of the fluorescence and light scatter properties of single particles (e.g. cells, nuclei, chromosomes, etc) during their passage within a narrow, precisely defined liquid stream. Forward scatter (FSC) approximates cell size whiles side scatter (SSC) detects cell complexity or granularity and fluorescence so as to investigate cell structure and function. It functions by the use of fluorescent lights and therefore uses tandem dyes known as fluorochromes. The fluorochrome absorbs energy from the laser and releases the absorbed energy by vibration and heat dissipation and emission of photons of a longer wavelength. The purpose of a fluorochrome-conjugated antibody, is to directly target an epitope of a cell of interest for its antigen density in relation to its biochemical properties. Flow cytometry enables

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several measurable parameters such as cell surface antigens (CD markers), intracellular antigens, DNA (cell cycle analysis, cell kinetics, proliferation etc), RNA, cell pigment such as chlorophyll or phycoerthyrin, volume & morphological complexity of cells, cell viability, enzymatic activity and so many other parameters. Flow cytometry is applied in so many branches of science research such as immunology, molecular biology, microbiology, pathology, plant biology, marine biology, protein engineering and several others.

2.12.3 The principle of flow cytometry

Flow cytometry involves the analysis of the fluorescence and light scatter properties of single particles (e.g. cells, nuclei, chromosomes) during their passage within a narrow, precisely defined liquid stream. The amount of laser light that is scattered off the cells as they pass through the laser can be measured, which gives information concerning the size of the cells. Flow cytometry tells us certain specific things about a cell so as to investigate cell structure and function. They are forward scatter (FSC) for the approximation of cell size, side scatter (SSC) for the detection of cell internal complexity or cell relative granularity, both of which are based on the light scattering (diffraction, refraction and reflection) properties of the cell and fluorescence intensity for detection of antigen density based on the fluorescent emission of dyes. The equipment used to perform a flow cytometry is known as the flow cytometer. It functions by the use of fluorescent lights and uses tandem dyes known as fluorochromes. The fluorochrome absorbs energy from the laser and releases the absorbed energy by vibration and heat dissipation and emission of photons of a longer wavelength. The purpose of a fluorochrome-conjugated antibody, is to directly target an epitope of a cell of interest for its antigen density in relation to its biochemical properties.

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Flow cytometry enables several measurable parameters such as cell surface antigens (CD markers), intracellular antigens, DNA (cell cycle analysis, cell kinetics, proliferation etc), RNA, cell pigment such as chlorophyll or phycoerthyrin, volume & morphological complexity of cells, cell viability, enzymatic activity and so many other parameters. Some software programs that can be used in flow cytometry for data interpretation are the CellQuest, Flowjo and WinMDI. Flow cytometry can be applied in so many branches of science research such as immunology, molecular biology, microbiology, pathology, plant biology, marine biology, protein engineering and several others.

2.12.4 Systems of the flow cytometer

A flow cytometer is made up of three systems. They are the fluidics, optics and electronics. The fluidics deals with hydrodynamic focusing of the sample thru the flow cell to produce a single stream of particles to the laser beam for examination. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

2.12.5 Analysis of flow cytometric data

Recorded form of data is collected on each cell or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analysed to provide information about subpopulations within the

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sample in the form of histograms, dot plots, density plot or contour plot. The histograms are represented as either one or two parameter histograms.

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CHAPTER THREE

METHODOLOGY

3.1 Chemicals, reagents and equipment

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

3.2 Study sites

This study was part of a major project that is aimed at investigating the role of endothelial progenitor cells and other endothelial biomarkers in the pathogenesis of cerebral malaria. Samples were collected from five hospitals and two schools in the Greater-Accra Region of Ghana. The study was done in collaboration with five hospitals of The Ghana Health Service:

Princess Marie Louise Children's Hospital (PML), Tema General Hospital (TGH), Ridge Hospital (RH), La General Hospital (LGH), Ledzokuku Krowor Municipal Assembly Hospital (LEKMA) all with referral units for paediatric cases including malaria. Two schools which are Hayward Nursery School and Osu Home School were also used for part of the study. Also the study was conducted at the Noguchi Memorial Institute for Medical Research (NMIMR) which has the capacity for cellular, serological, genetic and molecular analysis. Malaria is hyper endemic in Ghana, with a crude parasite rate ranging from 10-70% with *Plasmodium falciparum* dominating (UNICEF, 2007). Accra has a perennial and hyper-endemic malaria transmission with a seasonal peak occurring between May and October, which is just after the major rainy season.

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3.3 Study participants

The study participants for the first group included children with malaria between the ages of 1-12 years who reported for treatment at the PML, TGH, RH, LGH and LEKMA. These patients were clinically examined to select for those who met the inclusion criteria, which is defined in the next session (case definition). Study participants for the second group involved children without malaria between the ages of 1-12 years whose parents agreed to let them take part of the study in Hayward Nursery School and Osu Home School. The parents or guardians of the participants signed an informed consent form on behalf of their children after explaining to them (Appendix I).

3.4 Case definition

Case definition for malaria patients were; fever (axillary temperature $\geq 37.5^{O}$C) measured within 24 hours, malaria parasitaemia of 5 or more *P. falciparum* parasites per high power field (approx. 2500/µl) and at least one other sign of malaria (vomiting, diarrhoea, malaise etc). The patient also had to be sickling negative, with no other obvious cause found for the fever and show no signs of any other disease. Inclusion criteria for participants without malaria were: normal temperature, no malaria parasitaemia, sickling negative and no current presence of any other disease and no previous treatment of malaria within the past one month as at the time of recruitment.

The qualified participants were categorized into three groups; cerebral malaria (CM), uncomplicated malaria (UM) and healthy controls (HC). Participants in the cerebral malaria

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category included patients who were unconscious, with a Blantyre coma scale score (BCS) of ≤3 duration of coma of at least 60 minutes and any haemoglobin value. The patient should not have had any record of recent severe head trauma or other cause of coma or neurological diseases including meningitis/encephalitis (which was assessed by lumbar puncture). Those in the uncomplicated malaria category included patients who presented with clinical malaria without any of World Health Organization (WHO) criteria for severe malaria such as severe malaria anemia (SMA) or respiratory distress. Patients who had haemoglobin (Hb) values ≥8g/dL with malaria parasitaemia were fully conscious and not hospitalized. Healthy controls category included those who did not present with malaria or any other form of disease as at time of recruitment with haemoglobin (Hb) level greater than 8g/dL. Participants had no malaria parasitaemia and were fully conscious.

3.5 Sampling of blood

Venous blood was collected into a 1mL EDTA and Heparinised vacutainers from enrolled participants by a trained phlebotomist using butterfly needle (Beckton Dickenson) for parasitological, haematological and immunological studies. The collected blood was transported to the NMIMR and processed within two hours after collection. 400 µl of blood from the EDTA vacutainer was used to run flow cytometry described in session 3.9. The rest of the blood was spun and the plasma of both EDTA and Heparinised vacutainer tubes were collected and stored at -30°C until ready for use. The red blood cells (RBC) from the EDTA vacutainer was also stored at -30°C until ready for use.

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3.6 Ethical clearance

The study received ethical approval from the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR). It also received approval from Ghana Health Service and Ghana Education Service. The study was voluntary and participants were enrolled only after informed consent was obtained from their parents and guardians following their understanding of the objectives of the study (See appendix for consent form).

3.7 Laboratory investigations

All laboratory investigations were carried out at NMIMR. These included parasitological examination, haematological examination, and flow cytometry.

3.7.1 Parasitological examination

Thick and thin films of peripheral blood were prepared for plasmodium parasites examination. For the thick film, a drop of 6µl of blood was placed at one end of a microscope glass slide and evenly spread on the slide. For the thin blood films, 2µl of blood was placed at one end of the microscope glass slide and evenly spread out to cover almost the entire length of the slide using a smooth-edge spreader held at an angle of about 45° to the slide. The prepared blood films were air-dried thoroughly. The thin blood film was fixed with absolute methanol for species identification. The blood films were then stained with freshly prepared 10% Giemsa solution (in Phosphate buffer) and left to stain for 10 minutes, followed by careful washing under running tap water. The slides were air-dried and examined with immersion oil under light microscope (X100 objective) for the

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presence of plasmodium parasites and species identification. The plasmodium parasites were counted against 200 white blood cells (WBCs). Parasite densities were obtained when the total parasite counted within this range from the thick blood film slide was multiplied by 8000 and divided by the number of 200 WBCs counted.

3.7.2 Haematological examination

The haematological parameters were measured using an automated haematological analyser. The automated haematological analyser functions with the principle of hydrodynamic focusing. With this, diluted red blood cells are surrounded by a sheath fluid, which lines up the cells in a single file while passing through the detection aperture. After passing through the aperture, the cells are then directed away from the back of the aperture to eliminate the recirculation of cells. The number, size and volume of cells are then detected by the machine. The parameter measured includes haemoglobin level, platelets count, total white blood cell (WBC) counts, total red blood cell (RBC) counts, mean corpuscular volume (MCV), haematocrit (HCT), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin content (MCHC). Sickling test was done for each patient by using the Sodium Metabisulphite test and participants who tested sickling-positive were excluded from the study. For the sickling test, a drop of finger prick blood pipetted unto a clean microscope slide. An equal volume of the 2% sodium metabiosulphite solution was added to the blood on the slide and mixed thoroughly. It was then covered gently with a cover slip to avoid trapping air bubbles and to give a low oxygen tension and read under a microscope.

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3.8 Measuring the levels of biomarkers

Angiopoietin-1 and -2, soluble thrombomodulin and soluble endothelial protein c receptor levels were measured by employing the Enzyme-linked immunosorbent assay (ELISA).

3.8.1 Measurement of Angiopoietin-1

The levels of Angiopoietin-1 in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA Development kit) according to manufacturer's instructions. 96-well polystyrene microtitre plates (R&D) were coated with 100µl per well of mouse anti-human Ang-1 antibody (capture antigen) and incubated overnight at room temperature. The plate wells were aspirated and washed thrice using 400µl of wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against clean paper towel after the third wash to adequately remove excess unbound capture antigen. The plate wells were then blocked with 300µl per well of reagent diluent [1% Bovine Serum Albumin (BSA) in PBS] and incubated for an hour at room temperature. The plates were then aspirated and washed thrice as mentioned above.

100µl of plasma samples (20 fold dilution in PBS) were added per well. The first two rows of each plate had controls added to it. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 +/- 50 rpm. Each well was aspirated and washed three times by filling with wash buffer (400µl) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. 100µl of detection antibody diluted in reagent diluent with normal goat serum (NGS) were added to each well. The plates were covered with a different adhesive strip and

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incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as described above. 100µl of working dilution of Streptavidin-HRP was added to each plate well. The plates were covered with an adhesive strip and incubated for 20 minutes in the dark at room temperature. The plates were aspirated and washed three times as described above. 100µl of Substrate Solution was added to each well for colour development and incubated for 20 min at room temperature in the dark. Then 50µl of Stop Solution was added to each well to halt the colour change. The optical density was determined within 30 min, using the EL808 BioTek microplate reader set to 450 nm.

3.8.2 Measurement of Angiopoietin-2

The levels of Angiopoietin- 2 in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA Development kit) according to manufacturer's instructions. A 96-well polystyrene microtitre plate (R&D) was coated with 100µl per well of mouse anti-human Ang-2 antibody (capture antigen) and incubated overnight at room temperature. The plate wells were aspirated and washed thrice using 400µl of wash buffer

[0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against clean paper towel after the third wash to adequately remove excess unbound capture antigen. The plate wells were then blocked with 300µl per well of reagent diluent [1% Bovine Serum Albumin (BSA) in PBS] and incubated for an hour at room temperature. The plates were then aspirated and washed thrice as mentioned above.

100µl of plasma samples (20 fold dilution in PBS) were added per well. The first two rows of each

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plate had controls added to it. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 +/- 50 rpm. Each well was aspirated and washed three times by filling with wash buffer (400µl) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. 100µl of detection antibody diluted in reagent diluent with normal mouse serum (NMS) were added to each well. The plates were covered with a different adhesive strip and incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as described above. 100µl of working dilution of Streptavidin-HRP was added to each plate well. The plates were covered with an adhesive strip and incubated for 20 min in the dark at room temperature. The plates were aspirated and washed three times as described above. 100µl of Substrate Solution was added to each well for colour development and incubated for 20 minutes at room temperature in the dark. Finally, 50µl of Stop Solution was added to each well to arrest the colour change. The optical density was determined within 30 minutes, using the EL808 BioTek microplate reader set to 450 nm.

3.8.3 Measurement of soluble Thrombomodulin

The levels of soluble Thrombomodulin (sTM) in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA Development kit) according to manufacturer's instructions. A 96-well polystyrene microtitre plate (R&D) was coated with 100µl per well of mouse anti-human Thrombomodulin antibody (capture antigen) and incubated overnight at room temperature. The plate wells were aspirated and washed thrice using 400µl of wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against

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clean paper towel after the third wash to adequately remove excess unbound capture antigen. The plate wells were then blocked with 300µl per well of reagent diluent [1% Bovine Serum Albumin (BSA) in PBS] and incubated for an hour at room temperature. The plates were then aspirated and washed thrice as mentioned above.

100µl of plasma samples (20 fold dilution in PBS) were added per well. The first two rows of each plate had controls added to it. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 +/- 50 rpm. Each well was aspirated and washed three times by filling with wash buffer (400µl) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. 100µl of detection antibody diluted in reagent diluent were added to each well. The plates were covered with a different adhesive strip and incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as described above. 100µl of working dilution of Streptavidin-HRP was added to each plate well. The plates were covered with an adhesive strip and incubated for 20 min in the dark at room temperature. The plates were aspirated and washed three times as described above. 100µl of Substrate Solution was added to each well for colour development and incubated for 20 minutes at room temperature in the dark. Then 50µl of Stop Solution was added to each well to arrest the colour change. The optical density was determined within 30 min, using the EL808 BioTek microplate reader set to 450 nm.

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3.8.4 Measurement of soluble Endothelial Protein C Receptor

The levels of soluble Endothelial Protein C Receptor (sEPCR) in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA

Development kit) according to manufacturer's instructions. A 96-well polystyrene microtitre plate (R&D) was coated with 100µl per well of mouse anti-human EPCR antibody (capture antigen) and incubated overnight at room temperature. The plate wells were aspirated and washed thrice using 400µl of wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against clean paper towel after the third wash to adequately remove excess unbound capture antigen. The plate wells were then blocked with 300µl per well of reagent diluent [1%

Bovine Serum Albumin (BSA) in PBS] and incubated for an hour at room temperature. The plates were then aspirated and washed thrice as mentioned above.

100µl of plasma samples (pretreated with 1N HCl and 1N NaOH to release protein C) were added per well. The first two rows of each plate had controls added to it. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 +/- 50 rpm.

Each well was aspirated and washed three times by filling with wash buffer (400µl) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. 100µl of detection antibody diluted in reagent diluent were added to each well. The plates were covered with a different adhesive strip and incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as described above. 100µl of working dilution of Streptavidin – HRP was added to each plate well.

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The plates were covered with an adhesive strip and incubated for 20 min in the dark at room temperature. The plates were aspirated and washed three times as described above.

100µl of Substrate Solution was added to each well for colour development and incubated for 20 min at room temperature in the dark. Then 50µl of Stop Solution was added to each well to arrest the colour change. The optical density was determined within 30 min, using the EL808 BioTek microplate reader set to 450 nm.

3.9 Preparation of sample for flow cytometry

After labeling six BD FALCON tubes, different antibodies for endothelial progenitor cell (EPC) marking were put in them. The markers and their corresponding tubes for CEPC's Assay are shown in Table 1 below. On the addition of blocking monoclonal antibody (MAb) – IgG Chrome Pure, 2 or 4 µl (shown in Table 2) of IgG chrome was added to each tube. Following that, 50 or 100µl (shown in Table 2) of whole blood in EDTA was added to each tube. The tubes were gently vortexed twice for 10 sec each and incubated for 10 min at 4 ^{O}C. Addition of monoclonal antibodies was done in the dark. After the 10 min incubation period, monoclonal antibodies were added to the samples. About 3µl each of the monoclonal antibodies were added (Table 3). After addition of the antibodies, all the tubes were vortexed twice at high speed for 10 sec each. The rack containing the tubes was wrapped in Aluminium foil and incubated for 30 min at 4 ^{O}C. After 30 min of incubation, all tubes were gently vortexed and 2ml of FACS lysing buffer was added to all tubes. These were then vortexed gently and incubated at room temperature for 10min in dark. After lysing, all six tubes were centrifuged at a speed of 1500 rpm for 7 min at 4°C using the RSL-05A

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(Sakuma, Japan) model. The supernatant was decanted and the cells were made loose by gently rubbing each tube thrice on the rack. This was repeated after every wash and decantation of supernatant; for the second wash, the cells were re-suspended in 3ml Cold FACS FLOW and centrifuged at a speed of 1500 rpm for 7 minutes at $4^{O}C$ using the RSL-05A (Sakuma, Japan) model. For the third wash, the cells were re-suspended in 3ml Cold FACS Flow and the same procedure for the second wash was repeated. After the third wash, the loosened cells were re-suspended in 250µl cold FACS Flow and was acquired on the flow cytometer.

Table 1: Markers for detection of endothelial progenitor cells

Tube No.	Description
1	Unstained
2	Iso-type 1
3	Iso-type 2
4	15/14/4/8 FOR CD15/CD14/ CD4/CD 8
5	11/133/34/31 FOR CD11b/CD133/CD34/CD31
6	45/133/34/309 FOR CD45/CD133/CD34/CD309 (VEGF)

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Table 2: Monoclonal antibodies and their corresponding whole blood volumes

Tube No.	Vol. of IgG (µl)	Vol. of whole blood (µl)
1	2	50
2	2	50
3	2	50
4	2	50
5	4	100
6	4	100

Table 3: Monoclonal antibodies that were added to the whole blood for the detection of endothelial progenitor cells

Tube	Monoclonal antibodies (3 µl each)			
	FITC	PE	PerCP	APC
1	Unstained			
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 (VEGF-2)

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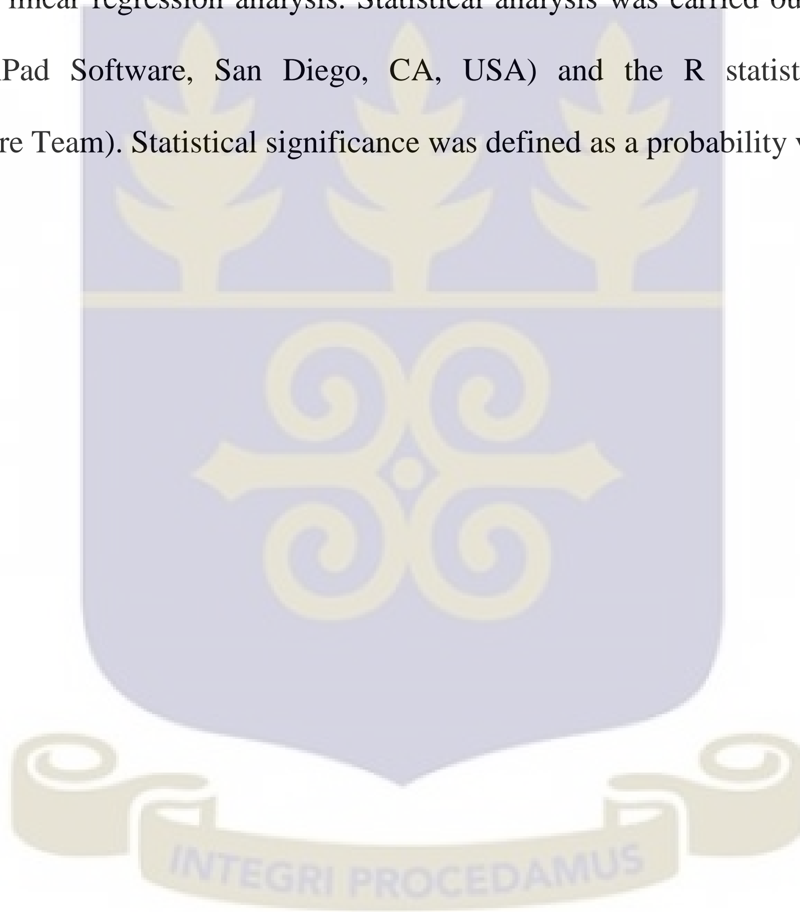
3.9.1 Statistical analyses

Comparison between the three study groups (CM, UM and HC) was performed using a Kruskal-Wallis test and the Dunn's Multiple Comparison Post-Hoc test where applicable. Correlation analysis between endothelial cells amongst the study groups were done using Spearman rank correlation test. Associations of endothelial cells to biomarkers amongst and within study groups were done using linear regression analysis. Statistical analysis was carried out using Prism 5.0 software (GraphPad Software, San Diego, CA, USA) and the R statistical software (R Development Core Team). Statistical significance was defined as a probability value below 0.05.



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CHAPTER FOUR

RESULTS

4.1 General Clinical Characteristics of Study participants

A total of 92 children between 1 and 12 years comprising 30 (32.61%) cerebral malaria cases, 28 (30.43%) uncomplicated malaria cases and 34 (36.96%) healthy individuals used as controls were included in the analysis for this study (Table 4.1). Study participants for uncomplicated malaria (4.2 years) were significantly younger than participants in healthy control group (6.5years, $P<0.05$) but not the cerebral malaria group (5.0 years). A statistical significance was observed between the median parasite densities of the cerebral malaria (840 parasites/uL/blood) and uncomplicated malaria (27,900 parasites/uL/blood) groups (*P= 0.0023)*.Also haemoglobin levels in cerebral malaria group (8.375 g/dL) was significantly lower than uncomplicated malaria (10.45 g/dL) and healthy control (11.20 g/dL) groups ($P < 0.05$, Table 4.1).

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Table 4.1: Characteristics of study participants

Characteristics	Study Participants			Pvalue (Kruskal-Wallis)
	Cerebral Malaria	Uncomplicated Malaria	Healthy Control	
N (%)	37(35.56)	30(28.85)	37 (35.56)	
Age (years)	5 (1-12)	#4.2 (1.4-10)	6.5(2-12)	0.0302
Haemoglobin (g/dL)	*8.375(5.9-12.10)	10.45 (6-14.10)	11.20 (5.3-13.70)	<0.0001
Parasite density (parasite/µl)	840(0-304000)	27900(1680-154000)		0.0023

Data was reported as median and interquartile range. P value was obtained by Kruskal-Wallis on Ranks. Dunns Multiple Test was used to detect significant differences between paired groups. *Indicates that Hb level in the CM group was significantly lower than UM and HC group ($P < 0.05$) following the Post-Hoc test. # Indicates that UM group were significantly younger than HC (P<0.05) at 95% CI. Hb=Haemoglobin

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4.2 Plasma levels of Angiopoietin-1 and Angiopoietin-2 in study participants

Plasma levels of Angiopoietin-1 (Ang-1) were measured and used as a marker for endothelial quiescence. Ang-1 levels in UM (7452 pg/mL, range 2358-25593) was significantly lower than levels in CM (12564 pg/mL (2561-60106)) and HC (14275 pg/mL (4683 – 33123), $P<0.05$, Figure 4.1) groups following Post-hoc test.Ang-1 levels between CM and HC however were not significantly different ($P > 0.05$)

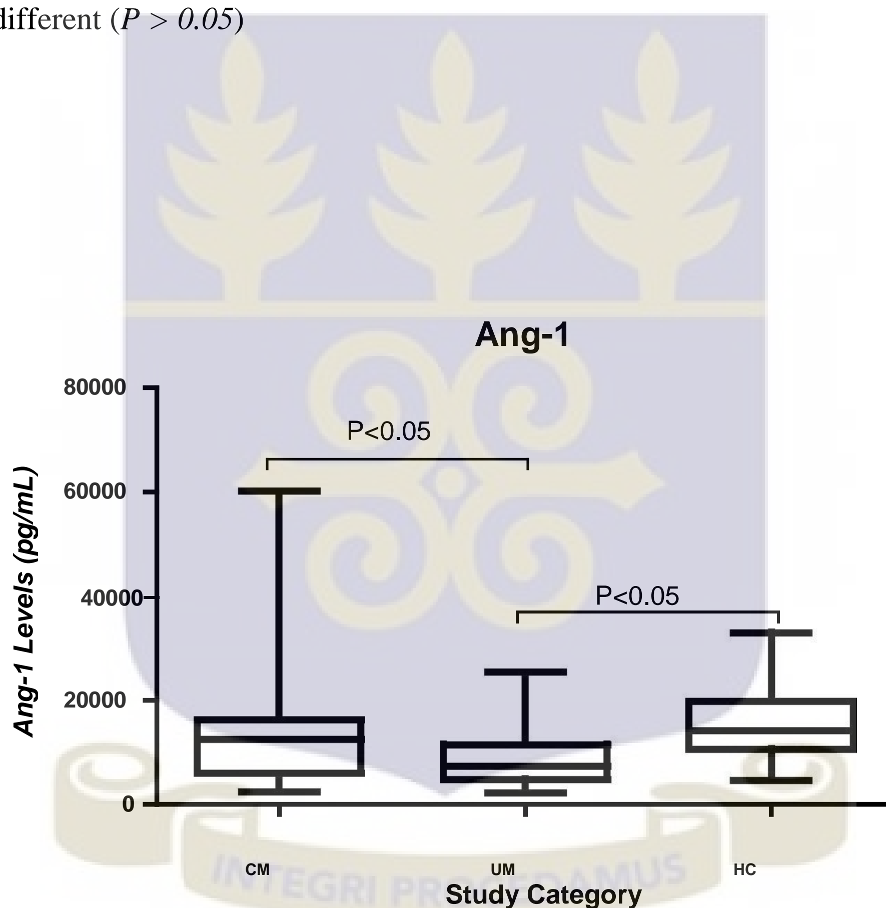

Figure 1.1: Median levels of Angiopoietin-1 in study participants

The middle lines in each box represent the median values of Ang-1. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper whiskers represent the 90th value and the lower whiskers represent the 10th value.

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Plasma levels of Angiopoietin-2 (Ang-2), was measured and used as a marker for endothelial damage. Ang-2 levels between CM, UM and HC were not significantly different (CM = 4932 pg/mL, range 1510–13776; UM = 4142 pg/mL, (1025–42218); HC 3312 pg/mL, (1346–20442), *P = 0.0928*, Figure 4.2).

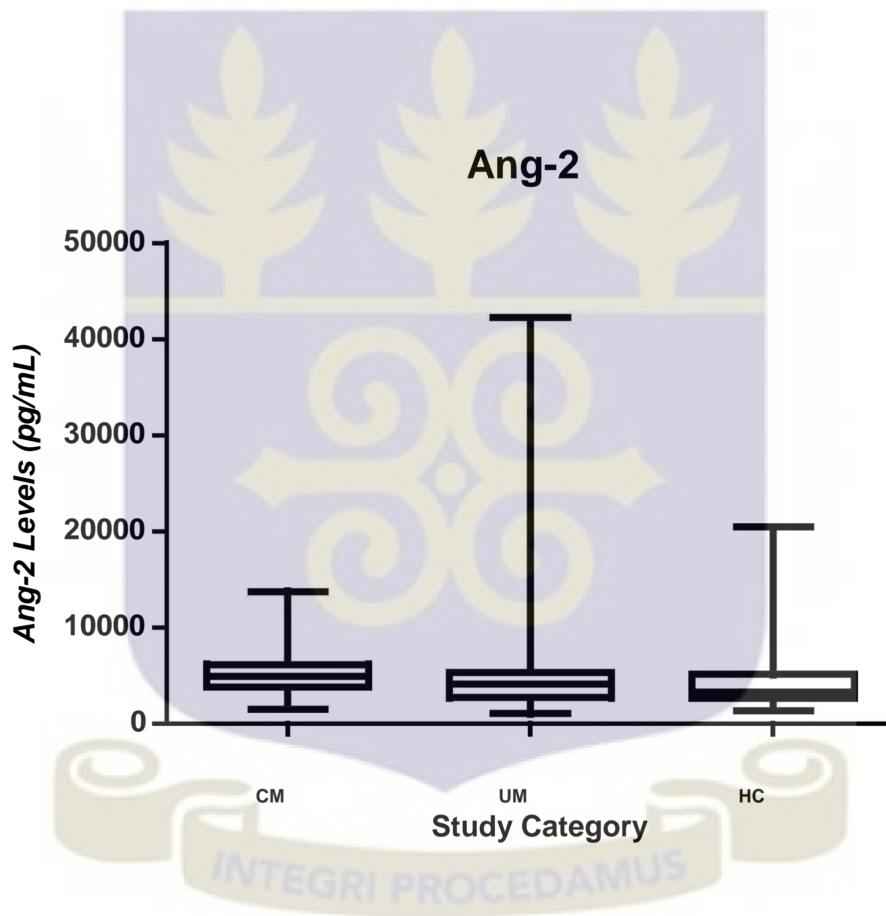

Figure 2.2: Median levels of Angiopoietin-2 in study participants

A statistical significant difference was not found between the study groups (*P = 0.0928*) when tested withKruskal-Wallis on Ranks. The middle lines in each box represent the median values of Ang-2. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper whiskers represent the 90th value and the lower whiskers represent the 10th value.

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Ang-2 levels toAng-1 levels was determined separately for the three study groups (CM, UM and HC) and the median values compared. Ang-2: Ang-1 ratio was significantly lower in HC (0.2336, range0.06125-1.840) than CM (0.4523 (0.1129-1.672), P < 0.05) and UM (0.5544 (0.08965 – 1.375), Figure 4.3) groups following Post-hoc test.

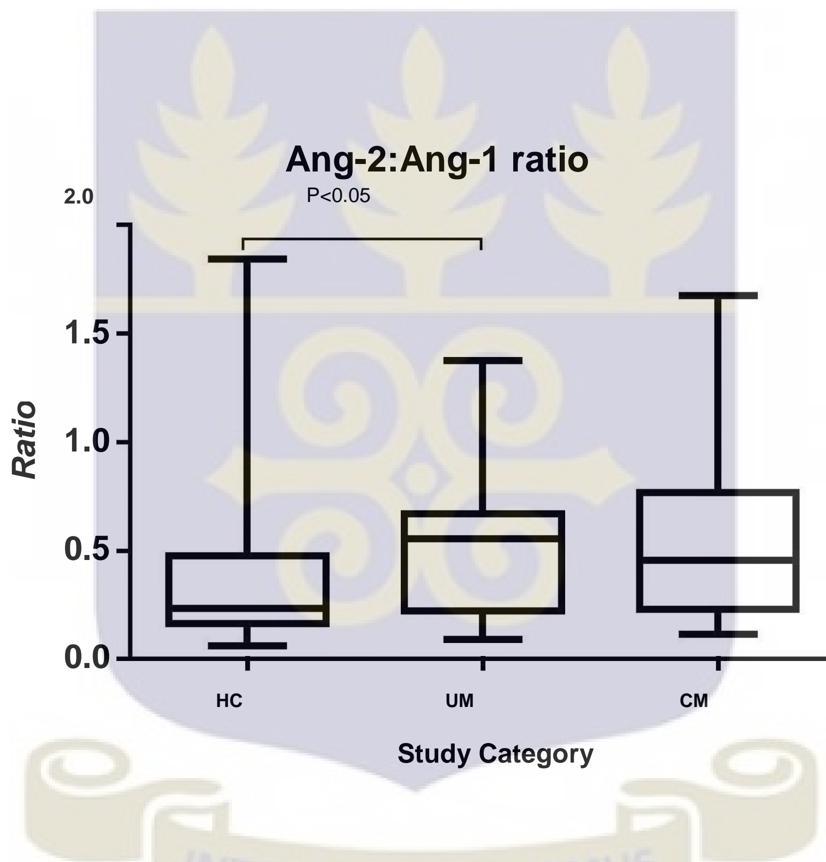

Figure 3.3: Median levels of Angiopoietin-2: Angiopoietin-1 ratio in study participants.

The middle lines in each box represent the median values of Ang-1. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper and lower whiskers represent the 90th value and the lower whiskers represent the 10th value.

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4.3 Plasma levels of soluble Endothelial Protein C Receptor and soluble Thrombomodulin in study participants

Plasma levels of soluble Endothelial Protein C Receptor (sEPCR) was measured and used as a marker for endothelial activation and damage. sEPCR levels between CM, UM and HC was not significantly different (CM = 19200 pg/mL, range 10500 – 35100; UM = 18200 pg/mL (11000 – 53900); HC = 18900 pg/mL (10900 – 79300), *P =0.5837,* Figure 4.4).

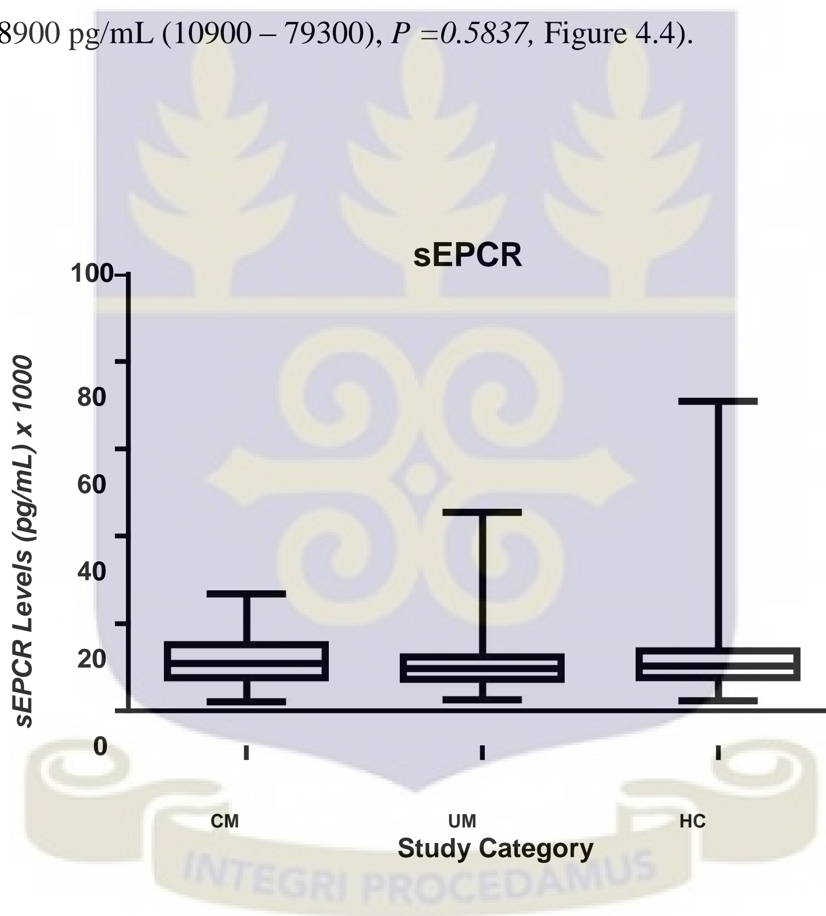

Figure 4.4: Median levels of soluble Endothelial Protein CReceptor in study participants.

A statistical significant difference was not found between the study groups (*P = 0.5837*) when tested with Kruskal -Wallis on Ranks. The middle lines in each box represent the median values of EPCR. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper whiskers represent the 90th value and the lower whiskers represent the 10th value.

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Plasma levels of soluble Thrombomodulin (sTM) was measured and used as a marker for endothelial activation and damage. sTMlevels was significantly higher in CM (6832pg/mL, range 3552-16687) than UM (6214pg/mL (3805-13559) and HC (4386pg/mL (3149-6232),P<*0.05,* Figure 4.4) groups following Post-hoc test.

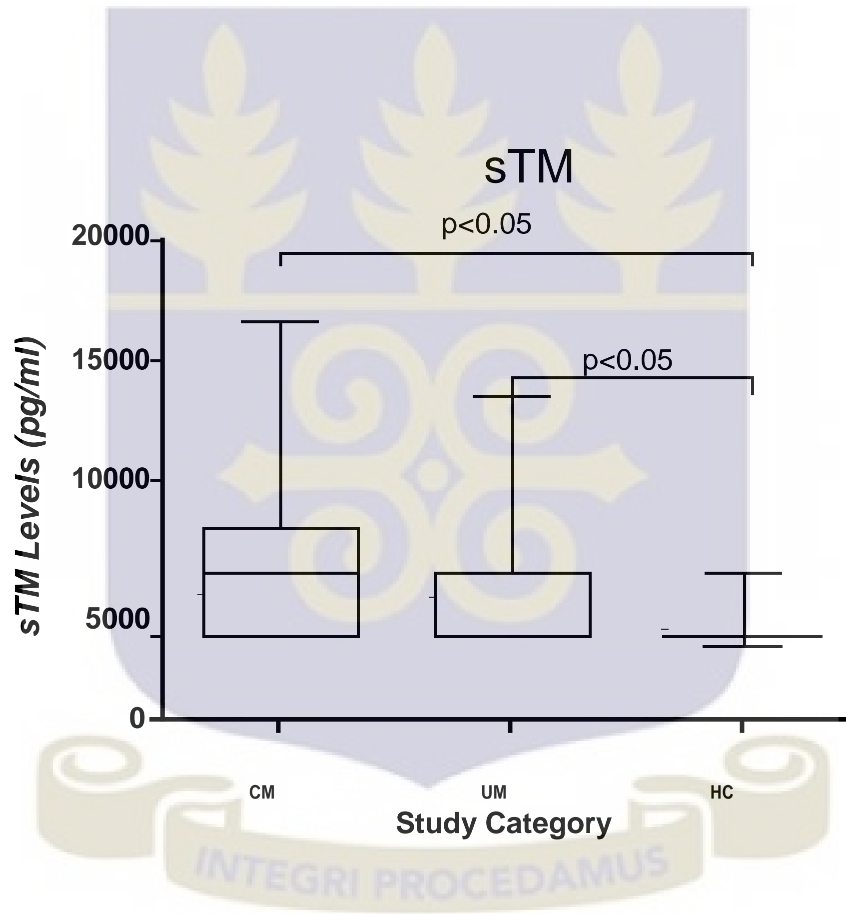

Figure 5.5: Median levels of soluble Thrombomodulin in study participants.

The middle lines in each box represent the median values of EPCR. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper and lower whiskers represent the 90th value and the lower whiskers represent the 10th value.

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4.4 Determination of EPC and CEC levels in study participants.

Whole blood stained with appropriate antibodies were analysed using a FACS Calibur to obtain graphical data in the form of dot-plots and percentages. The immature EPCs and mature CECs were obtained from three sub-populations of leukocytes (lymphocytes, monocytes and neutrophils) and were defined by the following combination of cell surface receptors; CD45dim/CD34+/CD133+/CD309+ for mature CECs and CD31dim/CD11b-/CD133+/CD34+ for immature EPCs (Figure 4.6).

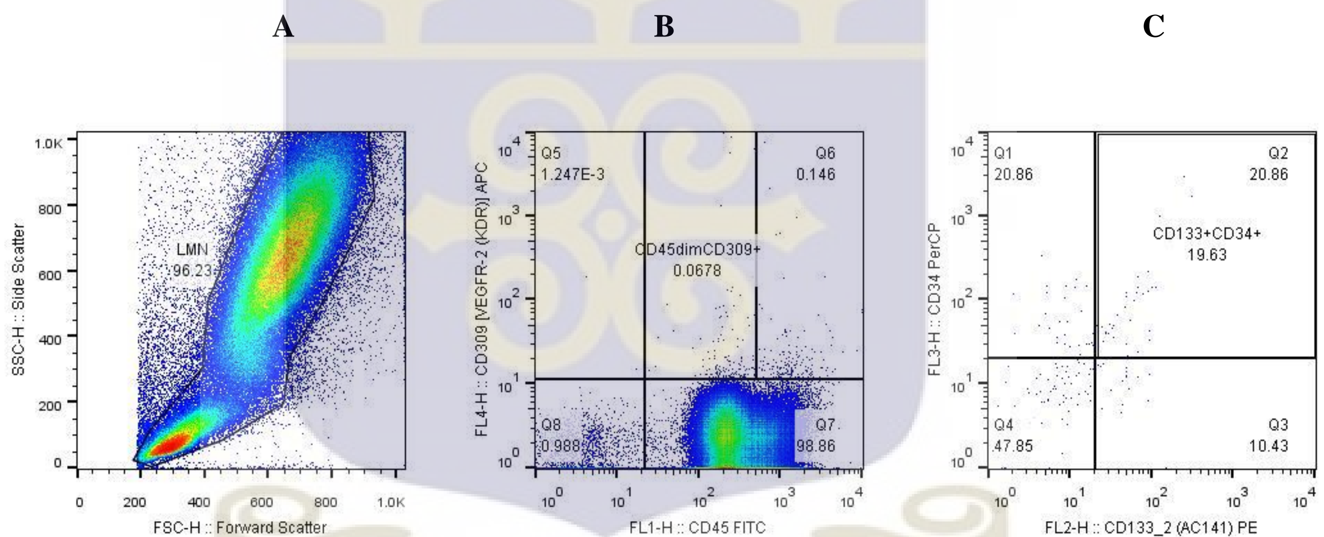

Figure 6.6: Enumeration of mature CECs

A. The three subpopulations of leukocytes (Lymphocytes, monocytes and neutrophils) were gated from the forward scatter/side scatter plot. B. Cells staining for CD45dim and CD309+ (within upper right quadrant) were gated from the obtained leukocyte population (lymphocyte monocyte neutrophil gate). C. Cells that stained double positive for CD34 and CD133 (upper right quadrant) were gated to give percentage of circulating CECs (CD45dimCD34+CD133+CD309+).

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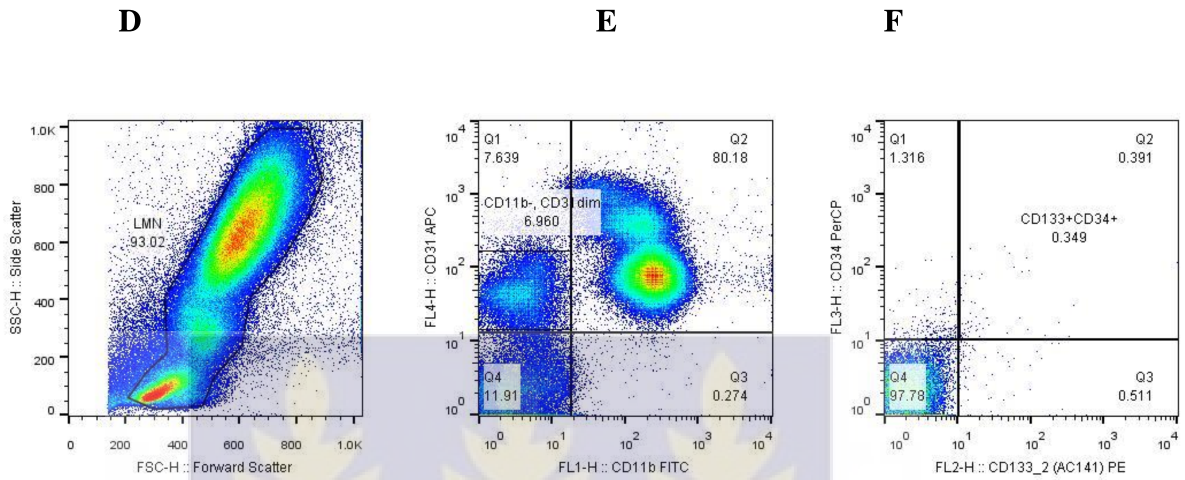

Figure 4.6 Enumeration of immature EPCs

D. The three subpopulations of leukocytes (Lymphocytes, monocytes and neutrophils) were gated from the forward scatter/sides scatter plot. E. Cells staining for CD311dim and CD11b- (within upper left quadrant) were gated from the obtained leukocyte population (lymphocyte monocyte neutrophil gate). F. Cells that stained double positive for CD34 and CD133 (upper right quadrant) were gated to give percentage of immature EPCs (CD31dimCD11b-CD133+CD34+).

The percentage of immature EPCs was compared amongst the three study groups. Levels of immature EPCs was significantly higher in CM (0.0103%, range 0-0.0543) than UM (0.001714% (0-0.0418)) and HC (0.008615% (0.001697-0.0296), *P<0.05*, Figure 4.7) groups following Post-hoc test.

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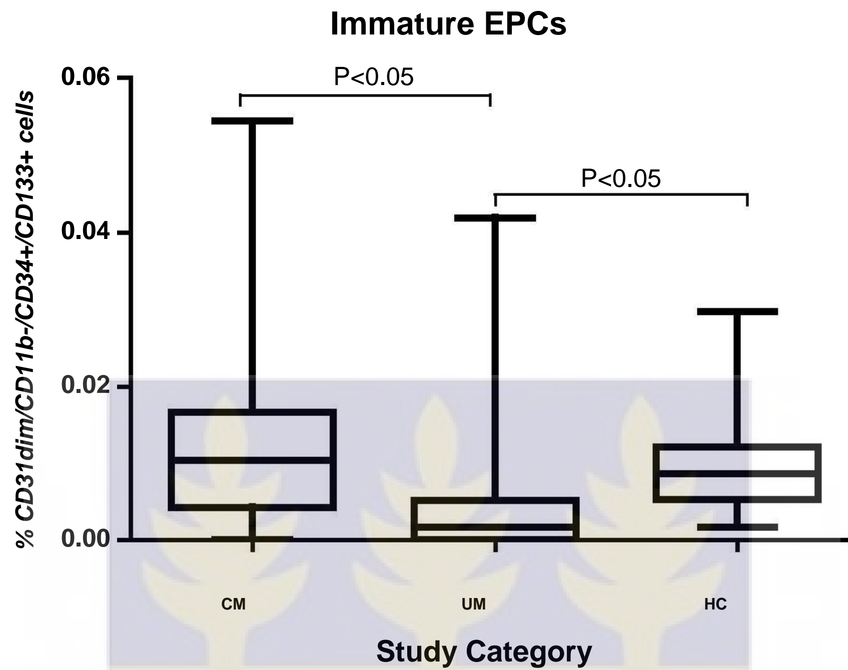

Figure 7.7: Levels of immature EPCs in study participants.

The middle lines in each box represent the median values of EPCR. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper whiskers represent the 90th value and the lower whiskers represent the 10th value.

The percentage of mature CECs were compared amongst the three study groups. Levels of mature CECs was significantly lower in CM (0.003857%, range 0 – 0.0304) than UM (0.005138% (0-0.0190)) and HC (0.007199% (0.001372-0.0317), *P<0.05*, Figure 4.8) groups following Post-hoc test.

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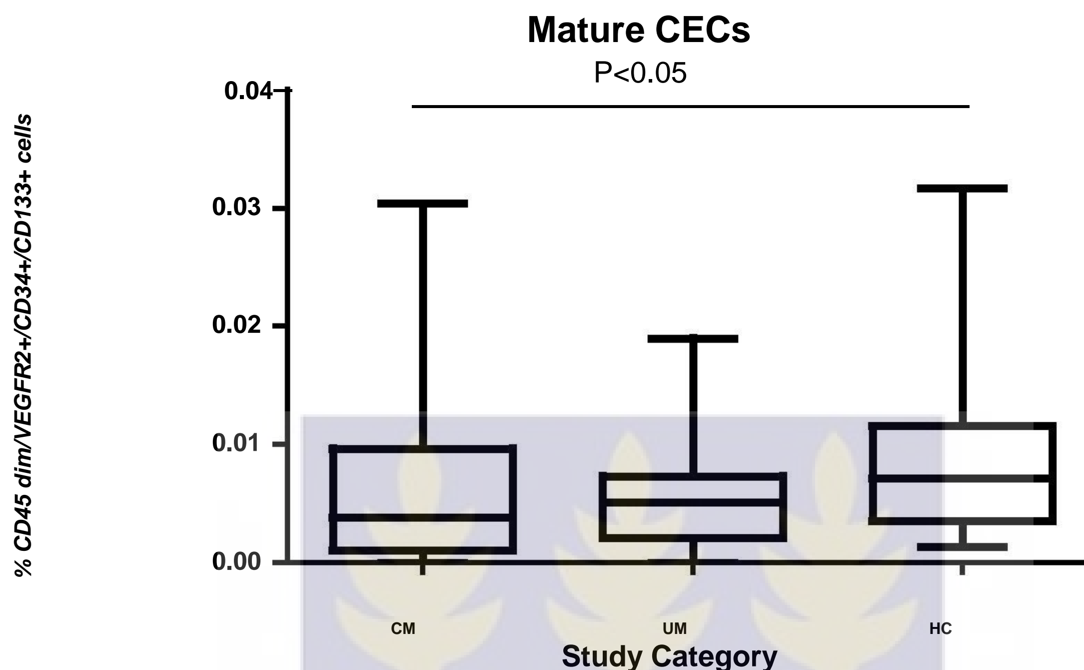

Figure 8.8: Levels of mature CECs in study participants.

The middle lines in each box represent the median values of EPCR. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper whiskers represent the 90th value and the lower whiskers represent the 10th value.

4.5 Relationship of endothelial cells to endothelial biomarkers and clinical characteristics of study participants

Correlation analyses were done between the immature EPCs and mature CECs with the measured biomarkers. There was no significant correlation between immature EPCs and the Ang-1 (r = 0.1971, *P = 0.0642*, *Spearman rank correlation*), Ang-2 (r = -0.0823, *P = 0.4416*), sTM (r = -0.0878, *P = 0.0430*) and sEPCR (r = -0.0128, *P = 0.9064*). There was also no significant correlation between immature CECs and Ang-1 (r = 0.1853, *P = 0.0821*), Ang-2 (r = 0.0299, *P = 0.7800*), sTM (r = -0.1061, *P = 0.3398*) and sEPCR (r = -0.1026, *P = 0.3445*).

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Regression analysis was used to predict the effect of Ang-1, Ang-2, sTM and sEPCR on immature EPCs and mature CECs. For the entire data set the levels of immature EPCs were significantly associated with Ang-1 levels (*P=0.0018*). Using multiple Stepwise Regression analysis involving data for the various study groups (CM, UM and HC) however, the significant association between proportions of immature CEPCs and Ang-1 was maintained in the healthy control group but not in the other categories (*P=0.016*). After correcting for age and parasite density however the association was lost and this presupposes that age and parasitaemia have an effect on the levels of Ang-1.

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CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

A compromised microcirculation with sequestration of parasitized red blood cells is known so far to be the main cause of the pathogenesis of the cerebral malaria (Dondorp *et al*., 2005a). Sequestration of infected red blood cells in the cerebral microvessels have been reported to result in capillary occlusion leading to obstruction of blood flow, reduction in tissue perfusion and reduced removal of waste products, all causing microvascular damage (Heyde *et al*., 2006). Many markers including adhesion molecules, cytokines and endothelial cells are involved in the sequestration process. It is believed that some biomarkers can serve as prognostic tools for cerebral malaria since their levels can determine whether there is vascular damage or not. Our study therefore hypothesized that the endothelial biomarkers, specifically Ang-1, Ang-2, sEPCR and sTM may have individual pathways or synergic pathways to determine the severity of *Plasmodium falciparum* infection in children. The study aimed to investigate the relationship between Ang-1, Ang-2, sEPCR and sTM and the pathogenesis of *P. falciparum* infection in children with circulating endothelial progenitor cells and circulating endothelial cells which are known to be responsible for endothelial repair when damage occurs.

In our study it was found that Ang-1 levels were significantly different amongst the CM, UM and HC groups ($P = 0.0002$). Ang-1 levels were found to be highest in HC compared to CM and UM groups ($P < 0.05$). CM and UM groups also had different Ang-1 levels with the CM group having the lowest Ang-1 level ($P < 0.05$, Figure 4.1). Angiopoietins are critical regulators of vascular

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structure and function and therefore serve as indicators of vascular injury. The higher Ang-1 levels found in our study corroborates what Lovegrove *et al*. (2009) found which shows that Ang-1 serves as a consistent diagnostic biomarker in individuals with malaria by its levels being able to best discriminate between cerebral malaria and uncomplicated malaria. Silver *et al.* (2010) had also reported that decreased Ang-1 levels are associated with *P. falciparum* malaria in pregnancy. This collectively means that irrespective of the organ involved, sequestration leads to dysregulation of Ang-1 and therefore means that sequestration leads to damage of the endothelium. Our findings therefore confirm what these studies have found regarding Ang-1.

No significant differences were observed in the levels of Ang-2 amongst the three study groups ($P = 0.0928$, Figure 4.2). Elevated Ang-2 levels have been associated with endothelial damage and a sign for severe malaria in several studies (Sato *et al*., 1995; Fiedler *et al*., 2006; Yeo *et al*., 2008). Contrary to our findings, in a work done by Conroy *et al*. (2010) using Malawian children, it was found that Ang-2 levels was highest in children with CM compared to children who presented with UM and those with decreased consciousness due to other causes. The reason for this disparity with our study is unclear, yet the observation in our study still implicates the dysregulation of angiopoietins which will eventually lead to the progression of malaria.

We further looked at the ratio between Ang-2 and Ang-1 because these two are known to regulate the endothelium. A dysregulation of the ANG-2/1 balance, may be associated with disease states that cause inflammation and vascular permeability. In our study it was found that the ratio of Ang-2/1 were significantly different amongst the three study groups ($P = 0.0417$).

The ratio of Ang-2/1 was lowest in the HC compared to UM and CM groups ($P < 0.05$). No

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significant difference was found in the Ang-2/1 ratio between CM and UM groups (*$P > 0.05$*, Figure 4.3). This observation still implicates the dysregulation of angiopoietins to be a contributing factor in malaria which eventually might lead to endothelial dysfunction and disease severity.

Contrary to our findings, Yeo *et al*. (2008) found that plasma Ang-2:1 levels were higher in patients with severe than non-severe *Plasmodium falciparum* malaria and were a better predictor of disease outcome.

The association between Ang-1 and Ang-2 is very important in the outcome of CM since Ang-2 is known to have antagonistic effect on Ang-1. Ang-2 is released from endothelial cells and antagonizes Ang-1's stabilizing effect on the vasculature during endothelial activation (Fiedler *et al*., 2004). Ang-1 and Ang-2 balance have been shown to regulate the functional responsiveness of the endothelium and also the ratio of Ang-2: Ang-1 have been shown to improve the specificity of Ang-1 and Ang-2 as indicators of endothelial function or destruction (Conroy *et al*., 2010).

In our study it was found that there were differences in the levels of sTM amongst the three study groups ($P < 0.0001$). The CM group had the highest level of sTM compared to UM and HC groups ($P < 0.05$, Figure 4.5). The UM and HC group also had different sTM levels with HC having the lowest. The high sTM levels found in the groups presenting with malaria and low sTM levels found in the HC groups confirms what Ohnishi, (1999) found which showed that Malian children presenting with uncomplicated and non-cerebral severe malaria had elevated sTM levels compared to healthy controls. Our findings with sTM also confirms what Mita-Mendoza *et al*. (2013) found which showed that patients with uncomplicated *P. falciparum* malaria had elevated sTM levels

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compared to uninfected control subjects. Maya *et al*. (2008) also found significant differences in levels of soluble TM when they investigated associations between markers of damage of vascular endothelial cells and plasma cytokine levels using ELISA in *falciparum* malaria patients and healthy people. The results obtained in this study suggest that sTM might be a useful marker of endothelium activation.

No significant differences were found in the levels of sEPCR amongst the three study groups ($P = 0.5837$, Figure 4.4). Contrary to this outcome Moxon *et al.* (2013) showed data that strongly suggested that cerebral malaria is associated with a localized disturbance of coagulation and inflammation caused by a local loss of EPCR and thrombomodulin initiated by sequestration of infected erythrocytes. Boomsma *et al*. (2002) also showed that sEPCR and sTM correlated with disease activity scores in patients with renal dysfunction.

Also the levels of circulating endothelial progenitor cells referred to in this study as immature EPCs (CD31dim/CD11b-/CD133+/CD34+) were significantly different amongst the study groups ($P < 0.0001$). CM group had the highest percentage (%) of immature EPCs than the UM and HC groups ($P < 0.05$, Figure 4.7). The immature EPCs levels also differed between the UM and HC groups with UM having the lowest percentage (%). The high immature EPC levels found in this study is in contrast to what was found by Gyan *et al*. (2009) in Ghanaian children presenting with CM and other malaria complications where they showed that the mean percentage of EPCs were significantly lower in CM compared to UM and controls. Also with regards to levels of endothelial cells being reflective of microvascular damage, Schmidt-Lucke *et al.* (2005) found that low levels of CEPCs correlated with cardiovascular disease progression and were predictive of cardiovascular events.

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In this present study the levels of endothelial cells may be said to be reflective of microvascular damage also. The high levels of immature EPCs observed in CM compared to UM and HC study groups can be suggested to be due to the mobilization of endothelial cells to sites of vascular injury after resulting coma.

The levels of circulating endothelial cells referred to in this study as mature CECs (CD45dim/CD309+/CD133+/CD34+) were significantly different amongst the study groups ($P = 0.0222$). The HC group had the highest percentage of mature CECS compared to the CM and UM groups ($P < 0.05$, Figure 4.8). The percentage (%) of CM and UM were not different. The high level of mature CEC in the HC group and low level of mature CEC in the CM group confirms what was found by Gyan *et al*. (2009) which showed that mean percentage of CECs were significantly lower in children with CM compared to the rest of their study categories.

Mature CECs replicate in order to replace damaged endothelial cells however they have a limited ability in migrating and proliferating to sites of endothelial damage thus immature EPCs support them by effectively migrating and proliferating from the bone marrow to endothelial injury sites in order to repair it (Urbich & Dimmeler, 2004). High levels of immature EPCs was found in our study possibly because those presenting with CM required recruitment of more immature EPCS to buffer the loss of mature CECs which might have been exhausted in an attempt to repair the endothelial damage. This is supported by low mature CEC levels found in this study.

The association of Ang-1, Ang-2, sTM and sEPCR with immature EPCs was assessed using a linear regression analyses. In our study only Ang-1 showed a significant association with immature EPCs ($P = 0.0018$).Our data suggests that Ang-1 may contribute to the production of immature

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EPCs primarily from the bone marrow for vascular repair. Our findings therefore confirms what Jeansson *et al*. (2011) found which showed that Ang-1 has a powerful ability to modulate the vascular response after injury when Ang-1 deficiency coupled with microvascular stress or injury in their mice model resulted in organ damage, accelerated angiogenesis, and fibrosis. Ang-2 has also been shown to liaise with Ang1in modulating endothelial development however this was not found in our study. Ang-1 was the only biomarker that showed an association because it is a marker for endothelial damage and immature EPC release whiles the rest with the exception of Ang-2 are only markers of endothelial damage.

After correcting for age and parasitaemia however, Ang-1 association with immature EPC levels was lost thus the levels was affected by age ($P < 0.0001$) and parasitaemia ($P = 0.0033$). This means that the difference in immature CEPCs levels may not necessarily be due to Ang-1 levels but may be due to age and /or parasitaemia. The loss of association between Ang-1 and immature EPCs after correcting for parasitaemia gives further proof that there is a dysregulation in the level of Ang-1 and Ang-2 in the endothelium of the CM and UM groups but the levels are regulated healthy individuals. This shows that parasitaemia is actually a factor that affects the levels of Ang-1 and Ang-2 in the malaria cohort since healthy controls individuals did not have parasites as at time of recruitment. The parasitaemia obtained in this study however is only from peripheral blood thus the extent to which it causes damage cannot be ascertained since it is the sequestered parasites in endothelial vessels may be responsible for the dysregulation of Ang-1 and Ang-2 in cerebral malaria and uncomplicated malaria.

Also the loss of association between Ang-1 and immature EPCs after correcting with age supports the notion that EPC function is impaired as one ages. Heiss *et al*. (2005) compared the number and

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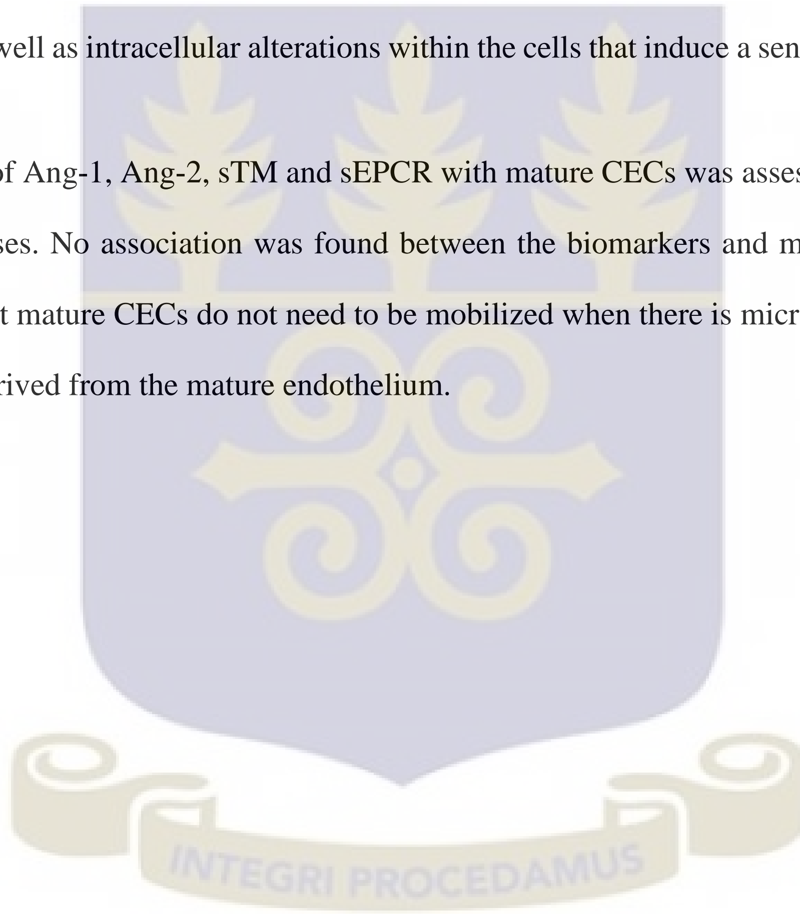
function of early EPCs isolated from the peripheral blood of healthy young and old individuals and found that EPCs from the old subjects were found to be significantly impaired in terms of proliferation, migration, and survival. Also Rauscher *et al.* (2003) suggested based on their study with rats that the athero-protective properties of EPCs are diminished with increasing age. It is likely that the age-associated impairment of EPC number and function is due to a variety of environmental changes that impair EPC generation, mobilization from the bone marrow, homing, and function, as well as intracellular alterations within the cells that induce a senescent phenotype.

The association of Ang-1, Ang-2, sTM and sEPCR with mature CECs was assessed using a linear regression analyses. No association was found between the biomarkers and mature CECs. This results shows that mature CECs do not need to be mobilized when there is microvascular damage since they are derived from the mature endothelium.



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5.2 Conclusion

Ang-1 levels were higher in the healthy children compared to children who had uncomplicated or cerebral malaria and was associated with immature EPC levels ($P < 0.05$). We have shown that Ang-1 but not Ang-2 is a predictor of endothelial damage and EPC recruitment. Also sTM was highest in children in the cerebral malaria group compared to uncomplicated malaria and healthy control groups ($P < 0.05$). sTM was found to be a predictor of endothelial damage. sEPCR levels did not indicate whether there was endothelial damage or not. CM study group had the least level of mature CECs and the highest level of immature EPCs because the CM group needed repair most.

5.3 Recommendation

Prospective measurements of EPC and CEC levels before and after disease state in individuals are needed to confirm the trend observed in this study.

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APPENDIX I

CONSENT FORMS

A. Consent for children with malaria

Title: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben Gyan, PhD

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

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

Dear Volunteer,

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

INTEGRI PROCEDAMUS

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

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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.

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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

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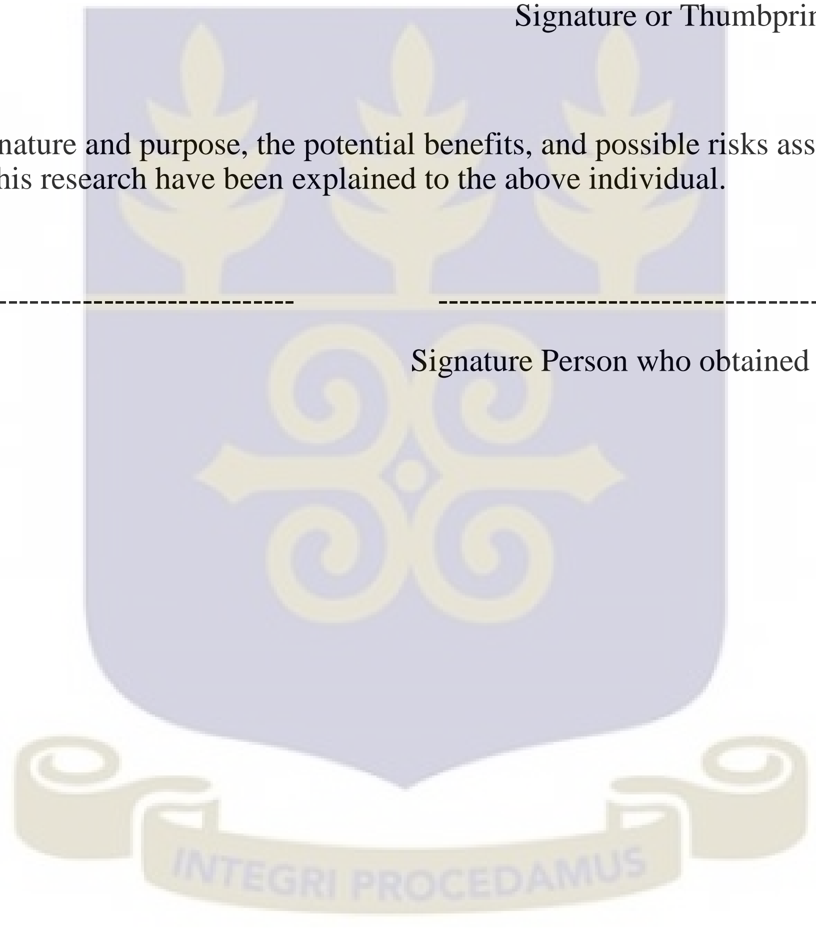
If volunteer's Parent/Guardian cannot read the form themselves, a witness must sign here:

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

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B. Consent for healthy controls

Title: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben Gyan, PhD

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

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

Dear Volunteer,

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

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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.

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General Information and your part in the study

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

Possible Benefits

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

Possible Risks

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

Withdrawal from study

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

Visits

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

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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)

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VOLUNTEER AGREEMENT

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--- ---

Date Signature or Thumbprint of volunteer

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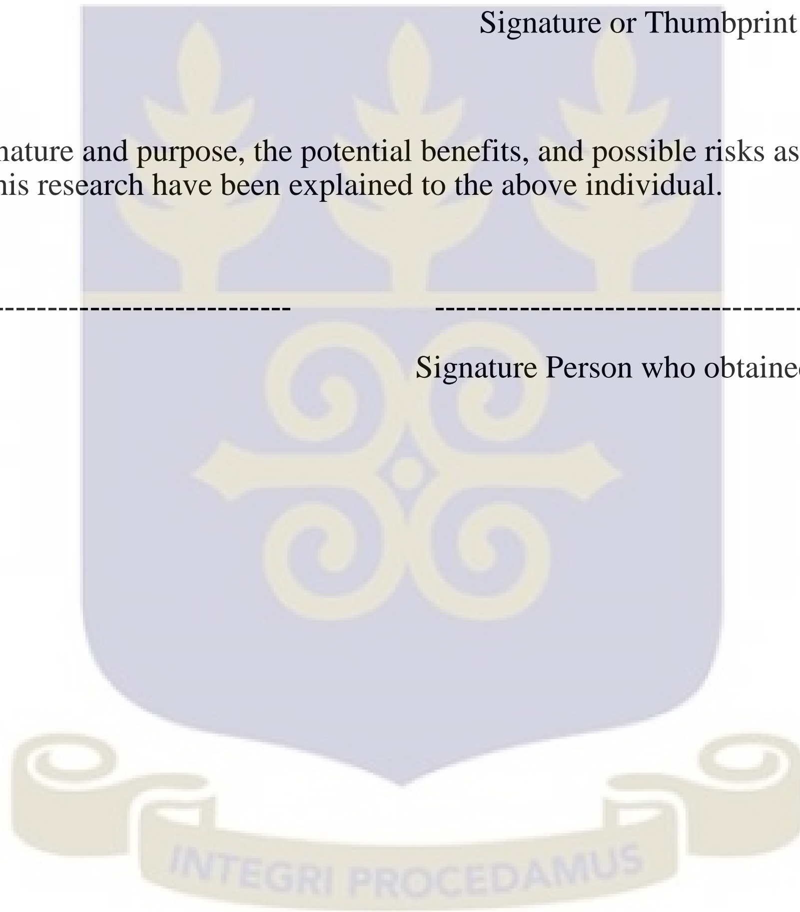
If volunteer's Parent/Guardian cannot read the form themselves, a witness must sign here:

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.



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Date Signature Person who obtained Consent

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APPENDIX II

BUFFERS AND REAGENTS

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

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

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

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

IV. Reagent diluent — 1% BSA in PBS.

B. Giemsa Buffer for parasite staining

Na_2HPO_4	1.0g
KH_2PO_4	0.7g
Distilled water	1 litre

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

C. Sickling test Buffer

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

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D. **Antibody and buffers for flow cytometry**

I.	Blocking antibody (IgG)	1part IgG Chrome to 4 parts 1X PBS.
II.	FACS Lysing Buffer	1 part FACS buffer to 9 parts distilled water.
III.	Acquisition of cell events	50,000 events in was acquired for tubes 1-4 whilst 250,000 events were acquired for tubes 5-6.

INTEGRI PROCEDAMUS

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IV. Fluorochromes used for each antibody

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

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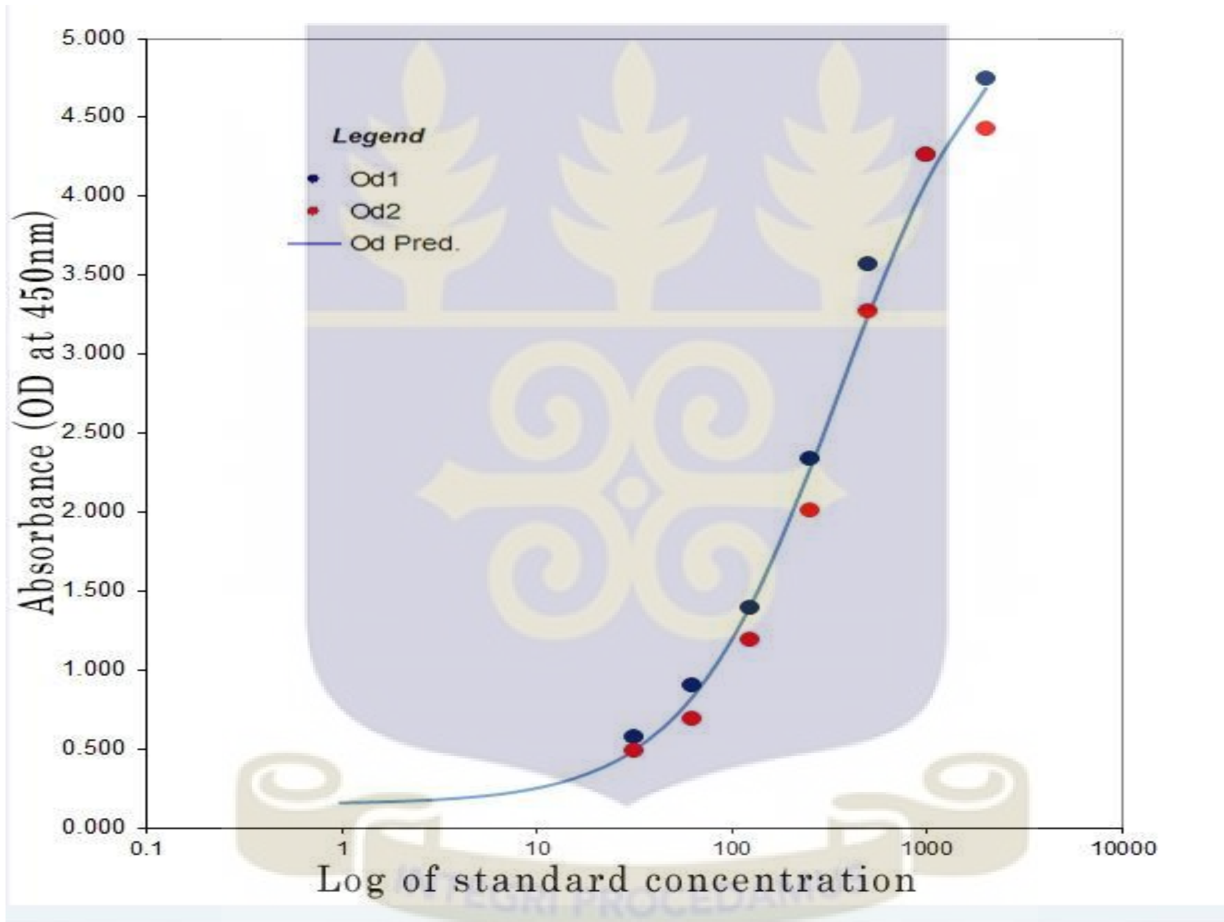
APPENDIX III

BIOMARKER CONCENTRATION CONVERSION

R square 0.986410

Mean Sq 11.49728

Max Od 5.38265



A sample standard fitting curve for ELISA (Ang-1, Ang-2, sTM and sEPCR)

The curve was obtained using the Adamsel ELISA software which uses the sample concentrations and corresponding absorbance values. The curve was generated for each plate read. R-squared value above 0.9700 showed that the regression line for that plate was good for generating the antibody units for the plasma samples measured in that plate.