THE ROLE OF MALARIA PARASITE BIOMASS AND IMMUNOHAEMATOLOGICAL INDICES IN THE PATHOGENESIS OF SEVERE MALARIA AMONG CHILDREN IN ACCRA METROPOLIS OF GHANA

BY

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DECLARATION

I hereby declare that this thesis except for references to other people's studies, which I have duly acknowledged, is the results of my own research performed at the Immunology department of Noguchi Memorial Institute for Medical Research (NMIMR) of the College of Health Sciences, University of Ghana and that it has neither in part nor in whole been submitted for another degree elsewhere. This work was done under the supervision of the persons mentioned below.

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DEDICATION

This work is dedicated to my parents Mr. John Addison and Ms. Dora Banful, and to my siblings.

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LIST OF ABBREVIATIONS

BBB	Blood Brain Barrier
BCS	Blantyre Coma Scale
CECs	Circulating Endothelial Cells
CM	Cerebral Malaria
CSF	Cerebrospinal Fluid
DIC	Disseminated Intravascular Coagulation
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPCs	Endothelial Progenitor Cells
FBC	Full Blood Counts
GPD	Gross Domestic Products
Hb	Haemoglobin
HC	Healthy Controls
HCT	Haematocrit
HIV	Human immunodeficiency Virus
ICAM-1	Intercellular adhesion molecule-1
IFN- α	Interferon-alpha
IFN- γ	Interferon-gamma
IL-12	Interleukin-12
IPTi	Intermittent Preventive Treatment in Infants
IRB	Institutional Review Board
IRS	Insecticide Residual Spraying

ITNs	Insecticide Treated Nets
LEKMA	Ledzokuku Krowo Municipal Assembly
LGH	La General Hospital
LLINs	Long Lasting Insecticide Nets
LYM	Lymphocytes
NMIMR	Noguchi Memorial Institute for Medical Research
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MDGs	Millennium Development Goals
MPV	Mean Platelets Volume
OPD	Outpatient Department
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
Pcirc	Circulating Parasite Biomass
PCT	Platelecrit
PDW	Platelets Distribution Width
PfHRP2	Histidine-Rich Protein 2
PLT	Platelets
PML	Princess Marie Louise Children's Hospital
Pseq	Sequestered Parasite Biomass
Ptot	Total Parasite Biomass
pRBCs	parasitized Red Blood Cells
RBC	Red Blood Cells

RDT	Rapid Diagnostic Test
RDW-CV	Red cell Distribution Width Coefficient of Variation
RDW-SD	Red cell Distribution Width Standard deviation
RH	Ridge Hospital, Accra
ROC	Receiver Operating Characteristic Curve
WHO	World Health Organization
WBC	White Blood Cells
SDGs	Sustainable Development Goals
SMA	Severe Malaria Anaemia
TGH	Tema General Hospital
TNF-α	Tumour necrosis factor-alpha
UM	Uncomplicated Malaria
VSA	Variant Surface Antigens

ABSTRACT

The pathogenesis of severe complications of *Plasmodium falciparum* malaria is still poorly understood in spite of its potentially fatal consequences. This poses practical challenges for malaria control efforts, especially, in discriminating children who are really at risk of severe malaria from those with incidental malaria in moderate and high malaria transmission areas. The search for reliable diagnostic and prognostic biomarkers for severe malaria is yet to produce encouraging results and this gives impetus to the pursuit of diagnostic/prognostic markers from perspective of little-explored indices like parasite biomass and composite malariometric index. This study explored the prognostic value of parasite biomass and immunohaematological indices in discriminating malaria phenotypes.

A total of 172 Ghanaian children aged 1 to 12 years with cerebral malaria (46), severe malarial anaemia (23), uncomplicated malaria (46) and healthy controls (57) in the Greater Accra metropolis were recruited. Giemsa stained slides were enumerated using microscopy and plasma HRP2 concentrations were measured using ELISA. Haematological indices were measured using Sysmex Automated Haematology Analyser and CD4 and CD8 counts were enumerated using a FACSCalibur. Total parasite biomass, circulating parasite biomass and sequestered parasite biomass estimates for each patient was evaluated from the plasma histidine-rich protein 2 (HRP2) concentration as well as haematocrit, patient's body weight and parasite density using a mathematical model. Principal Component Analysis (PCA) approach was employed in the construction of a composite malariometric index using parasitological and immunohaematological variables. A receiver operating characteristic curve was used in assessing

the sensitivity, specificity and ideal cut-off points of variables with diagnostic/prognostic potential. .

The total parasite biomass and sequestered parasite biomass were significantly higher in cerebral malaria than the uncomplicated and severe malaria anaemia groups ($p < 0.0001$). However, median parasite density did not differ among malaria phenotypes. ($p=0.188$). For haematological indices, individually, platelets, haematocrit and red cell distribution width coefficient of variation with a minimum cut-off point of 268, 32.750 and 14.450 respectively were the discriminators of malarial infection among children within Accra metropolis. Thrombocytopenia was found to be higher in cerebral malaria compared to other malaria phenotypes. The composite malariometric index was able to discriminate between uncomplicated malaria and severe malarial anaemia ($p=0.002$), and between cerebral malaria and severe malarial anaemia ($p=0.006$) but not between uncomplicated malaria and severe malarial anaemia ($p=1.000$).

Total parasite biomass and a composite malariometric index are promising indices that can be further explored as a prognostic/diagnostic marker for severe malaria in moderate and high malaria transmissions areas.

CHAPTER ONE

INTRODUCTION

1.1 Background of study

In spite of recent successes in control, malaria is still an important parasitic disease and a leading cause of mortality in the world (Mathers & Loncar, 2006). Malaria control efforts in the last two decades have harnessed insights from malaria research, upscaled effective intervention, and committed substantial funds to lessen the burden of disease globally. These achievements notwithstanding, malaria remains a global public health threat, particularly in children under five years and pregnant women living in Sub-Saharan Africa. The latest WHO malaria report (2016) stipulates that about 3.2 billion individuals could be at risk of the disease in 91 countries and regions with malaria transmission. The report also estimates the global tally of malaria episodes to be 212 million with 429,000 deaths. All these statistics represent a reduction in malaria burden and therefore, a welcomed news. However, the discomforting aspect of this statistics is the fact that about 90% of the current burden of disease occur in the African region. Of the several *Plasmodium* spp., only six routinely infect humans and *P. falciparum* is the most severe of the six and liable for 99% of the malaria mortality cases globally. In Ghana, malaria is responsible for nearly 1800 admissions of the out-patients department and 10 deaths for every 100,000 population (GHS, 2014). The main vector responsible for transmitting the disease is the female *Anopheles* mosquito as it does so during its blood meal. An infection with *P. falciparum* could end in a disease with varying degrees of severity. Whereas the majority of children may remain asymptomatic or have mild clinical disease, a minority of children develops severe complications like cerebral malaria and severe malaria anaemia (Pongponratn *et al.*, 2003). Factors responsible for the progression from mild to severe malaria is still not fully understood and extensive

research to unravel the pathogenesis of the disease and identify prognostic markers of severe disease are ongoing.

Cerebral malaria (CM) is the deadliest neurological complication of malarial infection caused by *P. falciparum.* It has a case fatality of about 20% and about 7% of children surviving from CM develops long-term neurocognitive impairments (Birbeck *et al.*, 2010). Cerebral malaria is an acute encephalopathy and has a clinical syndrome characterized by impaired consciousness with coma, seizures and the demonstration of parasitaemia on a stained blood film (Idro *et al.*, 2010). Although the pathophysiology of CM is still not fully understood, current knowledge suggests that the disease results from a combination of vascular and inflammatory immune system dysfunction as well as microparticle formation and homeostasis disruption (Souza *et al.*, 2015; Wah *et al.*, 2016). This is mostly due to microvasculature damage owing to the sequestration of parasitized red blood cells (pRBCs), vascular occlusion or a cytokine-mediated sepsis-like syndrome which results in alterations in the blood brain barrier (BBB) (Bland & Altman, 1995).

Good prognostic markers can potentially reduce mortality due to CM, however, these markers are ominously lacking in malaria endemic areas (Lucchi *et al.*, 2011; von Seidlein *et al.*, 2012). Several factors, both immunological and parasite-related, have been studied in an attempt to find good prognostic markers for CM (Lucchi *et al.*, 2011; von Seidlein *et al.*, 2012). A recent review paper in this area identified several molecules that had been suggested as either diagnostic or prognostic CM biomarkers but found no suggestions for early biomarkers that can predict febrile patients who will succumb to CM (Lucchi *et al.*, 2011; Sahu *et al.*, 2015). Parasite density has consistently turned out to be an unreliable CM prognostic marker and this observation probably

finds explanation in some of the pathophysiologic events associated with CM pathogenesis such as sequestration. Sequestered parasitized erythrocytes mostly harbour the matured parasites (trophozoites and schizont) within the deep vessels of microvasculature compromises microcirculation has been proposed as the underlying factor for disease pathogenesis, especially CM (White & Ho, 1992). Parasitized erythrocytes containing the mature trophozoites and schizont are found only in deep vascular beds. This facilitates asexual growth and division and also prevents pRBCs from being destroyed by the spleen (Marsh *et al.*, 1988; Newton *et al.*, 2000). As a result, only the less pathogenic and immature ring stage parasites are found in the peripheral blood. Thus, estimation of parasitaemia by peripheral blood parasite could therefore be misleading and a relatively poor predictor of the actual parasite burden since mature parasitized erythrocytes remains unavailable to be counted via microscopy. Thus, an approach to parasite estimation that accounts for sequestered mature parasites could be a more accurate estimator of parasite burden and perhaps a better prognostic CM marker.

A parasite-related molecule which holds promise as a CM prognostic/diagnostic marker is the *P. falciparum* histidine-rich protein 2 (PfHRP2). This protein is secreted by sequestered matured trophozoites and schizont into the plasma compartment and is released as the schizont rupture (Fox *et al.*, 2013). About 90% of the protein is liberated as the schizont ruptures (Desakorn *et al.*, 2005). Upon release, the protein can circulate freely or bound to other proteins. These proteins can be released both *in vivo* and *in-vitro*. Available evidence suggests that, soluble PfHRP2 concentrations could be used to access individual's total parasite burden, and a good prognostic marker for malaria severity. Few studies have demonstrated that PfHRP2 can indeed be used as a prognostic marker (Dondorp *et al.*, 2005; Kariuki *et al.*, 2013; Kariuki & Newton, 2014; Storm

& Craig, 2014). However, these studies did not evaluate the usefulness of this marker when used in combination with other malariometric and immunohaematological indices.

Prompt clinical diagnosis of the severe forms of malarial infections still remains a major challenge in malaria-endemic countries owing to the unspecific nature of the signs and symptoms, overlapping with acute febrile ailments in these countries (Jairajpuri *et al.*, 2014). This mostly impairs diagnostic specificity resulting in either delayed treatment or indiscriminate use of anti-malarial drugs. Although the former possess an eminent danger in the potential loss of lives, the emergence of drug resistance due to the latter cannot be discounted. Many have turned to haematological indices in search of markers that may discriminate live threatening malaria from other febrile illnesses. Infection with *Plasmodium* parasites targets red blood cells, which are capable of inducing haematological alterations. Alterations in haematological indices are one of the primary manifestations of malaria infection and this greatly influences disease outcome of the host (Gansane *et al.*, 2013). Several blood cell constituents comprising RBCs, PLT, WBCs, RDW, MCH, MCHC, PDW, HCT, Hb are affected during malaria infection. These haematological abnormalities may result in anaemia, thrombocytopenia, leucopenia, leucocytosis, splenomegaly, and lymphocytosis and rarely disseminated intravascular coagulation (DIC) (Facer, 1994; Murphy & Oldfield, 1996). However, malariologists are yet to leverage changes in haematological indices to better predict malaria diagnosis and prognosis.

Besides haematological changes, *P. falciparum* causes the activation, dysfunction and reallocation of T-cells and lymphopenia during the malarial infection (Anstey *et al.*, 2011; Kemp *et al.*, 2002). Available evidence shows that, CD 8^+ T-cells, cytokines, interferon-gamma (IFN-γ), transforming growth factor alpha (IFN- α) elicit immune response which confer protection

against pre-erythrocytic parasites within the liver cells (Kemp *et al.*, 2002), whereas CD 4^+ T-cells elicit immune response that restrains the growth of blood stage parasites by the activation of macrophages, secretion of cytokines and the activation and direction of humoral immune responses (Schmidt *et al.*, 2011). Some studies have explored the interaction between haematological and immunological indices in predicting changes in disease severity (Gudo *et al.*, 2012; Gudo *et al.*, 2013). The idea of using immunohaematology to enhance disease management and to avert severe complications parameters of malaria thrives on the fact that measurable blood factors vary with respect to demography, nutritional status, haemoglobinopathy, malaria endemicity and the immune status (Erhart *et al.*, 2004; Petel *et al.*, 2004; Price *et al.*, 2001; Wickramasinghe & Abdalla, 2000). Its potential notwithstanding, immunohaematological indices are difficult to interpret because they are numerous and diverse.

1.2 Justification of study

A better understanding of the pathogenesis of severe malaria may prompt a holistic approach to improve clinical management of the disease, treatment, supportive care and immediate interventions. Unfortunately, unravelling the pathogenesis of severe malaria is very challenging since studies involving individuals mostly focus on associations, and hardly demonstrate causality, whereas the appropriateness of studies from experimental animal models remain contentious. As a result, factors responsible for the progression from mild to severe malaria is still not fully understood. Malaria studies often churn out a worth of malariometric and immunohaematological data that is sometimes underutilized. These variables are sometimes analyzed individually or subjected to multivariate analyses but results of such studies have been difficult to interpret. However, a composite index that combines the various variables may give a

holistic measure that could be easier to interpret. Data dimension reduction strategies such as factor analysis and principal component analysis (PCA) may come in handy in this endeavour. In this exploratory data analysis approach, sets of potentially correlated variables are orthogonally transformed into linearly uncorrelated variable referred to as principal components that best explains the variance in the dataset (Krishnan, 2010). Thus, various malariometric and immunohaematological variables may be first subjected to principal component analysis (PCA) and a composite malariometric index constructs from emerging principal components. Although this approach has been utilized in other disciplines, it is yet to be explored in malariometry and the pathogenesis of malaria.

Cerebral malaria is a fatal malaria complication and yet its pathogenesis is not fully understood. The ability to predict which child is likely to develop cerebral malaria or die from cerebral malaria has implication for disease management and malaria control. However, the lack of good diagnostic and prognostic markers makes the search for these markers imperative. Further exploration of parasite biomass and the development of a composite malariometric index using immunohaematological indices may offer insights on the search for diagnostic/prognostic CM marker. This study therefore pursues a CM diagnostic/prognostic marker from the perspective of parasite biomass and a composite malariometric index.

1.3 Study hypothesis

1. There is no difference in parasite biomass among children with different malaria phenotypes

2. There is no difference in immunohaematological indices among children with different malaria phenotypes
3. The combined effects of parasite biomass and immunohaematological indices does not influence the outcome of malaria infection

1.4 Study objectives

The objectives of the study were as follows;

1.4.1 General objective

The general objective was to determine the effects of parasite biomass, immunohaematological indices and a composite malariometric index on the outcome of *Plasmodium* malaria infection.

1.4.2 Specific objectives

The specific objectives were;

1. To determine the relationship between parasite biomass and the outcome of *Plasmodium falciparum* infection in children with different malaria phenotypes.
2. To determine the relationship between immunohaematological indices and the outcome of *Plasmodium falciparum* infection in children with different malaria phenotypes.
3. To evaluate the effects of the interaction between parasite biomass and immunohaematological indices on the outcome of *Plasmodium falciparum* infection.
4. To develop a composite immunohaematological malariometric index, using haematological, immunological and parasite parameters.

5. To determine the relationships between the composite malariometric index and malaria phenoytpes.

CHAPTER TWO

LITERATURE REVIEW

2.1 The disease malaria

Malaria is a poverty-related protozoan disease responsible for death of many, particularly children under five years and pregnant women in the tropical countries. It is caused by a protozoan parasite of the genus *Plasmodium*. Primarily, four plasmodia parasites are known to infect humans and cause clinical cases in humans. These are *P. falciparum, P. ovale*, *P. malariae* and *P. vivax* (Gal *et al.*, 2001). In recent times, two non-human primate *Plasmodium* parasites have been added to this group. These are *P. knowlesi,* which was originally identified to infect macaque monkeys in nature had been recorded nearly in all the Southeast Asian countries in recent years (WHO, 2014) and *P. cynomolgi*, which is closely related to infection with *P. vivax* with similar biological and genetic features (Ta *et al.,* 2014). The various species of *Plasmodium* differ in morphology, immunological response and different geographical patterns (Tuteja, 2007). Infection with *P. falciparum* and *P. vivax* are the most deadly forms of malarial infection with deleterious impact on health management with *P. falciparum* being the most virulent and predominant in the African regions responsibly for nearly all the complicated malarial cases. This results in the number of deaths accounting for more than 96% of all malaria-attributable infections in the - sub Sahara Africa (Gal *et al.*, 2001). Globally, *P. vivax* is widely distributed geographically as compared to *P. falciparum* because *P. vivax* are able to develop in the infected anopheles mosquito at lower temperatures and survive at higher altitudes and cooler climatic conditions (WHO, 2014). But infection with *P. vivax* may be restricted in African continent due to the absence of the Duffy gene, which produces the protein necessary for the invasion of the erythrocytes. *Plasmodium vivax* infection can relapse after initial treatment to the dormant liver

stage, hypnozoite which can be activated months later. Infection with *Plasmodium knowlesi* and *P. vivax* are mostly less fatal, with acute febrile illness causing mild morbidity but rarely death and are mostly restricted to the West Africa, Asia and Latin America. Malaria parasites are usually transmitted to humans by the infective bite of female *Anopheline* mosquito. The parasite is rarely transmitted through transfusion of infected blood products and also through the placenta or during delivery (Malhotra *et al.*, 2006). Over 400 *Anopheles* mosquitoes have been identified, out of these only 30 are capable of transmitting the malaria parasite. Malaria is usually characterized by fever, chills, sweating and headache. A number of these infections, may result in severe or complications such as muscle aches, metabolic acidosis, respiratory distress, hyperlactatemia, hypoglycaemia, kidney failure, severe anaemia and cerebral malaria (Marsh *et al.*, 1995).

2.2 Global malaria burden

The global burden of malaria has seen a significant reduction between 2000 and 2015. Globally, the incidence of malaria has reduced by 37% since 2000 and the risk of dying also declined by 60%. Despite this remarkable achievement, malaria still causes significant morbidity and mortality worldwide threatening the lives of millions of peoples, especially children and pregnant women in the tropical countries. In estimating the leading cause of death from infectious diseases, malaria ranked second in Sub Saharan Africa and fifth globally (Mathers & Loncar, 2006) . The year 2015 marked the beginning of a new global agenda for human health and prosperity known as the Sustainable Development Goals (SDGs) and the end of the era of Millennium Development Goals (Cibulskis *et al.*, 2016). However, like the MDGs, malaria remains a significant threat to the SDGs. According to the 2016 World Malaria Report, decline

in malaria cases in the various African and some Asian countries has not been wholesale, since the malaria situation in Bangladesh, Pakistan and India showed a little change. The 2016 malaria report shows that approximately 3.2 billion individuals are at risk of malaria in 91 countries and territories with malaria transmission. In sub-Sahara Africa, the proportion of the population at risk declined from 17% in 2010 to 13% in 2015. In 2000, the number of malaria cases globally was estimated to be 262 million, however in 2015, the number of cases declined to 212 million representing 22%. Disaggregating malaria burden by region shows that a greater percentage of these cases in 2015 occurred in the African Regions (90%), followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%). With respect to the population growth, the incidence of malaria decreased by 37% between 2000 and 2015. Close to 76% of the estimated malaria cases occurred in only13 countries. Globally, the number of malaria deaths by WHO region declined from an estimated 839,000 in 2000, to 429,000 in 2015, a percentage decrease of 50% and 22% since 2010. In 2015, the WHO African Region recorded the highest number of malaria deaths at a percentage of 92%. This was followed by the WHO South-East Region (6%) and the WHO Eastern Mediterranean Region (2%).

Plasmodium falciparum caused most of the deaths, a percentage of 99% in the WHO African Region. Globally, the estimated number of malaria death among children under 5 years was 3,100 in 2015, representing 70% of all the mortality cases. With respect to the population growth, the number of mortality cases among children decreased by 60% since 2,000 and 29% since 2010 (WHO, 2016). Despite the tremendous effort in eliminating the disease, malaria remains a major killer of children - killing a child every 2 minutes (WHO, 2016). With respect to these figures, malaria ceased to be the leading cause of death among children in the sub- tropical

regions and only account for 10 % of child's mortality, which place malaria as the fourth highest cause of death in children (WHO, 2015). Malaria does not only inflicts the health of individuals, it also puts a heavy economic burden on endemic countries and contributes to the cycle of poverty people face in these countries. The economic burden of malaria is felt in a household of individuals and health systems in endemic countries (Sicuri *et al.*, 2013). Globally, the total funding for malaria control and elimination was estimated at US$ 2.9 billion in 2015, an increased by US$0.06 billion since 2010.

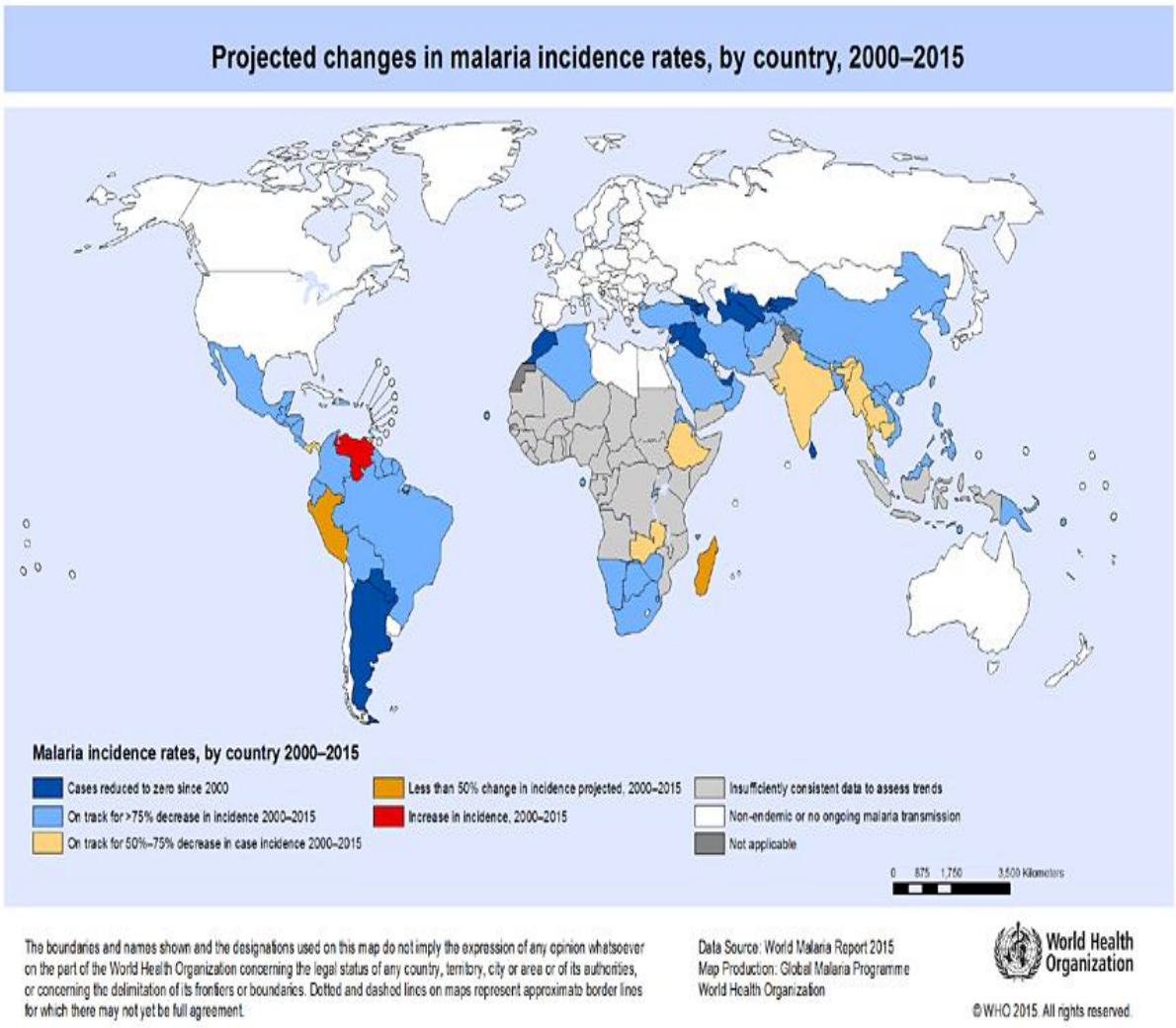

Figure 1: Worldwide Malaria Incidence rate. Adapted from WHO Malaria Report 2016

2.3 Malaria situation in Ghana

Malaria is one the major causes of morbidity and mortality worldwide, especially in the sub-Saharan regions of Africa. In Ghana, malaria is considered as endemic and perennial in all the regions, with seasonal variations that are more pronounced in the northern sector of the country (Afari *et al.*, 1995; Dery *et al.*, 2010). Ghana has a high transmission area with case to population

ratio of 1:1000. The period of the transmission season varies by the geographical pattern and this depends on the length of the dry season (December-February). Malaria transmission in the northern sector mostly last for 6-7 months with the highest number of cases occurring between July and November, however transmission period in the southern sector can travel beyond 9 months with a minimal peak period between May and June and a maximum peak period between October and November. However, spatial and temporal variability with respect to climate, topography and human settlement influence the level of transmission and the risk of infection (Abeku *et al.*, 2004; Snow & Marsh, 2002). *Plasmodium falciparum* is accountable for almost all malaria cases in the country accounting for 80%-90% and the vectors responsible for transmission are mostly *An. gambiae, An. funestus* and *An. Arabiensis* (WHO, 2013).

Malaria is a principal public health problem and the leading causes of morbidity and mortality in the country accounting for over 32.5% of all outpatient attendance and 48.8% of deaths in children under five years. Even though, the entire populace of about, 24.2 million is at risk of the infection, children under five years of age and pregnant women are at higher risk of severe illness. Different mechanisms have been proposed to the explain increase susceptibility to infection and severe disease in children and pregnant women (Sahu *et al.*, 2015). Most children who survive severe malaria often have some neurological damage that might hinder their development (UNICEF, 2007).

2.4 Socioeconomic burden of malaria

Malaria imposes a substantial economic burden on health care systems and households especially children and pregnant women among the Sub - Saharan countries (Sicuri *et al.*, 2013). Individual

households in African countries spend close to $25 on treatment and almost $20 on disease prevention monthly (Oluyole *et al.,* 2011). The socioeconomic costs of malaria associated with consultations, treatments, disease managements, hospitalizations, prevention and declining quality of life are immeasurable resulting into loss of productivity and reduced Gross Domestic Products (GPD). Individuals affected with severe forms of the disease can result in neurological disorders and disabilities when treatment is delayed. School-going children affected with malaria absent themselves from school, which eventually affects pupil's academic performance (Fernando *et al.*, 2003). Reducing childhood malaria may reduce school dropout at a relatively low cost to boost productivity (Zuilkowski & Jukes, 2014). It is generally understood that malaria is responsible for the highest loss of number of days of healthy life in Ghana although reliable information on the impact of malaria on labour productivity and the economy is absent (Asenso-Okyere & Dzator, 1997). Up to 60% of out-patient clinical cases in Ghana is as a result of malaria. The disease burden is quite high and is a major concern for health workers and scientists within the country.

2.5 Treatment, control and prevention of malaria

Currently the drugs used to treat unconfirmed and *P. falciparum* malaria in Ghana are quinoline and Artemether-Lumefantrin combination therapy. The available methods of diagnosis are the rapid diagnostic test (RDT) and microscopy. The RDT available in the country however is able to diagnosis *P. 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, and elimination is obtained from Global fund, 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 (WHO, 2013).

All efforts and programs put in place with regards to malaria in Ghana are targeted towards control (WHO, 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 through the organization and hosting of workshops in the field of malaria and several others (Asante & Asenso-Okyere, 2003). In addition to the ongoing national programs, smaller communities are also participating in the fight against malaria. One example is that in 2005, Anglogold Ashanti implemented an integrated malaria control 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, 2009).

2.6 Life cycle of the *Plasmodium falciparum*

All the over one hundred *Plasmodium* parasites known have similar life cycles, with two hosts - a vertebrate and an invertebrate hosts. However, the pathology attributed to *P falciparum* is different from any of those that infect humans. *Plasmodium falciparum* has a complex life cycle which involves the mosquito vector and a human host. Malaria infection occurs when the human

host is bitten by an infective female anopheles mosquito through a blood meal. The infection involves a wide spectrum of morphological and pathophysiological changes involving both sexual and asexual stages. The parasite goes through various stages of development, starting from fertilization, meiosis, formation of ookinete, oocyst formation and finally sporogony to complete its cycle in the invertebrate host. The vertebrate host involves stages of pre-erythrocytic schizogony, erythrocytic schizogony and finally erythrocytic gametogony.

2.6.1 Vertebrate host

Infection with human and for that matter vertebrates with the parasite begins with a bite from an infective female anopheles mosquito to the human host during feeding. Sporozoites from the salivary glands of the mosquito are injected into the skin of the human host through a blood meal. It is approximated that 100 sporozoites are injected per each infective bite. These sporozoites circulate in the bloodstream and migrate to the liver in less than an hour to invade the liver cells (hepatocytes) beginning the asexual reproduction cycle called tissue schizogony. This characterizes the asymptomatic phase of the malaria infection with little or no pathology. Within the hepatocytes, the parasite multiplies asexually to form infective liver merozoites. The hepatocytes rapture to release over 20,000 merozoites into the bloodstream to invade the erythrocytes (Jones & Good, 2006). Approximately, the tissue schizogony can last for 6-10 days in *P. falciparum*, 8 days in *P. vivax*, 13 days in *P. malariae* and 9 days in both *P. knowlesi* and *P. ovale*, however dormant stages known as hypnozoites from infection with *P. vivax* and *P. ovale* can persist in the liver for months or years and are capable of causing relapses of the disease. This stage initiates the erythrocytic schizogony.

Merozoites released from the raptured hepatocytes quickly recognize, attach, and invade the red blood cells (RBCs) to begin the blood stage cycle known as blood schizogony and is responsible clinical symptoms of the disease (Miller *et al.*, 2002). Within the red blood cells, the merozoites develop to a 'ring' stage (early trophozoites) and then to a trophozoite by losing its ring shape. It then replicates asexually to form a multinucleated schizont, which contains an average of 10 merozoites (Greenwood *et al.*, 2008). Finally, after a period of maturation of the schizont, the erythrocyte raptures to release the newly formed merozoites into the peripheral blood which in turn infect new erythrocytes to perpetuate the cycle. The asexual erythrocytic cycle approximately lasts for 48 hours in *P. falciparum*, *P. vivax* and *P. ovale* infection while in *P. malariae* and *P. knowlesi* the cycle takes 72 hours and 12 hours respectively (Cox-Singh *et al.*, 2008; Cox, 2010; Ng, 2008). The periodic fever paroxysms that accompany malaria are generally perpetuated by synchronous parasite growth, release of new merozoites, rupture of infected erythrocytes, release of malaria antigens and waste products.

After the rapture of the erythrocytes to release merozoites, some of the erythrocytic parasites differentiate into male and female gametocytes which are the sexual forms of the parasite which subsequently re-infect the mosquito during a blood meal to initiate the sexual reproduction (sporogony) in the mosquito's midgut. The fusion of the gametocytes forms a zygote to propagate the sexual stage of the malaria life cycle within the mosquito midgut. After a period of maturation, the zygote differentiates into an ookinetes, oocysts and sporozoites.

2.6.2 Invertebrate host

Within the mosquito's gut the male gametocyte nucleus undergoes exflagelation where the nucleus undergoes a number of DNA replication to produce eight motile male microgametes. The female gametocytes also mature to form a macrogamete. The male and female gametes fuse to form a zygote (ookinete). At this point, recombination can easily take place if the mosquito ingests two genetically distinct parasites clones resulting into the emergence of novel genotypes. The motile ookinetes burrow into the wall of the mosquito midgut to form an oocyst. Asexual division takes place within the oocyst to produce several sporozoites. After the sporogenic phase of 8–15 days (Eichner *et al.*, 2001), the oocyst bursts to release the sporozoites which migrate through the body cavity of the mosquito to the salivary glands (Ferguson & Read, 2004). These sporozoites are transmitted to a susceptible human host in a blood meal and the life cycle begins again.

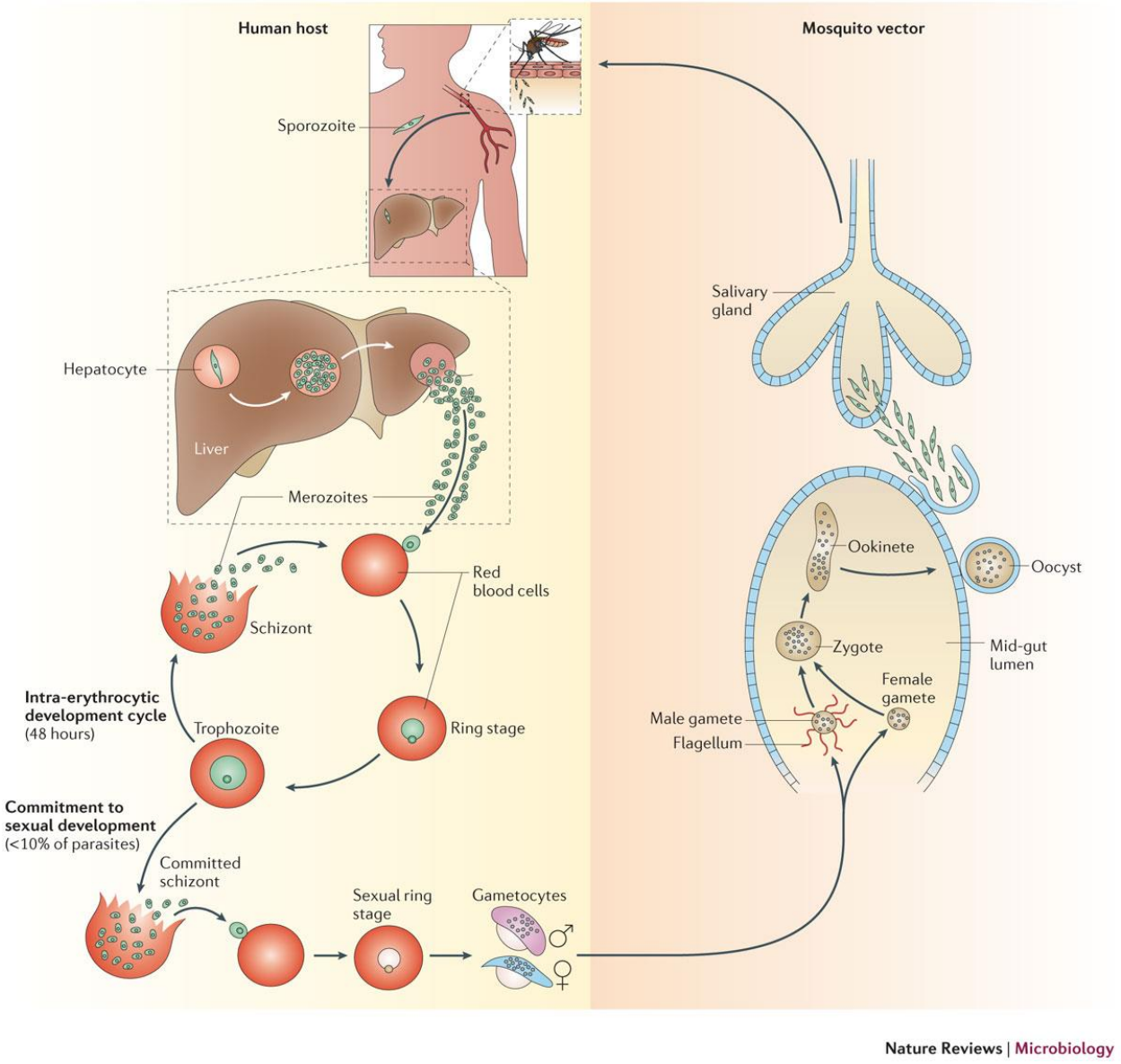

Figure 2: The life cycle of *Plasmodium falciparum*. Adapted from Nature Review/ Microbiology (Josling & Llinas, 2015)

2.7 *Plasmodium falciparum* infection outcome

Plasmodium infections are accompanied by a pre-patent period and an incubation period. The pre-patent period is the time of sporozoite inoculation by the mosquito to a detectable parasiteamia, a period that can last for nearly 5-10 days. Incubation period is the time from

sporozoites inoculation to the commencement of clinical manifestations which can also range from 6-14 days (Trampuz *et al.*, 2003). Infection with *P. falciparum* malaria may lead to disease with varying degrees of severity. Whereas some infected individuals actually remain asymptomatic, others develop uncomplicated or severe complications of the disease such as cerebral malaria, severe malaria anaemia, respiratory distress etc. (Langhorne *et al.*, 2008; Marsh & Kinyanjui, 2006).

2.7.1 Asymptomatic *Plasmodium falciparum* infection

Asymptomatic infection is the commonest part of malaria infection, characterized by very low levels of chronic parasitaemia and without any manifestation of clinical symptoms among the population living in high malaria endemic areas (Hamad *et al.*, 2000; Smith *et al.*, 1994). Children living in these areas often tolerate the malaria parasite and serve as potential reservoirs of gametocyteamia resulting to persistent transmission (Bousema *et al.*, 2004). Continuous exposure of individuals to these parasites in malaria endemic regions frequently leads to partially acquired immunity (Bousema *et al.*, 2004; Staalsoe & Hviid, 1998).

2.7.2 Uncomplicated malaria

Uncomplicated malaria refers to individuals with fever and any of the following symptoms; dizziness, nausea/vomiting, headaches, muscular and joint pains, loss of appetite, sweating, coldness with shivering, and occasionally diarrhoea, fatigue, and abdominal pains (Ewerbeck, 2012). Uncomplicated cases are mostly non-specific and could resemble several childhood illness such as bacterial or viral diseases. Clinical malaria diagnosis must be accompanied by the

presence of parasitamia on a stained blood smears or parasite antigens identified by a rapid diagnostic test.

2.7.3 Severe Malaria

Exclusively, *P. falciparum* is responsible for almost all forms of severe malaria and is only in rare cases that *P. vivax* or *P. ovale* species may cause severe forms of the disease (Svenson *et al.*, 1995). Clinical manifestation of severe malaria may include haemoglobinuria resulting from haemolysis; severe respiratory distress; low blood pressure; severe renal dysfunction; hyperparasitaemia; metabolic acidosis due to undue acidity in the blood; severe malaria anaemia due to massive destruction of erythrocytes and cerebral malaria resulting from impaired microvasculature and other neural complications which may results in death (Breman *et al.*, 2001). Severe malaria is therefore defined by the occurrence of one or more of these clinical manifestations of the disease in individuals without any apparent cause of the disease other than *Plasmodium* infection (Ferreira *et al.*, 2008). The two most common forms of severe malaria are severe malaria anaemia and cerebral malaria.

2.7.3.1 Severe malarial anaemia (SMA)

According to WHO, SMA can be defined as a malaria infection with haemoglobin concentrations <5.0 g/dl (or a haematocrit <15.0%) with identifiable parasitaemia. Severe anaemia is a major public health problem associated with *P. falciparum* infection and is mostly confined to young children living in malaria holoendemic and high transmission areas mainly in the sub-Saharan Africa (Björkman, 2002; Perkins *et al.*, 2011). Globally, severe anaemia is the leading cause of malaria morbidity and mortality with devastating health implications (Trampuz

et al., 2003). The development of malarial anaemia is a multifactorial which involves nutritional deficits, erythrocytes defects such as glucose-6-phosphate dehydrogenase, suppression of erythropoiesis, and dyserythropoiesis, interactions with common haemoglobinopathies, bone marrow dysfunction, the immune and non-immune mediated haemolysis of parasitized and non-parasitized red blood cells and altered cytokine, chemokines, growth factors and effector molecules balance (Chang & Stevenson, 2004; Ghosh & Ghosh, 2007; Helleberg *et al.*, 2005). Spleen and phagocytic cells are implicated in the destruction of parasitized and non-parasitized erythrocytes owing to malarial IgG activities. (Awah *et al.*, 2011; Awah *et al.*, 2009; Waitumbi *et al.*, 2000). Tumour necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) are potent anti-parasitic properties that are known to play a major role in conferring protection against malaria and also stimulate the production of monocytes/macrophages (Akanmori *et al.*, 2000; Casals-Pascual *et al.*, 2006; Jafarshad *et al.*, 2007; Robinson *et al.*, 2009). However, excessive production of these cytokines and endogenous nitric oxide levels can contribute to the disease pathogenesis (Clark & Cowden, 2003). Also, malarial pigment homozoin in the bone marrow contribute to the pathogenesis of the through impaired erythropoiesis (Aguilar *et al.*, 2014).

2.7.3.2 Cerebral malaria

Cerebral malaria (CM) is mostly caused by *P. falciparum* and is the most severe neurological complication of malarial infections and remains a major cause of mortality in endemic populations (Idro *et al.*, 2010). Patients who survive from CM mostly sustain brain injury and may experience short- or long-term neuro-cognitive impairment (Newton & Krishna, 1998). According to WHO, CM can be defined as a clinical syndrome characterized by unarousable coma approximately 60 minutes after cessation of a seizure or correction of hypoglycaemia,

confirmation of parasitaemia on a stained blood films and barring other causes of encephalopathy especially bacterial meningitis and viral encephalitis . Other symptoms of CM may include stiffness of the neck, pallor, dehydration, respiratory distress, convulsions and in some cases jaundice (Idro *et al.*, 2005). Studies involving the histopathological examination of brain tissue from CM fatalities at autopsy has revealed large numbers of accumulated *P. falciparum* parasitized RBCs sequestered in the cerebral vessels with localized haemorrhages and cerebral oedema (Aikawa *et al.*, 1990; MacPherson *et al.*, 1985; Mishra & Newton, 2009; Pongponratn *et al.*, 1991; Porta *et al.*, 1992; Taylor *et al.*, 2004b). Accumulation of parasitized RBCs has been found to be more extensive in CM fatalities as compared to other severe malaria syndromes, also accumulated parasitized RBCs are greater in the brain than any other organs (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991; Pongponratn *et al.*, 2003; Riganti *et al.*, 1990). In addition, leucocyte infiltration and inflammatory markers and increased intercellular adhesion molecule-1 (ICAM-1) are found in areas of sequestered RBCs parasites (Porta *et al.*, 1992). Sequestration hypothesis suggests that cerebral malaria pathophysiology is due to the interaction between parasitized RBCs and the endothelial cells leading to a mechanical blockade of cerebral microvasculature owing to parasitized erythrocytes deformity (Coltel *et al.*, 2004; MacPherson *et al.*, 1985; Miller *et al.*, 1972). It can also result from the adhesion of pRBCs to a receptor expressed on endothelial cell surface causing mechanical obstruction of blood flow and hypoxia of the surrounding brain parenchyma cells (Luse & Miller, 1971; Turner, 1997) as well as metabolic competition between pRBCs and host cells.

2.8 Immunity to malaria

The level of immunity to clinical malaria depends on age and intensity of exposure to the parasite leaving children under the age of five at a higher risk (Baird, 1995; Doolan *et al.*, 2009; Hviid, 2005). Malaria immunity is attributable to low-level parasitaemia especially among adult leaving in a malaria endemic regions and a state of sterile immunity is unattainable (Doolan *et al.*, 2009). The parasite express a variety of proteins at different stages of its complex and multi-stage life cycle and these proteins keep changing resulting in only a partial and short-lived immunity (Garcia *et al.*, 2006). As a result, natural immunity is unattainable and partial immunity cannot not protect one against new infection.

The mechanisms by which protective immunity is acquired is not entirely understood and remains a major subject of discussions (Crompton *et al.*, 2014; Doolan *et al.*, 2009). However, there is enough evidence suggesting that antibody repertoires against malaria parasites' variant surface antigens (VSAs) with time are answerable for the steady development of immunity (Day & Marsh, 1991; Kinyanjui *et al.*, 2004; Marsh & Howard, 1986; Staalsoe & Hviid, 1998) Clinical immunity against malaria can be grouped into natural/innate and acquired/adaptive immunity.

Most studies of immune defence mechanisms acting against malaria parasite are mostly focused on the blood stage infection and the potential for natural or innate immunity to confer protection has been largely neglected (Liehl *et al.*, 2015; Stevenson & Riley, 2004). However, recent studies in both animal models and humans have repeatedly implicated the actions of pro-inflammatory cytokines, especially IL-12, TNF- and IFN- mediators to confer protective

immunity to blood-stage malaria infection and these cytokines can be elicited by either the innate which limits the initial growth all blood-stage parasites or adaptive arm of the immune response (FAVRE *et al.*, 1997; Langhorne *et al.*, 2008; Marsh & Kinyanjui, 2006; Stevenson *et al.*, 1995).

2.8.1 Natural immunity

Innate or natural malaria immunity is an intrinsic refractoriness of the host that interrupt the establishment of infection and also elicit inhibitory response against the introduction of the pathogen. Innate immune responses is not dependent on any previous infections and can also influence the development of the adaptive immunity (Kadowaki *et al.*, 2000). Cells of the innate immunity includes macrophages, monocytes and neutrophils which are responsible for parasites clearance and also reducing malaria associated clinical symptoms (Sherman, 1998; Zhou *et al.*, 2012). Also the spleen, alterations in haemoglobin structures, certain thalassemia, haemoglobin E, homozygote haemoglobin C, ovalocytosis carrier status and certain enzymes have been reported to confer protective immunity against malaria parasites. In addition, sickle cell haemoglobin and Glucose 6 phosphate dehydrogenase deficiency are known to confer protective immunity against severe forms of malaria (Carter & Mendis, 2003; Doolan *et al.*, 2009).

2.8.2 Adaptive immunity

Adaptive immunity is an antigen-specific immunity that develops with time as a result of continuous exposure to the pathogen. Adaptive or acquired immunity can be classified as active or passive immunity (Doolan *et al.*, 2009). Active immunity enhances the defence mechanism of the host due to a previous encounter with the pathogen and passive immunity is acquired as a results of prenatal or postnatal passage of immune substances from the mother to the child or by

injection of such substances (Doolan *et al.*, 2009). It involves cell-mediated immunity which is mediated by T lymphocytes and humoral immunity mediated by B lymphocytes.

It is widely accepted that the blood stage of the malarial parasite is answerable for the pathophysiology of malarial infection and this has informed researchers to formulate a vaccine that will result in long-term immunological memory (Taylor-Robinson, 2010). B cells and CD4 cells are well accepted to play a very important roles in the immune response against the asexual blood stage of malarial infection by controlling of the parasite replication and clearance while CD8 cells are triggered during the pre-erythrocytic stage of the malaria life cycle (Chandele *et al.*, 2011; Good & Doolan, 1999; Jayawardena *et al.*, 1982). However, the immune response of CD8 cells remains controversial (Miyakoda *et al.*, 2008). Some studies have shown that protective immunity can be conferred by CD8 cells to asexual blood stage infections whereas other studies have suggested that CD8 cells do not elicit any protective immunity against blood stage infection (Chandele *et al.*, 2011; Langhorne *et al.*, 1998; Miyakoda *et al.*, 2008; Mogil *et al.*, 1987; Vinetz *et al.*, 1990).

In contrast to these studies, other studies from rodent model of *Plasmodium berghei* ANKA suggest that CD8 cells contribute to the pathogenesis of severe malaria, especially CM and malaria mortality cases (Belnoue *et al.*, 2002; Miyakoda *et al.*, 2008; Nitcheu *et al.*, 2003; Rénia *et al.*, 2006; Yanez *et al.*, 1996). Also studies in outbred-rat model have shown that CD8-dependent parasite clearance and erythrocyte removal in the spleen could predispose individuals to severe malaria anaemia as a result of removal of uninfected-erythrocytes (Safeukui *et al.*, 2015). Notwithstanding the role of CD8 cells, CD4 cells have been shown to confer protective

immunity against the liver stage of the plasmodium life cycle by assisting B cells to elicit anti-malarial antibodies that helps to induce a CD8 cell responses and indirectly inhibit the establishment of the liver stage of the parasite (Carvalho *et al.*, 2002; Tsuji & Zavala, 2003; Vampouille *et al.*, 2010). In another experimental studies, CD4 cells were shown to confer protective immunity by eliciting an immune response that interrupts the development of the blood stage parasites (Pierrot *et al.*, 2007; Weiss *et al.*, 1993). Tse *et al.* (2010) also demonstrated that CD8 cells are the first line of immune responses against liver stage malaria infection. Again, the expression of CD4 cells on CD8 cells modulates cytotoxic T lymphocytes to elicit immune response against the malaria parasite (Kitchen *et al.*, 2005). Due to these overwhelming evidence, CD4 cells are known to play a significant role in the development of CD8 cells during malaria infection and both CD4 and CD8 cells are promising candidates for malaria vaccine development in humans.

2.9 Malaria parasite biomass

Diagnosis of severe malaria in children is very challenging in high malaria transmissions areas where a greater percentage of children are parasitaemic at any point in time (Hendriksen *et al.*, 2012). Prompt and reliable prognosis of the disease is essential for effective management. The two most widely prognosis approaches used currently for disease management and intervention are clinical diagnosis and microscopy examinations. However, these forms of prognosis are non-specific and do not allow satisfactory prognosis of severe malaria (Iqbal *et al.*, 2002). Clinical diagnosis based on symptoms is the most widely diagnostic tool used in clinical procedures, however clinical symptoms show very less specificity owing to overlapping symptoms with other severe childhood diseases such severe pneumonia (English *et al.*, 1996; Verma *et al.*,

2013). As a result, clinical diagnosis of malaria is therefore unpredictable and should be accompanied by laboratory investigation if possible.

Microscopy examination of stained blood smears is another cornerstone for malaria diagnosis and estimation of parasitaemia. This form of malaria diagnosis has been the gold standard for centuries in malaria research and still remains the most appropriate and accessible diagnostic tool in clinical research and epidemiological studies (Verma *et al.*, 2013; Wu *et al.*, 2015). Although microscopy examination is relatively simple and cost effective, it shows poor reproducibility, variable sensitivity, unacceptable high false-positive rates and requires skilled labours for accurate examination (Verma *et al.*, 2013). In resource poor-countries, where malaria commonly occurs, it becomes very challenging to maintain good quality microscopy and results based on microscopy examination is sometimes misleading (Verma *et al.*, 2013). Importantly, peripheral blood parasitaemia does not represent the sequestered parasite burden, which is very pivotal to the pathophysiology of severe *falciparum* (Hendriksen *et al.*, 2012).

In another study, it was demonstrated that sequestration of asexual parasites occurs in the second half of the erythrocytic stage of the life cycle and this makes mature trophozoite and schizont stages unavailable in the peripheral circulations on blood smears (Silamut & White, 1993). This mostly underestimate the number of falciparum parasites in severely ill children resulting in high morbidity and mortality rate (Reyburn *et al.*, 2004). In addition, autopsies of cerebral malaria have demonstrated 26–40 times the burden of *P. falciparum* parasites in the deep tissue circulation of the brain compared to peripheral blood (Pongponratn *et al.*, 2003; Silamut & White, 1993). These occurrences may possibly explain the poor association between malaria

severity and parasitaemia measured by peripheral blood microscopy. Autopsy studies conducted by Taylor *et al*. (2004) revealed that 23% of children dying with positive blood smears with clinically defined cerebral malaria were due to other possible causes of coma. Their findings suggest that a percentage of children perceived to have fatal cerebral malaria mostly die due to other possible cause of diseases and not necessary clinically defined cerebral malaria. These children probably had incidental parasitaemia and were more likely to recover if the primary clinical problem is addressed (Taylor *et al.*, 2004a).

The pathological hallmark of severe malaria had to do with the sequestration of parasitized erythrocytes which compromise the integrity of microcirculatory flow to vital organs (Dondorp *et al.,* 2005). Another study also reported the widely use of peripheral blood parasitaemia as an assessment of disease severity in malaria has a weak prognostic significance of mortality in falciparum malaria (Marsh *et al.*, 1995). Since the more pathogenic sequestered mature parasitized red blood cells are not seen and therefore cannot be counted whiles the less pathogenic circulating young ring-form parasites stages are counted (Dondorp *et al.*, 2005), this does not account accurately the sequestered parasite burden (Hendriksen *et al.*, 2012).

As a result of these findings, a good parasite biomarkers for estimating the total parasite burden should be measureable in either whole blood, serum or plasma at any location of the parasite (Kifude *et al.*, 2008). Promising candidates for these biomarkers are histidine-rich protein 2 (HRP2) for detection of only *P. falciparum* and parasite-specific lactate dehydrogenase (pLDH) as well as *Plasmodium* aldolase, both of which are detectable in all species (Moody, 2002). Out of these parasite biomarkers, HRP2 is the most successful and have been proven to be highly

sensitive and specific for falciparum detection as compared to pLDH and *Plasmodium* aldose-detecting assays (Iqbal *et al.,* 2004). These proteins provide evidence of recent and active malaria infection and they are being utilized for both malaria diagnostic and surveillance purposes since the 1990s (Bell *et al.,* 2006).

Plasmodium falciparum histidine-rich protein 2 (PfHRP2) is a water-soluble histidine-and-alanine-rich protein released from infected erythrocytes *in vivo* and *in-vitro* (Fox *et al.*, 2013). The HRP2 protein was initially discovered in the avian malaria parasite *P. lophurae* (Kilejian, 1974) and was later identified to be produced by only *P. falciparum* (Aikawa *et al.*, 1990; Parra *et al.*, 1991). Histidine-rich protein 2 antigen can be assayed in plasma, serum, erythrocytes, urine and cerebrospinal fluid (Kifude *et al.*, 2008). The antigen has a repetitive B-cell epitopes that are synthesized by the parasite and this allows for easy detection by a rapid diagnostic kit (Beadle *et al.*, 1994; Garcia *et al.*, 1996; Taylor & Voller, 1993). Histidine-rich protein 2 is located in the cytoplasm or the food vacuole of the parasite and can also be found in the host cytoplasm as well as the red blood cell membrane. These proteins can either freely circulate or bound to antibodies or other proteins found in the plasma compartment (Howard *et al.*, 1986; Kifude *et al.*, 2008). Production of these antigens peak throughout the trophozoite stage and almost 90% are secreted at schizont rapture (Desakorn *et al.*, 2005). This protein can be distributed throughout the total plasma volume, plasma concentration of this antigen can be regarded as a better measure of patient's total parasite burden and therefore serves as an accurate prognostic marker for falciparum identification (Desakorn *et al.*, 2005; Dondorp *et al.*, 2005). In addition, HRP2 is very useful in detecting placental malaria even at very low levels of placental paeasitaemia (Leke *et al.*, 1999). Also PfHRP2 antigenaemia has a long half-life can be detected

in circulation for at least three weeks, even after effective antimalarial usage (Mayxay *et al.*, 2001).

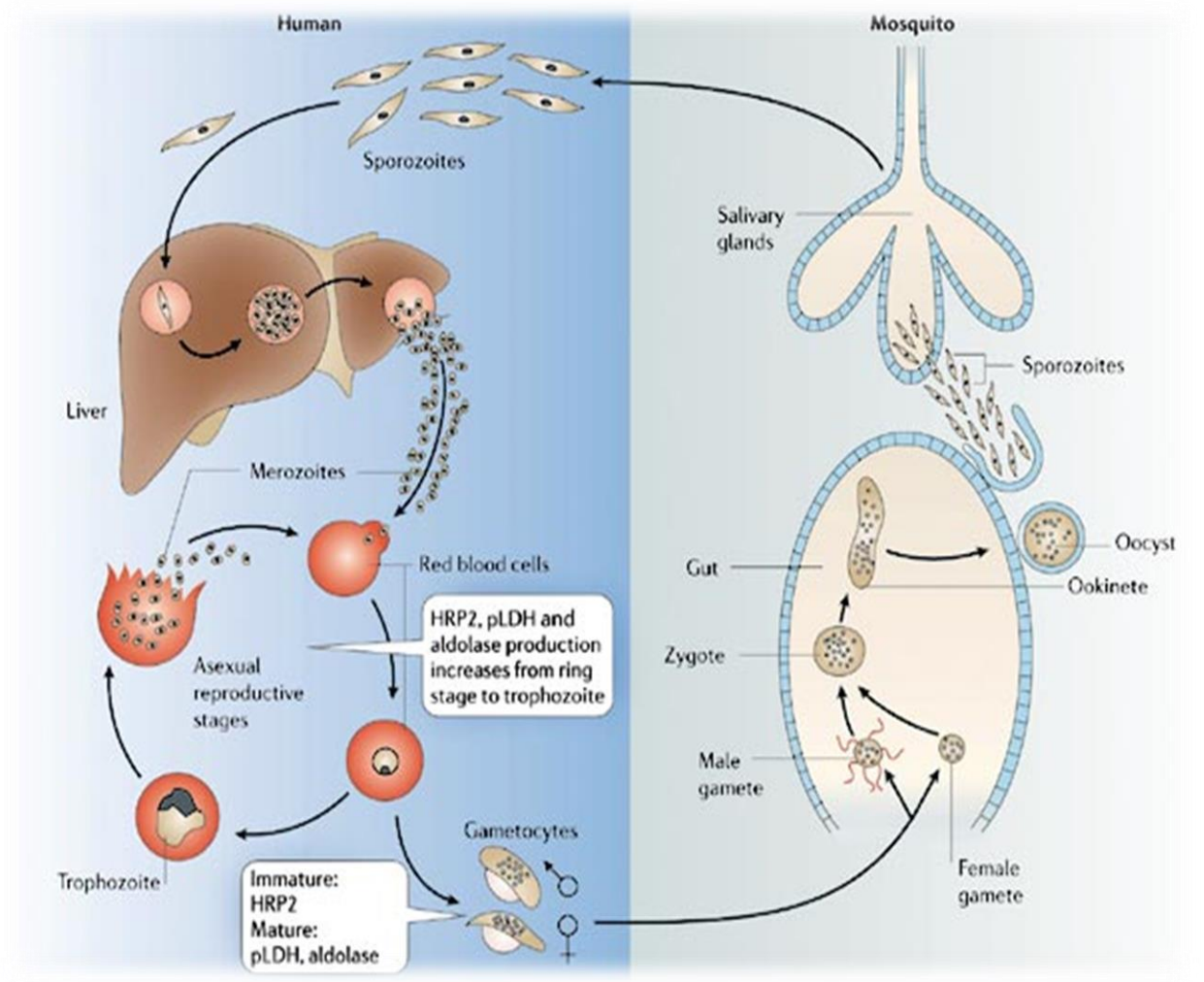

Figure 3: Stages of development of *Plasmodium falciparum* antigens (Bell *et al.*, 2006)

2.10 Haematological parameters and malaria infection

A number of clinical malaria involving severe malaria anaemia (SMA), cerebral malaria (CM), and uncomplicated malaria (UM), that progresses to severe forms of the disease have been studied (Shantsila *et al.*, 2007). Most of these studies were done using the blood tissue because blood is the most accessible diagnostic tissue for disease examinations. One of these diagnostic

examinations is the use of haematological parameters. Haematological changes are mostly affected by any disease syndrome and this can have a direct effect on haemopoetic physiology at any level (Maina *et al.*, 2010). This has been the case in high malaria transmission areas which mostly influences the host homeostasis with different disease manifestations and poses a key concern to public health due to its high morbidity and mortality rates (Kotepui *et al.*, 2014; Maina *et al.*, 2010). These changes account for most of the common malarial complications and also play a significant role in the pathophysiology of the disease progression (Kotepui *et al.*, 2014). Also changes in haematological parametes involve prominent cell lines such as red blood cells (RBC), leukocytes and thrombocytes (Kotepui *et al.*, 2015; Maina *et al.*, 2010; Yetişkinlerinde & Parametreler, 2013). Alterations in heamatological profiles vary with respect to demographic factors such as age, nutritional status, haemoglobinopathy, malarial endemicity and the immune status of the individuals with the malarial infection, both malaria immune and naïve individuals (Erhart *et al.*, 2004; Price *et al.*, 2001; Wickramasinghe & Abdalla, 2000). Individuals infected with malaria mostly have low platelets counts, haemoglobin concentrations lymphocytes counts, white blood cell counts and red blood cell counts whereas neutrophils and monocytes count are mostly higher in healthy individuals (Adedapo *et al.*, 2007; Erhart *et al.*, 2004; Gérardin *et al.*, 2002; Maina *et al.*, 2010; van Wolfswinkel *et al.*, 2013; Yetişkinlerinde & Parametreler, 2013). Another study reported higher WBCs count in malaria-infected individuals as compared non-infected malaria individuals (Ladhani *et al.*, 2002). The most frequent malaria-associated haematological complications are thrombocytopenia and severe anaemia with thrombocytopenia been known as the key indicator of malaria in febrile individuals (Erhart *et al.*, 2004; Khan *et al.*, 2012; Mahmood & Yasir, 2008; Moulin *et al.*, 2003). Individuals with platelets counts < 150,000 µl are reported to be 12-15 times more likely to have malarial

infection (Erhart *et al.*, 2004). Immunological and non-immunological depletion and dysfunction of platelets are known as possible causes of thrombocytopenia (Gupta *et al.*, 2013). Undue sequestration of platelets in the spleen, excessive platelets coagulation disturbances oxidative stress and antibody mediated platelets destruction are mostly found to be the underlying factors of thrombocytopenia in malaria (Gupta *et al.*, 2013). As a result, platelets structure, number and function are compromised in malaria-infected patients and these are mostly observed by the changes in the volume and other features of platelets cells (Greisenegger *et al.*, 2004). Platelets are known to be involved in haemostasis and inflammatory responses during malaria infections (Mirsaeidi *et al.*, 2010). Platelets morphology and functions can be assessed using mean platelets volume (MPV), platelet distribution width (PDW) and plateletcrit (PCT) and these indices are considered as markers of platelets activation.

2.11 The principle behind haematological analyser

The principle of the automated haematological analyser is based on hydrodynamic focusing. 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 (Meintker *et al.*, 2013). Parameters that are measured include haemoglobin (Hb) level, platelets count (PLT), 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), lymphocyte (LYM) counts.

CHAPTER THREE

METHODOLOGY

3.1 Chemicals, reagents and equipment

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

3.2 Study sites

This study was part of a longitudinal study that is aimed at investigating the role of circulating endothelial cells (CECs), endothelial progenitor cells (EPCs), and the pathogenesis of cerebral malaria. Study participants were recruited from five collaborating hospitals within the Accra metropolis of Ghana under the Ghana Health Service: Tema General Hospital (TGH), Ledzokuku Krowo Municipal Assembly (LEKMA) Hospital, La General Hospital (LGH), Princess Marie Louise Children's Hospital (PML) and Ridge Hospital (RH). These hospitals serve as referral centres for paediatric cases within the metropolis (Figure 4). Uninfected individuals as controls for the study were recruited from three collaborating schools under the Ghana Education Service: Hayward Nursery School, Osu Home School and LEKMA cluster of Schools in Teshie, all within the Accra metropolis and also the well-baby centre of the Tema General Hospital (TGH). Sample processing and analysis were conducted at the Noguchi Memorial Institute for Medical Research (NMIMR).

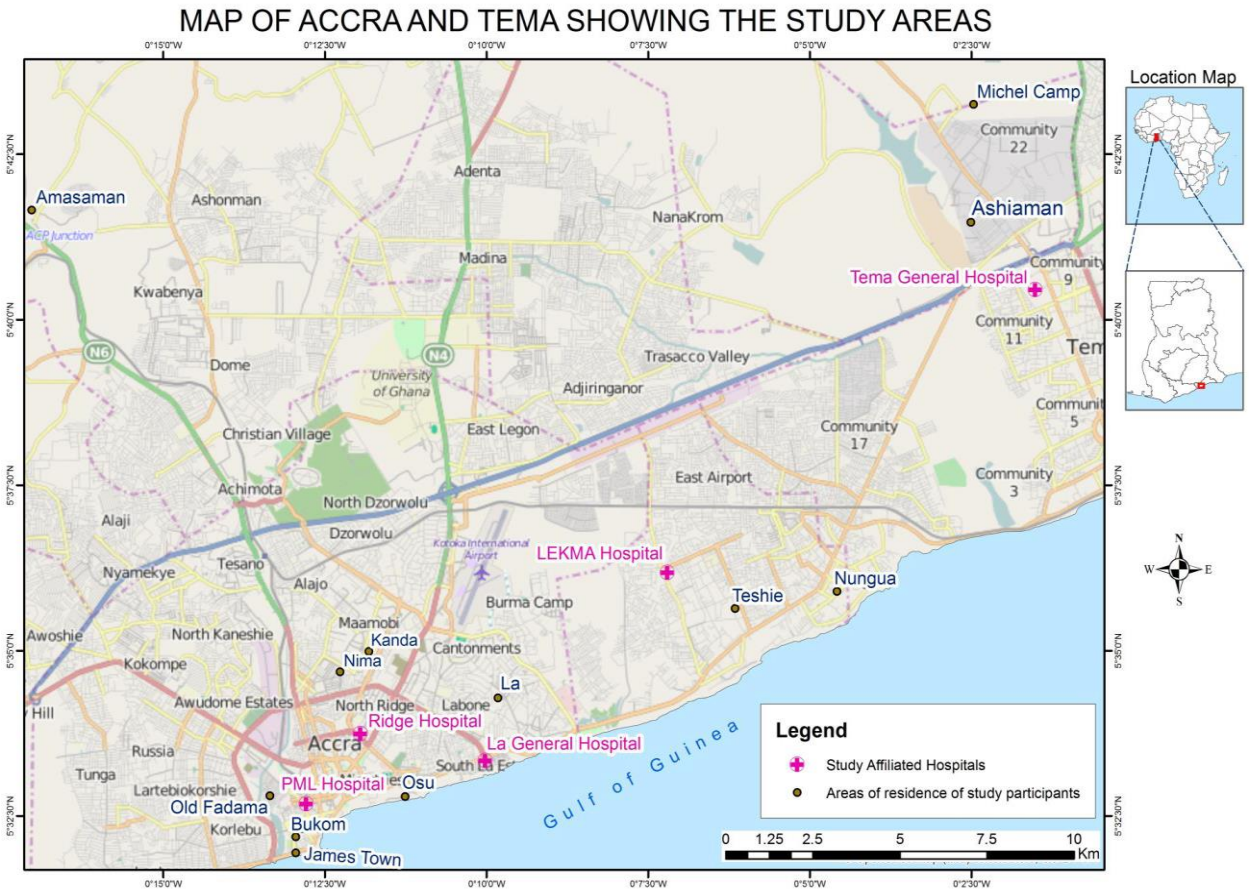

Figure 4: Map of Greater Accra showing the various study sites

3.3 Study design and groups

This is a longitudinal cohort study conducted between May, 2012 and July, 2016. Study participants were children between the ages of 1-12 years who reported to any of the collaborating hospitals with symptoms of malaria for treatment. Children with exclusive *P. falciparum* infection were qualified to partake in this study. Study participants underwent a detailed clinical assessment by a qualified paediatrician before recruitment. Detailed clinical information about previous malaria infections and time of symptom onset were collected from all the study participants. A total of 174 children were recruited. The study participants were divided

into three cohorts: Cerebral Malaria (CM), Uncomplicated Malaria (UM) and Severe Malaria Anaemia (SMA). Patients who reported with coma to the emergency unit of any of the collaborating hospitals were examined for cerebral malaria, also patients who presented with anaemia of haemoglobin (Hb) $\leq$ 5g/dl prior to transfusion were examined for severe malaria anaemia whereas patients reporting to the Outpatient Department (OPD) of any of the hospitals were examined for uncomplicated malaria. Uninfected healthy individuals within the same age bracket were recruited from the community schools within the Accra metropolis and the well-baby centre of Tema General Hospital. Patients recruited at the hospitals were classified as malaria group and those without malaria as Uninfected Healthy controls group (HC).

Peripheral whole blood sample was collected on the first day of clinical presentation at baseline sample, at the time of recovery from coma, and at 7 and 14 days post the recovery for children presenting with cerebral malaria. Whole blood samples were taken at baseline, 7 and 14 days post- baseline for patients reporting with uncomplicated malaria. Only baseline whole blood samples were taken for patients reporting with severe malaria anaemia before blood transfusion. Whole blood samples were taken at baseline, 7 and 14 days post- baseline for uninfected healthy individuals. Each study participant received a unique identification code to simplify data collection and identification.

3.4 Ethical clearance

The study was approved by the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR). Approval for the study was also sought from Ghana Health Service and Ghana Education Service. Parents or legal guardians of the study participants

signed informed consent following briefing of the objectives of the study before they were enrolled into the study (See appendix for consent form). The study was voluntary and participants could easily opt out of study at any point in time.

3.5 Case definition

This is a set of uniform criteria that is used to define a disease of public health interest. Below are the criteria that were set for selecting the study participants.

3.5.1 Inclusion criteria

Inclusion criteria for clinical malaria patients were; history of fever (axillary temperature ≥ 37.5°C) examined within 24-48 hours of admission with malaria parasitaemia of ≥5 *P. falciparum* parasites per high power field (approx. 2500 parasites/µl of blood) and at least one other sign of malaria (vomiting, diarrhoea, malaise, etc). Specific inclusion criteria for cerebral malaria was based on a patient being unconscious, with a coma score of ≤3 on the Blantyre coma scale (BCS) and being in coma for at least 60 minutes, no record of recent severe head trauma or other causes of coma or neurological diseases including meningitis/encephalitis (as assessed by lumbar puncture) and any haemoglobin value. Severe malaria anaemia was defined as clinical malaria with haemoglobin (Hb) ≤ 5 g/dl prior to blood transfusion. Uncomplicated malaria was defined as clinical malaria without any of World Health Organization (WHO) criteria for severe malaria such as severe malaria anaemia or respiratory distress. Whereas uncomplicated malaria (UM) patients were not hospitalised, CM and SMA patients were hospitalised. Uninfected healthy controls were defined as no evidence of parasitaemia assessed by thin and thick blood smears, normal temperature, sickling negative and no previous treatment of malaria and any other disease within the past one month as at the time of recruitment.

3.5.2 Exclusion Criteria

Study participants were excluded from the study if parent, guardian or proxy was unable to give signed informed consent and/or unwilling to comply with requirements of the study protocol. Participants were also excluded on the evidence of concomitant infection at time of enrollment including meningitis/encephalitis. Clinical history of any underlying disease that compromised the diagnosis and outcome of the illness including HIV infection were also excluded. Other disease or conditions such as recent severe bleeding, sickle cell disease or trait, evidence of bacterial or viral infection, history of diabetes mellitus, cardiovascular disease, hypercholesterolemia, surgery within 1 month, bone fracture within 3 months, major trauma within 1 month (e.g., car accident), blood transfusion within 3 months were also excluded from the study. Also participants who were receiving antimalarial treatment before being referred to the hospital were also excluded from the study. Patients with severe malaria anaemia that received blood transfusions prior to recruitment were also excluded. All malaria group cases without demonstration of plasmodium parasite by microscopy were also excluded from the study. For the healthy control group, any suspected sick individuals were excluded from the study and referred to the nearest health facility for prompt and adequate treatment at the expanse of the study.

3.6 Blood sample collection

About 5 ml of venous blood was collected aseptically from each study participant in the malaria group by qualified phlebotomists. Two millilitres of the venous blood was put into ethylenediaminetetraacetic acid (EDTA) tubes for full blood count (FBC), sickling test and blood smears for malaria parasite estimation and identification for routine clinical procedures. One

millilitre of the blood was put into another EDTA tube, while another 1 ml was placed in heparinized tube. The remaining 1 ml of blood was put into 40 ml BD BACTEC Peds Plus/F culture vials. These blood samples were used for laboratory assays and immunological studies. Cerebrospinal fluid (CSF) were collected via lumbar puncture by qualified paediatricians for examination in cases of cerebral malaria. Uninfected non-malaria group participants were physically examined by study clinicians and a total of 3 ml of venous blood were collected, of which 2 ml was put into EDTA tubes and 1 ml in heparinized tubes for laboratory assay and immunological studies.

3.7 Blood sample processing and storage

Blood samples for FBC, sickling test and blood smears for malaria parasite estimation and identification were immediately taken to the hospital laboratories. Whereas CSF samples were transported to either the Immunology Department of Korle Bu Teaching Hospital or the Lancet laboratory for examinations. Also, all other blood samples for further assays were transported on ice to the laboratories of the Immunology Department and Bacteriology Department, both of NMIMR for processing.

About 400 µl of blood from the EDTA vacutainer was used for flow cytometry. The rest of the blood and the heparinized blood were spun at 1,000 x g for 15 minutes to separate the plasma from the red blood cells (RBC). The plasma was again spun at 10,000 x g for 10 minutes to remove platelets. Platelet-free plasma and packed RBCs were stored in separate cryovials at -30°C until ready for further analysis. In addition, another set of blood smears for malaria parasite estimation and identification were done at NMIMR as a quality control.

3.8 Parasitological Evaluation

Thick and thin films of peripheral blood were made from anticoagulated EDTA blood sample for malaria parasites examination according to WHO protocol (WHO, 1988; WHO, 1991). Slides were labelled with study participant's identification code together with the date that the sample was obtained using a pencil. Briefly, thick film was made by dropping about 6 µl of blood on one end of a clean and lint-free microscope glass slide and was uniformly spread with a spreader in a circular manner at a diameter of about 1 cm on the slide. For the thin blood films, about 2 µl of blood was placed at the centre of 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 $45^{\circ C}$ to the slide. The slides were thoroughly air-dried. After drying, the thin film was fixed by immersing in absolute methanol for species identification. Both thin and thick films were then stained with freshly prepared 10% Giemsa stain (in Phosphate buffer) and left to stain for 15 minutes, followed by careful washing under running tap water. The slides were air-dried and examined with immersion oil under light microscope using X 100 objective lens for the presence of plasmodium parasites and species identification. The plasmodium parasites were counted against 200 white blood cells (WBCs). The number of parasites per microlitre of blood were estimated using the WHO guidelines (WHO, 2010). Parasite densities were obtained by multiplying the total parasite counted within this range from the thick blood film slide 8000, divided by the number of 200 WBCs counted.

3.9 Haematological evaluation

The haematological parameters were measured using an automated haematology analyser (Sysmex Automated Haematology Analyser Model KX-21N) at initial clinical presentation and

subsequent reviews. Control sample was run along each series of blood tests. Blood samples treated with EDTA. The number, size and volume of cells were detected by the machine and the following parameters were measured: Haemoglobin (Hb) level, platelets count (PLT), 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), lymphocyte (LYM) counts.

3.10 Bacteraemia evaluation

Bacteremia analysis was done according to (Cheesbrough, 1984). The 1 ml of blood which was placed into 40 ml BD BACTEC Peds Plus/F culture vials containing broth medium was kept in BACTEC FX incubator at 37°C and observed daily for seven days for any signs of haemolysis, production of gas, coagulation of the broth and turbidity above the red cell layer. Any sign of possible bacterial growth was then sub-cultured on solid media and examined after 24 - 48 hours for bacteria growth and also to determine the kind of the species.

3.11 Sickling evaluation

Sickling test was done for each patient by using the sodium metabisulphite test. For the sickling test, a drop of blood was placed on a clean microscope slide. An equal volume of the 2% sodium metabisulphite 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.

3.12 *Plasmodium falciparum* histidine-rich protein 2 determination

Cryovials containing heparinized plasma for both malaria cases and non-malaria cases were identified and retrieved from the stored samples. These plasma samples were thawed in batches and HRP2 levels were assessed using malaria antigen Celisa kit (Cellabs, Sydney, New South Wales, Australia) employing a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions with little modifications. Plasma samples and controls were diluted with 1% phosphate-buffered saline (PBS) in a ratio of 1:500 and 1:5.5 respectively with the controls at a starting concentration of 20 ng/ml. One hundred microliters of the plasma samples together with the positive and negative controls were plated in duplicate onto a plate pre-coated with anti-*P. falciparum* monoclonal antibody. The plates were incubated in a humidified chamber for 1 hour at room temperature. This step was followed with extensive washing with PBS/01% Tween. One hundred microliters of the anti- *P. falciparum* conjugated antibody in a 1:200 dilution was added and allowed to incubate in a humidified chamber for 1 hour at room temperature. The conjugate was extensively washed off and 100 µl chromogen substrate at a 1:20 dilution was plated. The assay was kept in the dark for 15 minutes at room temperature during which a visible colour was noticed. Fifty microliters of 2 M sulfuric acid (stopping solution) was added to stop the reaction and the plate was read spectrophotometrically at an optical density (OD) of 450 nm using Bio-Tek ELX 808 TM plate reader. A standardized curve was obtained from the recombinant HRP2. Readings from the diluted plasma samples were compared to the recombinant HRP2 curve to assess the HRP2 levels from the unknown samples. Optical density values that fell outside the linear range of the recombinant HRP2 were further diluted at either 1:10 or 1:1000. Optical density values that remained off scale (low) upon further dilution were given a value corresponding to the least possible detection value. The cut-off level

for defining a reading as positive was set at the absorbance value of the negative control plus 0.2 units, as recommended by the manufacturer of the test kit. A positive reading for PfHRP2 antigen was defined as an absorbance value above the cut-off level. A positive results indicate the presence of *P. falciparum*.

3.13 Estimation of malaria parasite biomass

Individual total parasite biomass (P_{tot}) was assessed from the estimated plasma HRP2 concentration using a mathematical model described by Dondord *et. al* in Asian adults in a study titled "Estimation of the Total Parasite Biomass in Acute Falciparum Malaria from Plasma PfHRP2" (Dondorp *et al.*, 2005). This model requires the use of parasite elimination half-life and parasite multiplication rate. This was adapted in a similar studies conducted among children living in Tanzania, Africa (Hendriksen *et al.*, 2012). The formula for one's total parasite burden was estimated as P_{tot} = 7.3*PfHRP2 *(1-Hct (%))*body weight (Kg)*10^{13}, with PfHRP2 in g/l (Dondorp *et al.*, 2005; Hendriksen *et al.*, 2012). The formula is dependent on plasma PfHRP2 half-life and the parasite multiplication rates due to one's immunity and the malaria transmission settings of the area. Circulating parasite biomass (Pcirc) from the peripheral blood was estimated using the formula: parasites/ µl x 10^6 x blood volume, with blood volume defined as 0.08 x body weight [kg] (Dondorp *et al.*, 2005; Hendriksen *et al.*, 2012). Sequestered parasite biomass (Pseq) was estimated as the difference between the total parasite biomass and the circulating parasite biomass.

3.14 Processing of whole blood samples for flow Cytometry

Flow cytometry was used to evaluate the levels of CD4 and CD8 in whole blood for all study participants using a FACSCalibur^{TM} flow cytometer (BECTON DICKINSON, USA). Two 5 ml

polystyrene falcon tubes (BD) were labeled (1-2) with permanent marker. Two microlitre of blocking buffer (Pure IgG Chrome and PBS at 1:5) was aliquoted to each tube and 50 µl of whole blood treated with EDTA was added to each tube. Each tube was gently vortexed for about 10 seconds and kept in the fridge for 10 minutes at 4^{o}C. Tube 1 was left unstained in the fridge whiles 3 µl each of monoclonal antibodies (CD4 and CD8) were added to tube 2. The sample was once again vortexed for 10 seconds and was added to tube 1 in a rack. Aluminum foil was used in wrapping the rack containing the sample tubes and was allowed to incubate for 30 minutes in the fridge at 4°C. Tubes were gentle vortexed at the end of the incubation period and 2 ml of lysing buffer (BD FACS lysing and distilled water at 1:10) was added to each tube. The tubes were gently vortexed and allowed to incubate in the dark at room temperature for 10 minutes to lyse the RBCs. Samples were gently vortexed and centrifuged at 4°C in a refrigerated centrifuge at a speed of 1500 rpm for 7 minutes. The supernatant was gently decanted and tube was carefully streak thrice against the rack to lose the cells stack at the bottom of the tube. Cells were washed by re-suspending the cells in 3 ml of Cold FACS FLOW. Cells were gently vortexed and centrifuged at 4°C in a refrigerated centrifuge at a speed of 1500 rpm for 7 minutes. The supernatant was once again gently decanted and tube was carefully streak thrice against the rack to lose the cells stack at the bottom of the tube and this is known as the second wash. All the process of washing was repeated and this is known as the third wash. Finally, the cells were re-suspended in 250 µl Cold FACS FLOW for cell acquisition using the FACSCalibur. To add up to the four panel colour of the FACSCalibur, CD 14 and CD15 were added.

3.15 Statistical analysis

All statistical analysis were done using IBM SPSS Statistics software, version 23. Exploratory data analysis first checked data distribution for the various variables after, which normally distributed variables were subjected to parametric test statistics whiles variables with kurtotic and skewed data were subjected to non-parametric statistics. In conducting analysis of variance, both parametric and non-parametric methods were employed. ANOVA, Mann-Whitney U test and Kruskal Wallis *t*-test were used where appropriate. Bonferroni's *t*-test for multiple comparisons was used in instances where post hoc pairwise comparisons were necessary. To determine the diagnostic potential of various haematological and immunological variables as binary classifiers a receiver operating characteristic curve, i.e. ROC curve were used. Principal Component Analysis approach was adopted in computing a composite malariometric index. Differences and associations were deemed significant at $p < 0.05$.

CHAPTER FOUR

RESULTS

4.1 General characteristics of study participants

A total of one hundred and seventy-two (172) study participants were recruited into the study. Of those, 115 (66.9%) were children aged between 1 and 12 years presenting with falciparum malaria in any of the five (5) collaborating hospitals who were recruited from May, 2012 to July, 2016 in the Greater Accra metropolis. According to WHO criteria for classifying clinical malaria adapted by Ghana Health Service, 46 (26.7%) of the children presented with uncomplicated malaria, 46 (26.7%) had cerebral malaria and 23 (13.4%) reported with severe malaria anaemia. In addition, 57 (33.1%) uninfected children within the same age brackets were also recruited as controls. Among the study participants, 99 (57.6%) were males and 73 (42.4%) were females and the mean age of the study participants was 5.2 years old as shown in Table 1.

Table 1: General characteristics of study participants

Malaria phenotype	**Study participants**	**Mean age**	**Sex**	
			Male	Female
UM	46 (26.7%)	5.902	28	18
CM	46 (26.7%)	4.924	32	14
SMA	23 (13.4%)	3.060	11	12
HC	57 (33.1%)	6.546	28	29
Total	172 (100%)			
P value; (F); [χ^2]		< 0.0001; (8.294)	0.140, [5.437]	

Values in curve brackets () is the F-statistics for an F-test. Values in square brackets [] is the chi-squared test.

4.2 Parasitological indices among malaria phenotypes

The parasitological indices among the various clinical malaria phenotypes are displayed in Table 2. These parasitological indices include Parasite density/ µl (P Density), Total parasite biomass (Ptot), circulating parasite biomass (Pcirc) and sequestered parasite biomass (Pseq). These parasitological indices varied with respect to disease severity. Median values for parasite burden was significant among the various malaria phenotypes ($p < 0.0001$). Using a Bonferroni post hoc comparison test, the difference among the various malaria phenotypes were observed between UM/CM and UM/SMA ($p < 0.0001$ and $p = 0.001$ respectively). It was observed that the difference between CM/SMA was not significance ($p = 1.000$). Conversely, the median parasite density was not statistically difference among the various malaria phenotypes ($p = 0.188$). Sequestered parasite differed significantly among the various phenotypes and the difference was observed in only UM/CM. Also there was no significant difference among the circulating parasite of the various malaria phenotypes ($p = 0.059$).

Table 2: Comparison of parasitological indices among malaria phenotypes

Variable	Malaria Phenotype			Unadjusted P values	Bonferroni adjusted P values		
	UM	CM	SMA		UM/CM	UM/SMA	CM/SMA
P Density	22190.000	1880.000	22200.000	0.188	-	-	-
Ptot (X 10^8)	345.100	10075.000	9021.571	< 0.0001	<0.0001	0.001	1.000
Pseq (X 10^8)	345.100	10075.000	9028.000	< 0.0001	<0.0001	0.595	0.056
Pcirc	150803.670	7499.752	11834.873	0.059	-	-	-

P Density = parasite density Ptot = Total parasite biomass Pseq= Total sequestered parasite biomass Pcirc = Total circulating parasite biomass

4.3 Estimation of CD4 and CD8 in whole blood

Circulating CD4 and CD8 cells were evaluated by surface staining with receptor-specific fluorescent-labelled antibodies and were expressed as a percentage of total number of cells acquired. Estimation of these cells were done by an initial gating of the major WBCs populations, namely lymphocytes, monocytes and granulocytes. Out of these CD 4 and CD8 positive cells were quantified as shown in Q1 and Q2 in Figure 5.

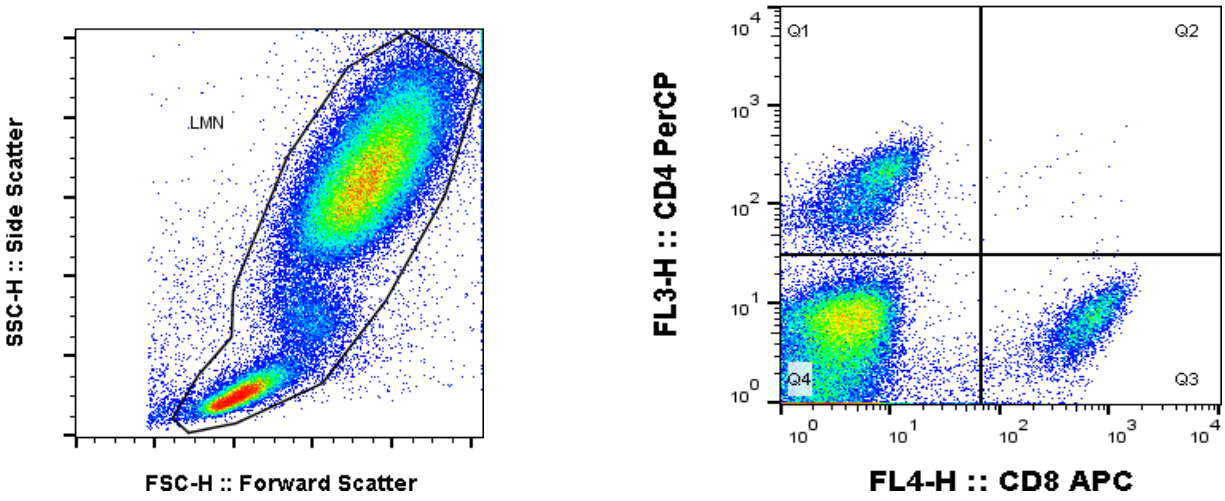

Figure 5: Enumeration of CD4 and CD8 cells

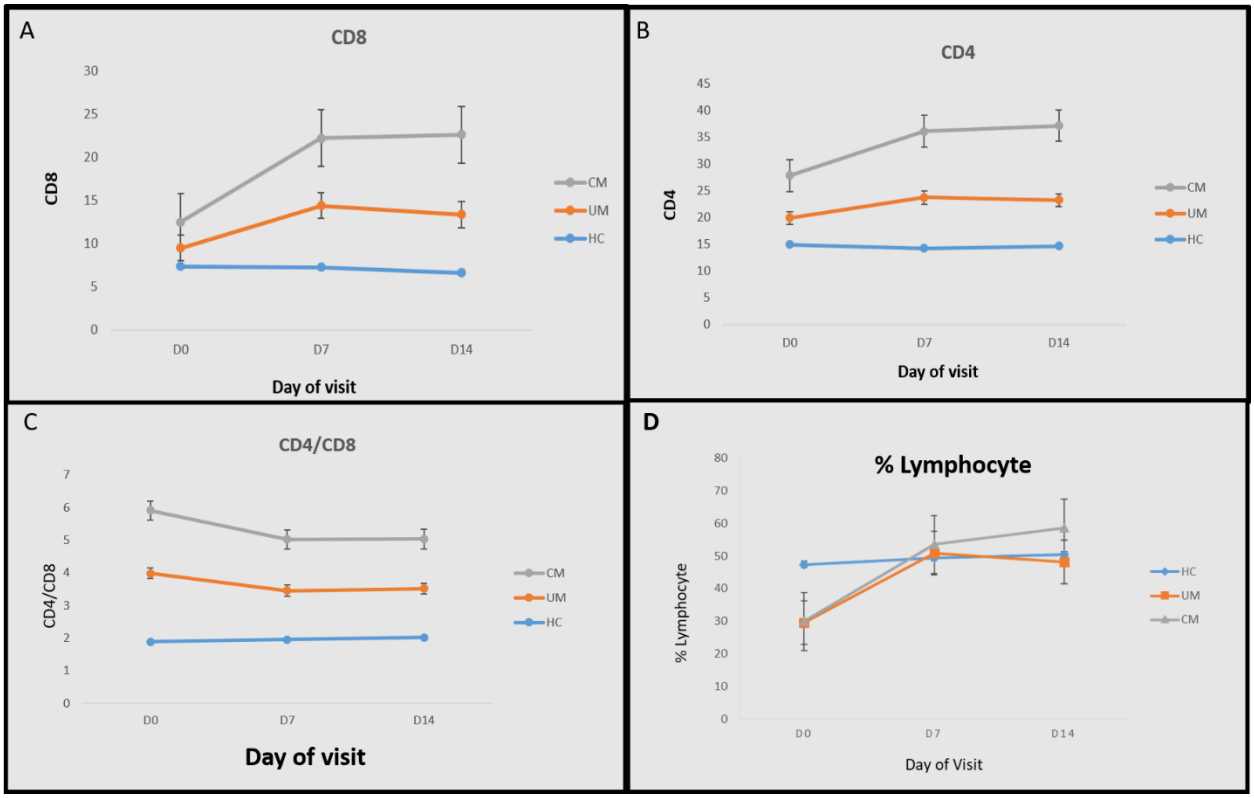

Figure 6: Mean/Median (95% CI) CD4, CD8, CD4/8 and lymphocyte among the various phenotypes at different time points

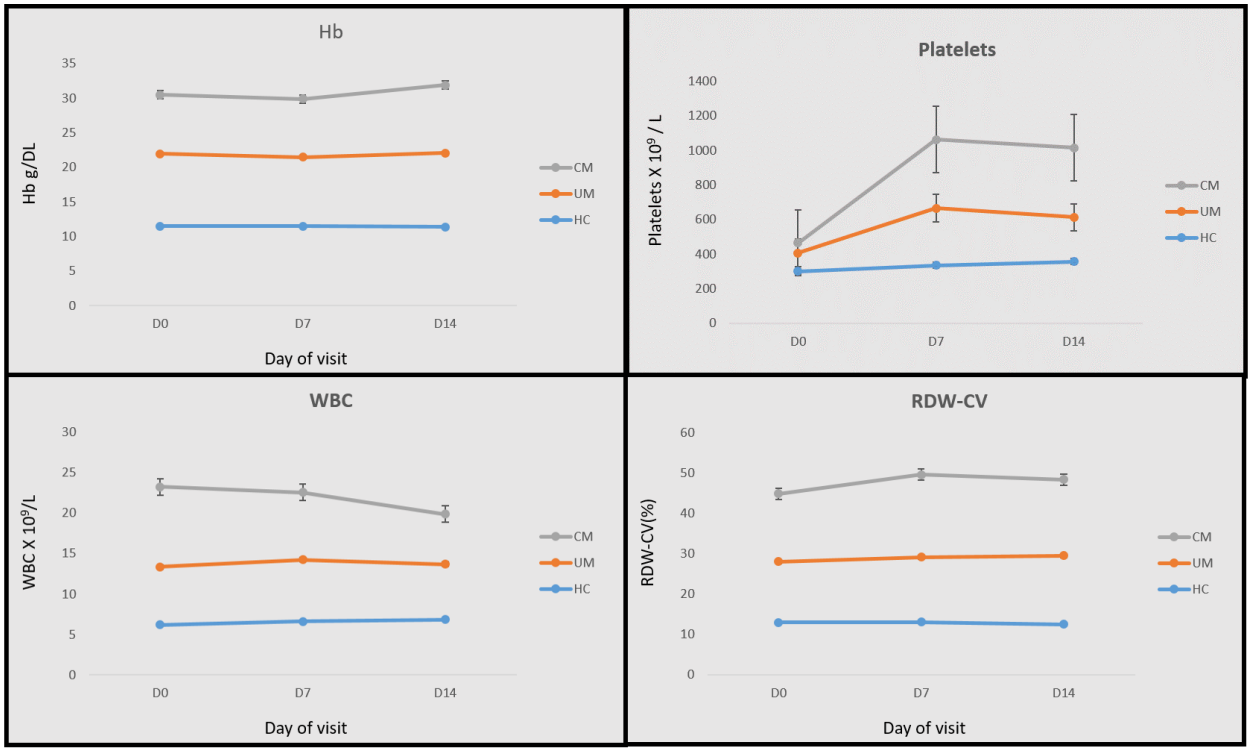

Figure 7: Mean/Median (95% CI) Hb, PLT, WBC and RDW-CV among the various phenotypes at different time points

4.4 Reliability of haematological indices

Reliability of haematological indices to predict the chances of malaria infection was tested on all the haematological indices using a Receiver Operating Characteristic (ROC) curve. Out of these, only five of the indices were found to be promising markers with an area under the ROC curve above 0.8 (80%) as shown in Table 3 and Figure 8 below.

Table 3: The reliability of haematological indices in predicting malaria

Variables	AUC	P-value	95% CI	Sen	Spec	Positive LR	Negative LR	Cut-off
PLT	0.939	0.001	0.900-0.901	93.9	80.7	4.865	0.076	268
HCT	0.920	0.001	0.878-0.962	86.8	80.7	4.497	0.164	32.750
RDW-CV	0.860	0.001	0.793-0.926	76.7	80.7	3.974	0.289	14.450
Hb	0.843	0.001	0.785-0.901	74.8	80.7	3.876	0.312	10.650
RBC	0.823	0.001	0.762-0.884	67.9	82.5	3.880	0.823	4.150

AUC = Area under the curve Sen = Sensitivity Spec = Specificity LR = Likelihood Ratio

4.5 Abnormal laboratory presentation in study participants

Table 4 below is a presentation of abnormal and normal laboratory finding among the various malaria phenotypes and healthy control group. Thrombocytopenia was found to be the commonest abnormal laboratory finding among CM cases (84.8%) and also UM cases (52.2%). The most common abnormal laboratory finding among HC group hypochromasia (26.3%).

Table 4: Abnormal laboratory finding in study participants

Variable		Malaria Phenotype				X^2	P-value
		HC	UM	CM	SMA		
HB	Normal	(56)98.2%	(40)87.0%	(21)45.7%	(0)0.0%	91.027	<0.0001
	Anaemic	(1)1.8%	(6)13.0%	(25)54.3%	(23)100%		
MCV	Normal	(56)98.2%	(38)82.6%	(39)84.8%	(23)100%	11.683	0.009
	Microcytosis	(1)6.2%	(8)17.4%	(7)15.2%	(0)100%		
MCHC	Normal	(42)73.7%	(43)95.6%	(42)91.3%	(22)95.7%	14.247	0.003
	Hypochromasia	(15)26.3%	(2)4.4%	(2)8.7%	(1)4.3%		
WBC	Normal	(53)93.0%	(39)84.4%	(37)82.2%	(11)47.8%	24.516	<0.0001
	Leukopenia	(2)3.5%	(3)6.5%	(2)4.4%	(3)13.0%		
	Leucocytosis	(2)3.5%	(4)8.7%	(6)13.3%	(9)39.31%		
PLT	Normal	(57)100.0%	(22)47.8%	(7)15.2%	(13)56.5%	77.582	<0.0001
	Thrombocytopenia	(0)0.0%	(24)52.2%	(39)84.8%	(10)43.5%		

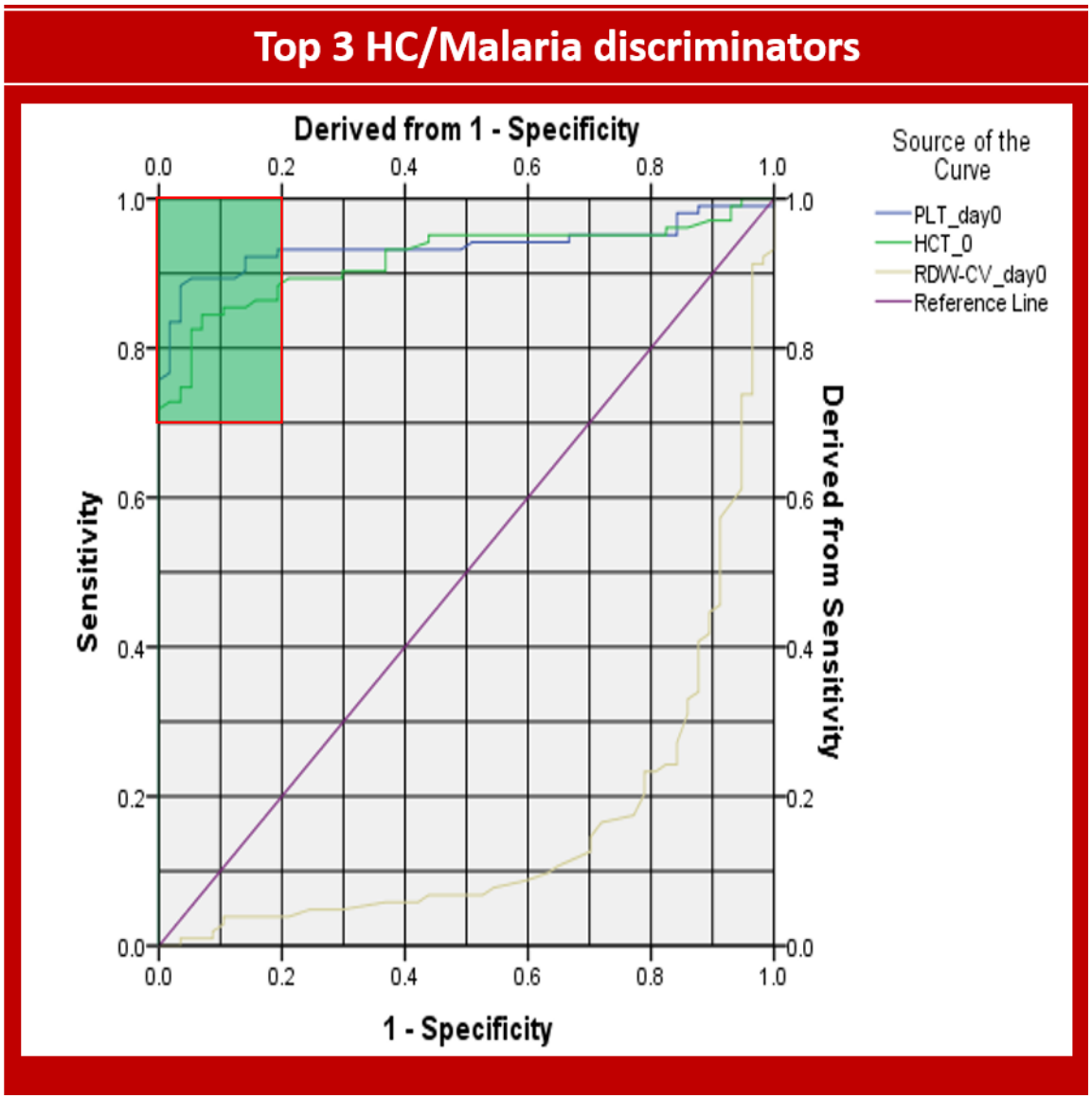

Figure 8: Receiver operating characteristic (ROC) curve and the associated reliability of haematological indices in predicting malaria infection in Accra metropolis, Ghana

4.6: Constructing of an Area-based Malariometric Index: Principal Composite Analysis (PCA) Approach (Appendix 3 A- D; Figure 9)

A composite index is an area-based method that is widely used in the field of social sciences. This index is derived from the PCA. Data on 18 variables measuring multiple aspects of haematological and parasitological indices (e.g., WBC, Hb and total parasite biomass) were utilized to extract underlying constructs. Several statistical tests (e.g., KMO, Bartlett's Test of Sphericity) were used to assess the appropriateness of using the PCA. Five factors were discovered which together explained 81 per cent of the total variation (Appendix 3). Factor scores were utilized to derive composite standardized indices and quintiles. As shown in Figure 10, ultimately the composite index was able to distinguish SMA from CM and SMA from UM but not CM and UM.

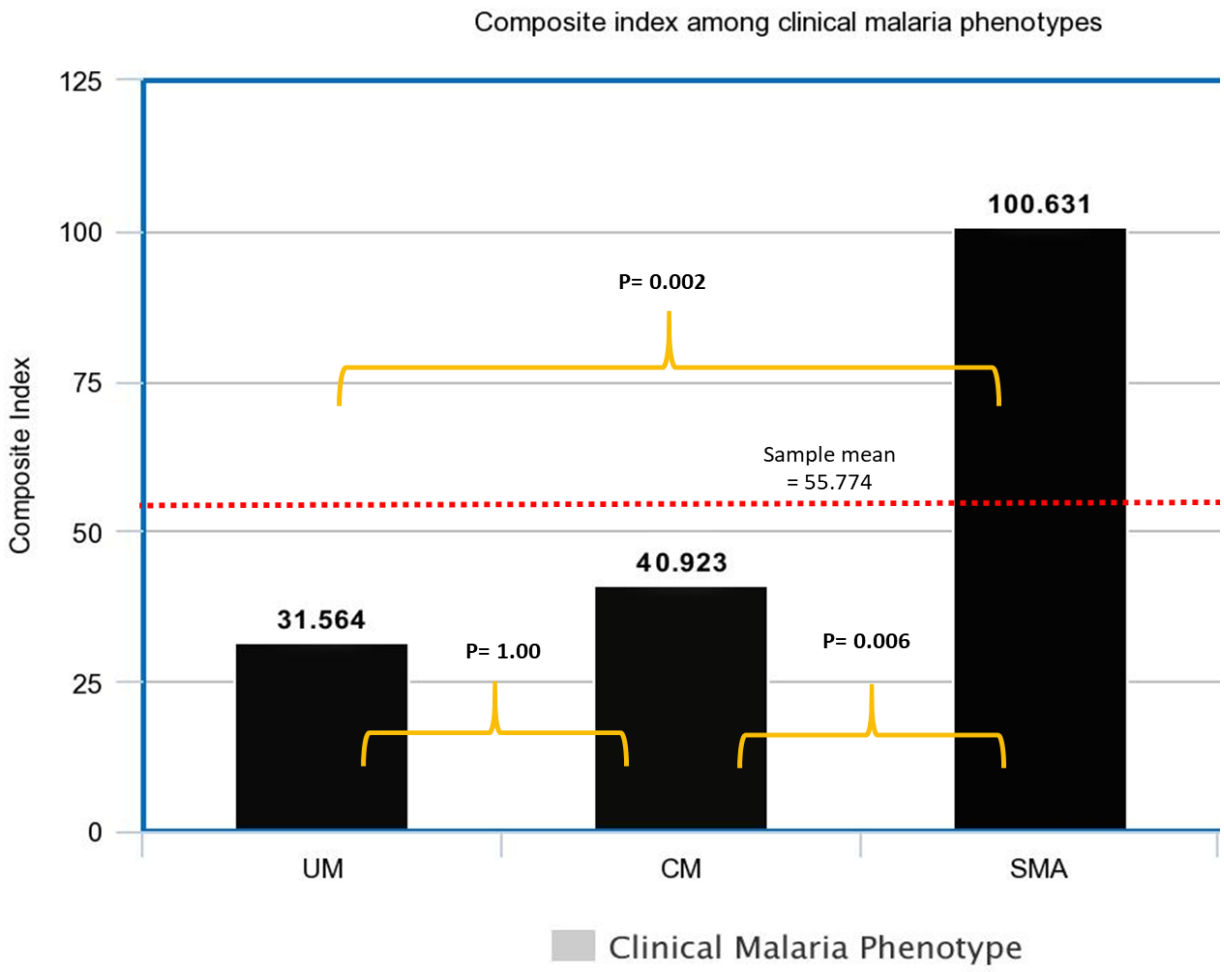

Figure 9: Composite index among clinical malaria phenotypes

CHAPTER FIVE

DISCUSSION

The data reported in this thesis investigated the diagnostic and prognostic markers for cerebral malaria from the perspective of parasite biomass and composite malariometric index. Such information equips one with the ability to predict which child is likely to develop cerebral malaria or die from cerebral malaria. This therefore has implication for disease management and malaria control. Limitations of the current study had to do with the immanent dependency of models based on assumptions; the use of concentrations of soluble PfHRP2 in estimating the total parasite biomass is directly related to the assumed parasite multiplication rate and this could differ in different transmission settings. In addition, the anticipated half-life of plasma PfHRP2 may differ between patients. Also different parasite strains may have different PfHRP2 half-life and the amount of the protein produced per parasite cycle may differ.

Although the pathophysiology of CM is still not fully understood, current knowledge suggests that the disease results from a combination of vascular and inflammatory immune system dysfunction as well as microparticle formation and homeostasis disruption (Souza *et al.*, 2015; Wah *et al.*, 2016). This occurs as a result of damage to the microvasculature caused by sequestration of parasitized red blood cells (pRBCs), vascular occlusion or a cytokine-mediated sepsis-like syndrome which results in alterations in the blood brain barrier (BBB) (Bland & Altman, 1995), but is not well elucidated if the same mechanisms underlie different severe malaria syndromes. In their study, Hendriksen *et al.* (2012) observed a “U-shaped” association between soluble PfHRP2 and the risk of death with PfHRP2 being a strong independent predictor

of death. Their findings contribute to the assumptions that, sequestration of pRBCs in the brain and other organs is the hallmark for nearly all the severe forms of malaria pathogenesis.

The present study was carried out to assess the role of the sequestration of pRBCs by estimating the total parasite burden using a mathematical modelling proposed by Dondrop *et al*. (2005) and a parasite-derived protein, PfHRP2 that is released into the peripheral blood after the rapture of matured trophozoite and schizont in acute falciparum malaria. If sequestration of pRBCs is the hallmark for the severe forms of the disease then we expect the estimated total parasite burden in CM cases to be higher than in SMA and UM cases. The estimated total parasite burden showed a strong prognostic value and it was strongly associated with malaria severity. In contrast, the estimated peripheral blood parasitaemia and the estimated total circulating parasites counts were not associated with malaria severity and could not discriminate among the various malaria phenotypes. Both the estimated peripheral blood parasitaemia and the derived circulating parasites showed a higher parasitaemia counts in UM cases and SMA cases than CM cases. This finding support the argument that there is a higher chances of sequestration of parasitized erythrocytes in CM compared to the other malaria phenotypes. It also implies that only the less pathogenic and immature trophozoites and schizont together with the ring stages of the asexual stages of the parasites are accessible in blood circulation to be measured by microscopy.

The more pathogenic and mature forms of the parasites which are the underlying factors for the impairment of the microvasculature, are scarcely seen in peripheral blood circulation. These pathogenic and mature forms of the parasite get sequestered in the deep vessels and are rarely accessible for peripheral blood parasitaemia estimation. In this study, the estimated sequestered

parasite biomass varied with malaria severity and this demonstrates the critical pathological role played by parasitized erythrocytes in the pathogenesis of severe malaria. These findings are supported by a quantitative analysis of microvascular sequestration of malaria parasites in the human brain studies which demonstrated a higher degree of sequestration in the deep vessels of vital organs (Silamut & White, 1993). In contrast, the use of conventional circulating peripheral blood parasite counts (parasite density) failed to discriminate among the various malaria phenotypes, and the median parasite count was higher in SMA cases than UM and CM. Surprisingly, the median parasite count was lower in CM cases as compared to SMA and UM, with UM parasite counts being lower than SMA cases. The differences among the various malaria phenotypes were not significant and this supports the assumption that, conventional peripheral blood parasite count is a weak prognostic maker to discriminate among the various malaria phenotypes since it cannot account for the matured sequestered parasites. In addition, the median circulating parasite counts in UM cases were higher than SMA and CM cases. This suggests that only the less pathogenic parasites comes into blood circulation to be measured by microscopy. As a result, the use of clinical malaria diagnosis still remains a challenge, especially in high endemic malaria transmission settings. This is mostly the result of the unspecific nature of clinical signs and symptoms which greatly overlaps with other febrile illness prevalent in those endemic malaria areas.

Although microscopic demonstration of parasitaemia is relatively cheaper and simple, it shows poor reproducibility and also requires skilled labour especially in resource poor-countries (Verma *et al.*, 2013). Alterations in haematological indices are considered as potential markers to diagnose clinical malaria especially in areas where microscopy expertise is unavailable. These

changes mostly have a direct effect on haemopoetic physiology and usually affect the host homeostasis with varying clinical manifestations (Maina *et al.,* 2010). Prompt and accurate diagnosis of malaria infected patients will prevent the indiscriminate use of drugs, and also prevent mild malaria infection from progressing to severe and fatal forms. Individuals infected with malaria usually have significantly lower levels of platelets, lymphocytes, eosinophils, WBCs, RBCs, and Hb (Adedapo *et al.*, 2007; Erhart *et al.*, 2004; Gérardin *et al.*, 2002). In contrast, Ladhani *et al* (2002) reported of higher WBCs in malaria infected individuals. The present study found lower levels of Hb, HCT, MCV, PLT, RBCs, and LYM and MXD at baseline compared to healthy controls. White blood cell counts were found to be higher in the malaria infected group compared to the non-malaria group. Also, higher levels of neutrophils, RDW-SD, RDW-CV, MCHC and PDW were observed in the malaria group compared to the uninfected group in this study. In addition, the present study also observed anaemia, microcytosis, hypochromasia, leucopenia, leucocytosis and thrombocytopenia among the study participants. The most pronounced ones were thrombocytopenia, leucopenia, leucocytosis and anaemia.

Thrombocytopenia is one of the commonest indicators of malaria in febrile patients and is more pronounce in cases of severe malaria (Erhart *et al.*, 2004; Eriksson *et al.*, 1989; Marsh *et al.*, 1995). In this study, individuals with thrombocytopenia were found to be more pronounced in CM (84.8%) as compared to UM (52.2%) and SMA (43.5%). This shows that thrombocytopenia is very common in CM cases as compared to other malaria phenotypes. This is consistent with studies that have reported more than 50% of cases having thrombocytopenia. A study by Ladhani *et al* (2002) reported 56.7% of hospital admissions to be due to cerebral malaria having

thrombocytopenia. Another study also recorded a little above 60% of admitted cerebral malaria cases having thrombocytopenia (D'Acremont *et al.*, 2002). A study by Amaral *et al* (2003) also reported thrombocytopenia as the commonest blood cell abnormalities among cerebral malaria. The pathogenesis of thrombocytopenia in malaria is mostly as a result of several factors. Studies from both clinical and animal experimental data have successively implicated the role of sequestration of platelets, destruction or sequestration of parasitized erythrocytes and immune factors as the underlying factors of thrombocytopenia (Moulin *et al.*, 2003). Other studies have also reported the association of higher number of sequestered platelets and erythrocytes in severe forms of malaria than other malaria phenotypes (Pain *et al.*, 2001). This study has also demonstrated the role of sequestered erythrocytes in the pathogenesis of cerebral malaria and this could account for the higher proportion of thrombocytopenia in cerebral malaria as compared to the other malaria phenotypes. None of the healthy control participants had thrombocytopenia and 15.2% of the cerebral malaria cases had normal platelets counts. Also 47.8% of uncomplicated malaria cases and 56.5% of severe malaria anaemia cases had normal platelet counts. Overtime, the median platelets counts increased in both UM and CM cases after treatment. The difference among the malaria phenotypes appeared to be higher in CM than UM patients at day 7 and even much higher at day 14. There was no significant increase in platelet counts among the healthy controls during the time course. Platelet counts decreased at day 14 but the difference was not significant, suggesting that malaria infected patients may require more than three weeks for full platelets recovery. This is consistent with studies that reported more than four weeks for full platelets recovery (Taylor *et al.*, 2008). This study has therefore confirmed the prominence of thrombocytopenia in cerebral malaria as compared to other malaria phenotypes and might be considered as a marker of malaria severity.

The proportion of WBC counts at initial presentation of malaria infection could be high, normal or low. Studies have reported lower WBC counts in acute malaria infection among adult populations (Erhart *et al.*, 2004; McKenzie *et al.*, 2005). Results from this study was in contrast with previous findings that WBC counts at initial presentation of acute malaria infection were lower than healthy controls but leucopenia was found to be less profound in cerebral malaria (Erhart *et al.*, 2004; McKenzie *et al.*, 2005). This difference could be due to the state of host immunity, degree of parasitaemia, acuteness of malarial infection and malarial severity (Abdalla & Pasvol, 2004). Leucopenia was found to be lower in CM casas (4.4%) as compared to cases of UM (6.5%) and SMA cases (13.0%). About 82.2% of CM patients have normal WBC counts with 13.3% having leucocytosis and 4.4% having leucopenia. Results from this study were similar with the study done by Khan and Malik (1996), which reported a higher proportion of CM cases having normal leukocytes. In addition, changes in WBC counts were similar in both malaria phenotypes. Although, patients with cerebral malaria are less likely to develop leucocytosis, however our findings showed a high WBC counts in children with cerebral malaria. This finding was in line with previous studies that had reported higher WBC counts in severe malaria compared to other malaria phenotypes (Ladhani *et al.*, 2002; Modiano *et al.*, 2001). Compared to the time of disease presentation, the median WBC counts declined at day 7 in both malaria phenotypes but there was no significant difference in the median WBC counts among the healthy controls during the time course. White blood cell counts decreased at day 14 but the difference was not significant, suggesting that malaria infected patients will require more than two weeks to attain normal WBC distribution.

Anaemia in malaria has a multifactorial and complex pathogenesis which mostly result from a combination of factors such as haemolysis of parasitized erythrocytes, clearance of both parasitized and non-parasitized erythrocytes, dysfunctional erythropoiesis and splenic phagocytosis. Studies have also considered anaemia in malaria as a measure of the cumulative impact of malaria on patients (Kotepui *et al.*, 2015). All the SMA cases in this study were anaemic, 54% of the CM cases were also anaemic. Thirteen per cent of UM cases and 1.8% of the HC group were found to be anaemic. Although haematological alterations affect the profile of circulating cells in peripheral circulation, these findings may vary among geographical locations depending on the nutritional status, malaria endemicity, immunity, genetic factors, ethnicity and sociodemographic factors of study participants (Hill *et al.*, 1991).

Lymphocytes, especially T cells are known to play a key role in immunity to malarial infection by activating other inflammatory cells and are also involved in the release proinflammatory of cytokines, such as interferon-gamma and tumour necrosis factor-alpha (Ladhani *et al.*, 2002). Nonetheless, immoderate secretion of these proinflammatory cytokines has been reported to contribute to malarial severity in humans (Biemba *et al.*, 2000; Ho *et al.*, 1998). This study observed a higher lymphocyte counts at day 7 among all the malaria phenotypes compared to the healthy controls. By day 14, lymphocyte counts in children with uncomplicated malaria declined but lymphocyte counts in children with cerebral malaria kept increasing. Excessive stimulation of proinflammatory and other cytokines with deleterious repercussions could accounts for this abnormalities.

The median CD4 and CD8 counts rose steadily from the time of disease presentation among the various malaria phenotypes till day 7. By day 14, the difference between CD4 and CD8 counts were deem insignificant between UM and CM patients and did not return to normal at the end of

the study. This finding supports the argument that both CD4 and CD8 cells confer protective immunity against the asexual blood stage infections (Chandele *et al.*, 2011). In addition, there was no correlation between CD4 and CD8 counts and the total parasite biomass.

From all the haematological indices obtained, only five of them (PLT, HCT, RDW-CV, Hb and RBCs) were found to be reliable markers to predict malaria infections with area under the ROC curve above 0.8 (80%). The three most promising ones were PLT, HCT and RDW-CV. It was observed that PLT, HCT and RDW-CV with cut-off point below 268, 32.750 and 14.450 respectively were the main predictors of malaria infections among children in the Accra metropolis. Platelet counts showed a sensitivity and specificity of 93.9% and 80.7% respectively. Haematocrit level assessment revealed a sensitivity and specificity of 86.7% and 80.7% respectively. Also RDW-CV showed a sensitivity and specificity of 76.7% and 80.7% respectively. However, other studies have reported PDW $\geq$ 14.55% and MPV $\geq$ 9.05 fl as the reliable markers for predicting malaria infections with area under the ROC curve as 0.6 and 0.7 respectively (Ali *et al.*, 2017). Their finding was inconsistent with the principle guiding ROC curve interpretation. An ROC curve with an area under the curve above 0.8 are considered as good test with favourable sensitivity and specificity characteristics (Ali *et al.*, 2017). Also area under ROC curve below 0.7 and 0.6 are considered fair but a poor test (Ali *et al.*, 2017). In addition PDW under the previous study showed a sensitivity and specificity of 72.8% and 56.8% respectively. Sensitivity and specificity for MPV was reported as 77.2% and 60.1% respectively. A lower MPV > 8.0 has also been reported by Chandra *et al* with sensitivity and specificity of 70.8% and 50.4% respectively (Chandra & Chandra, 2013).

To the best of my knowledge, for the first time in malariometric analysis, the study employed the use of a Principal Component Analysis (PCA) approach in computing a composite malariometric index among clinical malaria phenotypes. The composite mean for UM, CM and SMA were 31.564, 40.923 and 100.631 respectively, while overall sample mean was 55.774. Also, the composite index was deem meaningful and was able to discriminate SMA cases from UM cases ($p=0.002$). With respect to CM and SMA cases, the composite index was able to discriminate between these disease phenotypes ($p=0.006$). Although there was differences between UM and SMA cases, the composite index was unsuccessful in discriminating between these disease phenotypes ($p=1.00$).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study has shown that the estimated total parasite biomass from soluble PfHRP2 correlated with malarial severity among different clinical malaria phenotypes and provides a tool to delineate severe *falciparum* malaria from other forms of clinical malaria and other acute febrile illness with incidental malaria parasitaemia in moderate and high malaria endemic areas. In addition, thrombocytopenia was found to be more frequent in cerebral malaria cases than other forms of clinical malaria phenotypes. The study also revealed that a PLT, HCT and RDW-CV with cut-off point 268, 32.750 and 14.450 respectively were the most reliable haematological indices to predict *falciparum* malaria infection.

6.2 RECOMMENDATION

Further studies should estimate the parasite multiplication rate and half-life of soluble PfHRP2 within the study locality before incorporation into the model to reduce the immanent dependency of the model.

REFERENCES

Abdalla, S. H., & Pasvol, G. (2004). *Malaria: A hematological perspective* (Vol. 4): World Scientific.

Abeku, T., De Vlas, S., Borsboom, G., Tadege, A., Gebreyesus, Y., Gebreyohannes, H., Alamirew, D., Seifu, A., Nagelkerke, N., & Habbema, J. (2004). Effects of meteorological factors on epidemic malaria in Ethiopia: a statistical modelling approach based on theoretical reasoning. *Parasitology, 128*(06), 585-593.

Adedapo, A. D., Falade, C. O., Kotila, R. T., & Ademowo, G. O. (2007). Age as a risk factor for thrombocytopenia and anaemia in children treated for acute uncomplicated falciparum malaria. *Journal of Vector Borne Diseases, 44*(4), 266.

Afari, E., Appawu, M., Dunyo, S., Baffoe-Wilmot, A., & Nkrumah, F. (1995). Malaria infection, morbidity and transmission in two ecological zones Southern Ghana. *African Journal of Health Sciences, 2*(2), 312-315.

Aguilar, R., Moraleda, C., Achtman, A. H., Mayor, A., Quintó, L., Cisteró, P., Nhabomba, A., Macete, E., Schofield, L., & Alonso, P. L. (2014). Severity of anaemia is associated with bone marrow haemozoin in children exposed to Plasmodium falciparum. *British Journal of Haematology, 164*(6), 877-887.

Aikawa, M., Iseki, M., Barnwell, J. W., Taylor, D., Oo, M. M., & Howard, R. J. (1990). The pathology of human cerebral malaria. *The American Journal of Tropical Medicine and Hygiene, 43*(2_Part_2), 30-37.

Akanmori, B., Kurtzhals, J., Goka, B., Adabayeri, V., Ofori, M., Nkrumah, F., Behr, C., & Hviid, L. (2000). Distinct patterns of cytokine regulation in discrete clinical forms of Plasmodium falciparum malaria. *European Cytokine Network, 11*(1), 113-118.

Ali, E. A., Abdalla, T. M., & Adam, I. (2017). Platelet distribution width, mean platelet volume and haematological parameters in patients with uncomplicated plasmodium falciparum and P. vivax malaria. *F1000Research, 6*.

Anglogold. (2009).

Anstey, N. M., Douglas, N. M., Poespoprodjo, J. R., & Price, R. N. (2011). Plasmodium vivax: clinical spectrum, risk factors and pathogenesis. *Advances in Parasitology, 80*, 151-201.

Asante, F. A., & Asenso-Okyere, K. (2003). Economic burden of malaria in Ghana. *World Health Organization (WHO)*, 1-81.

Asenso-Okyere, W. K., & Dzator, J. A. (1997). Household cost of seeking malaria care. A retrospective study of two districts in Ghana. *Social Science & Medicine, 45*(5), 659-667.

Awah, N., Balogun, H., Achidi, E., Mariuba, L., Nogueira, P. A., Orlandi, P., TROYE-BLOMBERG, M., Gysin, J., & Berzins, K. (2011). Antibodies to the Plasmodium falciparum rhoptry protein RAP-2/RSP-2 in relation to anaemia in Cameroonian children. *Parasite Immunology, 33*(2), 104-115.

Awah, N. W., Troye-Blomberg, M., Berzins, K., & Gysin, J. (2009). Mechanisms of malarial anaemia: potential involvement of the Plasmodium falciparum low molecular weight rhoptry-associated proteins. *Acta Tropica, 112*(3), 295-302.

Baird, J. K. (1995). Host age as a determinant of naturally acquired immunity to Plasmodium falciparum. *Parasitology Today, 11*(3), 105-111.

Beadle, C., Long, G. W., McElroy, P., Hoffman, S., Weiss, W., Maret, S., & Oloo, A. (1994). Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. *The Lancet, 343*(8897), 564-568.

Bell, D., Wongsrichanalai, C., & Barnwell, J. W. (2006). Ensuring quality and access for malaria diagnosis: how can it be achieved? *Nature Reviews Microbiology, 4*(9), 682-695.

Belnoue, E., Kayibanda, M., Vigario, A. M., Deschemin, J.-C., van Rooijen, N., Viguier, M., Snounou, G., & Rénia, L. (2002). On the pathogenic role of brain-sequestered αβ CD8+ T cells in experimental cerebral malaria. *The Journal of Immunology, 169*(11), 6369-6375.

Biemba, G., Gordeuk, V. R., Thuma, P., & Weiss, G. (2000). Markers of inflammation in children with severe malarial anaemia. *Tropical Medicine & International Health, 5*(4), 256-262.

Birbeck, G. L., Molyneux, M. E., Kaplan, P. W., Seydel, K. B., Chimalizeni, Y. F., Kawaza, K., & Taylor, T. E. (2010). Blantyre Malaria Project Epilepsy Study (BMPES) of neurological outcomes in retinopathy-positive paediatric cerebral malaria survivors: a prospective cohort study. *The Lancet Neurology, 9*(12), 1173-1181.

Björkman, A. (2002). Malaria associated anaemia, drug resistance and antimalarial combination therapy. *International Journal for Parasitology, 32*(13), 1637-1643.

Bland, J. M., & Altman, D. G. (1995). Calculating correlation coefficients with repeated observations: Part 2—Correlation between subjects. *Bmj, 310*(6980), 633.

Bousema, J. T., Gouagna, L. C., Drakeley, C. J., Meutstege, A. M., Okech, B. A., Akim, I. N., Beier, J. C., Githure, J. I., & Sauerwein, R. W. (2004). Plasmodium falciparum

gametocyte carriage in asymptomatic children in western Kenya. *Malaria Journal, 3*(1), 18.

Breman, J. G., Egan, A., & Keusch, G. T. (2001). The intolerable burden of malaria: a new look at the numbers.

Carter, R., & Mendis, K. N. (2003). Evolutionary and Historical Aspects of the Burden of Malaria. *Clinical Microbiology Reviews, 16*(1), 173.

Carvalho, L. H., Sano, G.-i., Hafalla, J. C., Morrot, A., de Lafaille, M. A. C., & Zavala, F. (2002). IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nature Medicine, 8*(2), 166-170.

Casals-Pascual, C., Kai, O., Cheung, J. O., Williams, S., Lowe, B., Nyanoti, M., Williams, T. N., Maitland, K., Molyneux, M., & Newton, C. R. (2006). Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood, 108*(8), 2569-2577.

Chandele, A., Mukerjee, P., Das, G., Ahmed, R., & Chauhan, V. S. (2011). Phenotypic and functional profiling of malaria-induced CD8 and CD4 T cells during blood-stage infection with Plasmodium yoelii. *Immunology, 132*(2), 273-286.

Chandra, S., & Chandra, H. (2013). Role of haematological parameters as an indicator of acute malarial infection in Uttarakhand state of India. *Mediterranean Journal of Hematology and Infectious Diseases, 5*(1).

Chang, K.-H., & Stevenson, M. M. (2004). Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *International Journal for Parasitology, 34*(13), 1501-1516.

Cheesbrough, M. (1984). Medical laboratory manual for tropical countries vol11-microbiology.

Cibulskis, R. E., Alonso, P., Aponte, J., Aregawi, M., Barrette, A., Bergeron, L., Fergus, C. A., Knox, T., Lynch, M., & Patouillard, E. (2016). Malaria: global progress 2000–2015 and future challenges. *Infectious Diseases of Poverty, 5*(1), 61.

Clark, I. A., & Cowden, W. B. (2003). The pathophysiology of falciparum malaria. *Pharmacology & Therapeutics, 99*(2), 221-260.

Coltel, N., Combes, V., Hunt, N. H., & Grau, G. E. (2004). Cerebral malaria-a neurovascular pathology with many riddles still to be solved. *Current Neurovascular Research, 1*(2), 91-110.

Cox-Singh, J., Davis, T. M., Lee, K.-S., Shamsul, S. S., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J., & Singh, B. (2008). Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. *Clinical Infectious Diseases, 46*(2), 165-171.

Cox, F. E. (2010). History of the discovery of the malaria parasites and their vectors. *Parasites & Vectors, 3*(1), 5.

Crompton, P. D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L. S., Miller, L. H., Barillas-Mury, C., & Pierce, S. K. (2014). Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annual Review of Immunology, 32*, 157-187.

D'Acremont, V., Landry, P., Mueller, I., Pécoud, A., & Genton, B. (2002). Clinical and laboratory predictors of imported malaria in an outpatient setting: an aid to medical decision making in returning travelers with fever. *The American Journal of Tropical Medicine and Hygiene, 66*(5), 481-486.

Day, K. P., & Marsh, K. (1991). Naturally acquired immunity to Plasmodium faldparum. *Immunology Today, 12*(3), A68-A71.

Dery, D. B., Brown, C., Asante, K. P., Adams, M., Dosoo, D., Amenga-Etego, S., Wilson, M., Chandramohan, D., Greenwood, B., & Owusu-Agyei, S. (2010). Patterns and seasonality of malaria transmission in the forest-savannah transitional zones of Ghana. *Malaria Journal, 9*(1), 314.

Desakorn, V., Dondorp, A. M., Silamut, K., Pongtavornpinyo, W., Sahassananda, D., Chotivanich, K., Pitisuttithum, P., Smithyman, A., Day, N. P., & White, N. J. (2005). Stage-dependent production and release of histidine-rich protein 2 by Plasmodium falciparum. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 99*(7), 517-524.

Dondorp, A. M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., Newton, P. N., Pitisuttithum, P., Smithyman, A., & White, N. J. (2005). Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med, 2*(8), e204.

Doolan, D. L., Dobaño, C., & Baird, J. K. (2009). Acquired immunity to malaria. *Clinical Microbiology Reviews, 22*(1), 13-36.

Eichner, M., Diebner, H. H., Molineaux, L., Collins, W. E., Jeffery, G. M., & Dietz, K. (2001). Genesis, sequestration and survival of Plasmodium falciparum gametocytes: parameter estimates from fitting a model to malariatherapy data. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 95*(5), 497-501.

English, M., Punt, J., Mwangi, I., McHugh, K., & Marsh, K. (1996). Clinical overlap between malaria and severe pneumonia in African children in hospital. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 90*(6), 658-662.

Erhart, L. M., Yingyuen, K., Chuanak, N., Buathong, N., Laoboonchai, A., Miller, R. S., Meshnick, S. R., Gasser, R. A., & Wongsrichanalai, C. (2004). Hematologic and clinical indices of malaria in a semi-immune population of western Thailand. *The American Journal of Tropical Medicine and Hygiene, 70*(1), 8-14.

Eriksson, B., Hellgren, U., & Rombo, L. (1989). Changes in erythrocyte sedimentation rate, C-reactive protein and hematological parameters in patients with acute malaria. *Scandinavian Journal of Infectious Diseases, 21*(4), 435-441.

Ewerbeck, H. (2012). *Differential Diagnosis in Pediatrics: A Compendium of Symptoms and Findings*: Springer Science & Business Media.

Facer, C. A. (1994). Hematological aspects of malaria. *Infection and Hematology. Oxford: Butterworth Heinmann Ltd*, 259-294.

FAVRE, N., RYFFEL, B., BORDMANN, G., & RUDIN, W. (1997). The course of Plasmodium chabaudi chabaudi infections in interferon-gamma receptor deficient mice. *Parasite Immunology, 19*(8), 375-383.

Ferguson, H. M., & Read, A. F. (2004). Mosquito appetite for blood is stimulated by Plasmodium chabaudi infections in themselves and their vertebrate hosts. *Malaria Journal, 3*(1), 12.

Fernando, D., De Silva, D., & Wickremasinghe, R. (2003). Short-term impact of an acute attack of malaria on the cognitive performance ofschoolchildren living in a malaria-endemic

area of Sri Lanka. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 97*(6), 633-639.

Ferreira, A., Balla, J., Jeney, V., Balla, G., & Soares, M. P. (2008). A central role for free heme in the pathogenesis of severe malaria: the missing link? *Journal of Molecular Medicine, 86*(10), 1097-1111.

Fox, L. L., Taylor, T. E., Pensulo, P., Liomba, A., Mpakiza, A., Varela, A., Glover, S. J., Reeves, M. J., & Seydel, K. B. (2013). Histidine-rich protein 2 plasma levels predict progression to cerebral malaria in Malawian children with Plasmodium falciparum infection. *Journal of Infectious Diseases, 208*(3), 500-503.

Gal, S., Fidler, C., Turner, S., Lo, Y. D., Roberts, D. J., & Wainscoat, J. S. (2001). Detection of Plasmodium falciparum DNA in plasma. *Annals of the New York Academy of Sciences, 945*(1), 234-238.

Gansane, A., Ouedraogo, I. N., Henry, N. B., Soulama, I., Ouedraogo, E., Yaro, J.-B., Diarra, A., Benjamin, S., Konate, A. T., & Tiono, A. (2013). Variation in haematological parameters in children less than five years of age with asymptomatic Plasmodium infection: implication for malaria field studies. *Memorias do Instituto Oswaldo Cruz, 108*(5), 644-650.

Garcia, J. E., Puentes, A., & Patarroyo, M. E. (2006). Developmental biology of sporozoite-host interactions in Plasmodium falciparum malaria: implications for vaccine design. *Clin Microbiol Rev, 19*(4), 686-707. doi: 10.1128/CMR.00063-05

Garcia, M., Kirimoama, S., Marlborough, D., Leafasia, J., & Rieckmann, K. (1996). Immunochromatographic test for malaria diagnosis. *The Lancet, 347*(9014), 1549.

Gérardin, P., Rogier, C., Amadou, S. K., Jouvencel, P., Brousse, V., & Imbert, P. (2002). Prognostic value of thrombocytopenia in African children with falciparum malaria. *The American Journal of Tropical Medicine and Hygiene, 66*(6), 686-691.

Ghosh, K., & Ghosh, K. (2007). Pathogenesis of anemia in malaria: a concise review. *Parasitology Research, 101*(6), 1463-1469.

GHS. (2014). Ghana Health Service 2014 Annual Report. Ghana.

Good, M. F., & Doolan, D. L. (1999). Immune effector mechanisms in malaria. *Current Opinion in Immunology, 11*(4), 412-419.

Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H., Alonso, P. L., Collins, F. H., & Duffy, P. E. (2008). Malaria: progress, perils, and prospects for eradication. *The Journal of Clinical Investigation, 118*(4), 1266-1276.

Greisenegger, S., Endler, G., Hsieh, K., Tentschert, S., Mannhalter, C., & Lalouschek, W. (2004). Is elevated mean platelet volume associated with a worse outcome in patients with acute ischemic cerebrovascular events? *Stroke, 35*(7), 1688-1691.

Gudo, E. S., Prista, A., & Jani, I. (2012). Immunohematological indices among school children and adolescents living in Manhiça district. *Revista Científica da UEM: Série Ciências Biomédicas e Saúde Pública, 1*, 7-16.

Gudo, E. S., Prista, A., & Jani, I. V. (2013). Impact of asymptomatic Plasmodium falciparum parasitemia on the imunohematological indices among school children and adolescents in a rural area highly endemic for Malaria in southern Mozambique. *BMC Infectious Diseases, 13*(1), 244.

Gupta, N. K., Bansal, S. B., Jain, U. C., & Sahare, K. (2013). Study of thrombocytopenia in patients of malaria. *Tropical Parasitology, 3*(1), 58.

Hamad, A., El Hassan, I., El Khalifa, A., Ahmed, G., Abdelrahim, S., Theander, T., & Arnot, D. (2000). Chronic Plasmodium falciparum infections in an area of low intensity malaria transmission in the Sudan. *Parasitology, 120*(05), 447-456.

Helleberg, M., Goka, B. Q., Akanmori, B. D., Obeng-Adjei, G., Rodriques, O., & Kurtzhals, J. A. (2005). Bone marrow suppression and severe anaemia associated with persistent Plasmodium falciparum infection in African children with microscopically undetectable parasitaemia. *Malaria Journal, 4*(1), 56.

Hendriksen, I. C., Mwanga-Amumpaire, J., Von Seidlein, L., Mtove, G., White, L. J., Olaosebikan, R., Lee, S. J., Tshefu, A. K., Woodrow, C., & Amos, B. (2012). Diagnosing severe falciparum malaria in parasitaemic African children: a prospective evaluation of plasma Pf HRP2 measurement. *PLoS Med, 9*(8), e1001297.

Hill, A. V., Allsopp, C. E., Kwiatkowski, D., Anstey, N. M., Twumasi, P., Rowe, P. A., Bennett, S., Brewster, D., McMichael, A. J., & Greenwood, B. M. (1991). Common West African HLA antigens are associated with protection from severe malaria. *Nature, 352*(6336), 595-600.

Ho, M., Schollaardt, T., Snape, S., Looareesuwan, S., Suntharasamai, P., & White, N. J. (1998). Endogenous interleukin-10 modulates proinflammatory response in Plasmodium falciparum malaria. *Journal of Infectious Diseases, 178*(2), 520-525.

Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., Wellems, T. E., Rener, J., & Taylor, D. W. (1986). Secretion of a malarial histidine-rich protein (Pf HRP II) from Plasmodium falciparum-infected erythrocytes. *The Journal of Cell Biology, 103*(4), 1269-1277.

Hviid, L. (2005). Naturally acquired immunity to Plasmodium falciparum malaria in Africa. *Acta Tropica, 95*(3), 270-275.

Idro, R., Jenkins, N. E., & Newton, C. R. (2005). Pathogenesis, clinical features, and neurological outcome of cerebral malaria. *The Lancet Neurology, 4*(12), 827-840.

Idro, R., Marsh, K., John, C. C., & Newton, C. R. (2010). Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome. *Pediatric Research, 68*(4), 267-274.

Iqbal, J., Khalid, N., & Hira, P. R. (2002). Comparison of two commercial assays with expert microscopy for confirmation of symptomatically diagnosed malaria. *Journal of Clinical Microbiology, 40*(12), 4675-4678.

Iqbal, J., Siddique, A., Jameel, M., & Hira, P. R. (2004). Persistent histidine-rich protein 2, parasite lactate dehydrogenase, and panmalarial antigen reactivity after clearance of Plasmodium falciparum monoinfection. *Journal of Clinical Microbiology, 42*(9), 4237-4241.

Jafarshad, A., Dziegiel, M. H., Lundquist, R., Nielsen, L. K., Singh, S., & Druilhe, P. L. (2007). A novel antibody-dependent cellular cytotoxicity mechanism involved in defense against malaria requires costimulation of monocytes FcγRII and FcγRIII. *The Journal of Immunology, 178*(5), 3099-3106.

Jairajpuri, Z. S., Rana, S., Hassan, M. J., Nabi, F., & Jetley, S. (2014). An analysis of hematological parameters as a diagnostic test for malaria in patients with acute febrile illness: an institutional experience. *Oman Medical Journal, 29*(1), 12.

Jayawardena, A., Murphy, D., Janeway, C. A., & Gershon, R. (1982). T cell-mediated immunity in malaria. I. The Ly phenotype of T cells mediating resistance to Plasmodium yoelii. *The Journal of Immunology, 129*(1), 377-381.

Jones, M. K., & Good, M. F. (2006). Malaria parasites up close. *Nature medicine, 12*(2), 170-172.

Josling, G. A., & Llinas, M. (2015). Sexual development in Plasmodium parasites: knowing when it's time to commit. *Nature reviews. Microbiology, 13*(9), 573.

Kadowaki, N., Antonenko, S., Lau, J. Y.-N., & Liu, Y.-J. (2000). Natural interferon α/β–producing cells link innate and adaptive immunity. *Journal of Experimental Medicine, 192*(2), 219-226.

Kariuki, S. M., Gitau, E., Gwer, S., Karanja, H. K., Chengo, E., Kazungu, M., Urban, B. C., & Newton, C. R. (2013). Value of Plasmodium falciparum histidine-rich protein 2 level and malaria retinopathy in distinguishing cerebral malaria from other acute encephalopathies in Kenyan children. *The Journal of Infectious Diseases, 209*(4), 600-609.

Kariuki, S. M., & Newton, C. R. (2014). Retinopathy, histidine-rich protein-2 and perfusion pressure in cerebral malaria. *Brain, 137*(9), e298-e298.

Kemp, K., Akanmori, B., Adabayeri, V., Goka, B., Kurtzhals, J., Behr, C., & Hviid, L. (2002). Cytokine production and apoptosis among T cells from patients under treatment for Plasmodium falciparum malaria. *Clinical & Experimental Immunology, 127*(1), 151-157.

Khan, S. J., Abbass, Y., & Marwat, M. A. (2012). Thrombocytopenia as an indicator of malaria in adult population. *Malaria Research and Treatment, 2012*.

Kifude, C. M., Rajasekariah, H. G., Sullivan, D. J., Stewart, V. A., Angov, E., Martin, S. K., Diggs, C. L., & Waitumbi, J. N. (2008). Enzyme-linked immunosorbent assay for

detection of Plasmodium falciparum histidine-rich protein 2 in blood, plasma, and serum. *Clinical and Vaccine Immunology, 15*(6), 1012-1018.

Kilejian, A. (1974). A unique histidine-rich polypeptide from the malaria parasite, Plasmodium lophurae. *Journal of Biological Chemistry, 249*(14), 4650-4655.

Kinyanjui, S. M., Mwangi, T., Bull, P. C., Newbold, C. I., & Marsh, K. (2004). Protection against clinical malaria by heterologous immunoglobulin G antibodies against malaria-infected erythrocyte variant surface antigens requires interaction with asymptomatic infections. *Journal of Infectious Diseases, 190*(9), 1527-1533.

Kitchen, S. G., Whitmire, J. K., Jones, N. R., Galic, Z., Kitchen, C. M., Ahmed, R., & Zack, J. A. (2005). The CD4 molecule on CD8+ T lymphocytes directly enhances the immune response to viral and cellular antigens. *Proceedings of the National Academy of Sciences of the United States of America, 102*(10), 3794-3799.

Kotepui, M., Phunphuech, B., Phiwklam, N., Chupeerach, C., & Duangmano, S. (2014). Effect of malarial infection on haematological parameters in population near Thailand-Myanmar border. *Malaria Journal, 13*(1), 218.

Kotepui, M., Piwkham, D., PhunPhuech, B., Phiwklam, N., Chupeerach, C., & Duangmano, S. (2015). Effects of malaria parasite density on blood cell parameters. *PloS one, 10*(3), e0121057.

Krishnan, V. (2010). Constructing an area-based socioeconomic index: A principal components analysis approach. *Edmonton, Alberta: Early Child Development Mapping Project*.

Ladhani, S., Lowe, B., Cole, A. O., Kowuondo, K., & Newton, C. R. (2002). Changes in white blood cells and platelets in children with falciparum malaria: relationship to disease outcome. *British Journal of Haematology, 119*(3), 839-847.

Langhorne, J., Cross, C., Seixas, E., Li, C., & Von Der Weid, T. (1998). A role for B cells in the development of T cell helper function in a malaria infection in mice. *Proceedings of the National Academy of Sciences, 95*(4), 1730-1734.

Langhorne, J., Ndungu, F. M., Sponaas, A.-M., & Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nature Immunology, 9*(7), 725-732.

Leke, R. F., Djokam, R. R., Mbu, R., Leke, R. J., Fogako, J., Megnekou, R., Metenou, S., Sama, G., Zhou, Y., & Cadigan, T. (1999). Detection of the Plasmodium falciparumAntigen Histidine-Rich Protein 2 in Blood of Pregnant Women: Implications for Diagnosing Placental Malaria. *Journal of Clinical Microbiology, 37*(9), 2992-2996.

Liehl, P., Meireles, P., Albuquerque, I. S., Pinkevych, M., Baptista, F., Mota, M. M., Davenport, M. P., & Prudêncio, M. (2015). Innate immunity induced by Plasmodium liver infection inhibits malaria reinfections. *Infection and Immunity, 83*(3), 1172-1180.

Lucchi, N. W., Jain, V., Wilson, N. O., Singh, N., Udhayakumar, V., & Stiles, J. K. (2011). Potential serological biomarkers of cerebral malaria. *Disease Markers, 31*(6), 327-335.

Luse, S. A., & Miller, L. H. (1971). Plasmodium falciparum malaria. *The American Journal of Tropical Medicine and Hygiene, 20*(5), 655-660.

MacPherson, G., Warrell, M., White, N., Looareesuwan, S., & Warrell, D. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *The American Journal of Pathology, 119*(3), 385.

Mahmood, A., & Yasir, M. (2008). Thrombocytopenia: a predictor of malaria among febrile patients in Liberia. *Infect Dis J, 14*, 41-44.

Maina, R. N., Walsh, D., Gaddy, C., Hongo, G., Waitumbi, J., Otieno, L., Jones, D., & Ogutu, B. R. (2010). Impact of Plasmodium falciparum infection on haematological parameters in children living in Western Kenya. *Malaria Journal, 9*(3), S4.

Malhotra, I., Mungai, P., Muchiri, E., Kwiek, J. J., Meshnick, S. R., & King, C. L. (2006). Umbilical cord–blood infections with Plasmodium falciparum malaria are acquired antenatally in Kenya. *Journal of Infectious Diseases, 194*(2), 176-183.

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Warn, P., & Peshu, N. (1995). Indicators of life-threatening malaria in African children. *New England Journal of Medicine, 332*(21), 1399-1404.

Marsh, K., & Howard, R. J. (1986). Antigens induced on erythrocytes by P. falciparum: expression of diverse and conserved determinants. *Science, 231*, 150-154.

Marsh, K., & Kinyanjui, S. (2006). Immune effector mechanisms in malaria. *Parasite Immunology, 28*(1-2), 51-60.

Marsh, K., Marsh, V., Brown, J., Whittle, H., & Greenwood, B. (1988). Plasmodium falciparum: the behavior of clinical isolates in an in vitro model of infected red blood cell sequestration. *Experimental Parasitology, 65*(2), 202-208.

Mathers, C. D., & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med, 3*(11), e442.

Mayxay, M., Pukrittayakamee, S., Chotivanich, K., Looareesuwan, S., & White, N. J. (2001). Persistence of Plasmodium falciparum HRP-2 in successfully treated acute falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 95*(2), 179-182.

McKenzie, F. E., Prudhomme, W. A., Magill, A. J., Forney, J. R., Permpanich, B., Lucas, C., Gasser Jr, R. A., & Wongsrichanalai, C. (2005). White blood cell counts and malaria. *The Journal of Infectious Diseases, 192*(2), 323-330.

Meintker, L., Ringwald, J., Rauh, M., & Krause, S. W. (2013). Comparison of automated differential blood cell counts from Abbott Sapphire, Siemens Advia 120, Beckman Coulter DxH 800, and Sysmex XE-2100 in normal and pathologic samples. *American Journal of Clinical Pathology, 139*(5), 641-650.

Miller, L. H., Baruch, D. I., Marsh, K., & Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature, 415*(6872), 673-679.

Miller, L. H., Chien, S., & Usami, S. (1972). Decreased deformability of Plasmodium coatneyi-inîected red cells and its possible relation to cerebral malaria. *American Journal of Tropical Medicine and Hygiene, 21*(2), 133-137.

Mirsaeidi, M., Peyrani, P., Aliberti, S., Filardo, G., Bordon, J., Blasi, F., & Ramirez, J. A. (2010). Thrombocytopenia and thrombocytosis at time of hospitalization predict mortality in patients with community-acquired pneumonia. *Chest Journal, 137*(2), 416-420.

Mishra, S. K., & Newton, C. R. (2009). Diagnosis and management of the neurological complications of falciparum malaria. *Nature Reviews Neurology, 5*(4), 189-198.

Miyakoda, M., Kimura, D., Yuda, M., Chinzei, Y., Shibata, Y., Honma, K., & Yui, K. (2008). Malaria-specific and nonspecific activation of CD8+ T cells during blood stage of Plasmodium berghei infection. *The Journal of Immunology, 181*(2), 1420-1428.

Modiano, D., Sirima, B. S., Konaté, A., Sanou, I., & Sawadogo, A. (2001). Leucocytosis in severe malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 95*(2), 175-176.

Mogil, R. J., Patton, C. L., & Green, D. (1987). Cellular subsets involved in cell-mediated immunity to murine Plasmodium yoelii 17X malaria. *The Journal of Immunology, 138*(6), 1933-1939.

Moody, A. (2002). Rapid diagnostic tests for malaria parasites. *Clinical Microbiology Reviews, 15*(1), 66-78.

Moulin, F., Lesage, F., Legros, A., Maroga, C., Moussavou, A., Guyon, P., Marc, E., & Gendrel, D. (2003). Thrombocytopenia and Plasmodium falciparum malaria in children with different exposures. *Archives of Disease in Childhood, 88*(6), 540-541.

Murphy, G. S., & Oldfield, E. C. (1996). Falciparum malaria. *Infectious Disease Clinics of North America, 10*(4), 747-775.

Newton, C. R., Hien, T. T., & White, N. (2000). Cerebral malaria. *Journal of Neurology, Neurosurgery & Psychiatry, 69*(4), 433-441.

Newton, C. R., & Krishna, S. (1998). Severe falciparum malaria in children: current understanding of pathophysiology and supportive treatment. *Pharmacology & Therapeutics, 79*(1), 1-53.

Ng, L. C. (2008). Naturally Acquired Human Plasmodium knowlesi Infection, Singapore-Volume 14, Number 5—May 2008-Emerging Infectious Disease Journal-CDC.

Nitcheu, J., Bonduelle, O., Combadiere, C., Tefit, M., Seilhean, D., Mazier, D., & Combadiere, B. (2003). Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate

experimental cerebral malaria pathogenesis. *The Journal of Immunology*, *170*(4), 2221-2228.

Oluyole, K., Ogunlade, M., & Agbeniyi, S. (2011). Socio-economic burden of malaria disease on farm income among cocoa farming households in Nigeria. *American-Eurasian JAgric& Environ Science, 10*, 696-701.

Pain, A., Ferguson, D. J., Kai, O., Urban, B. C., Lowe, B., Marsh, K., & Roberts, D. J. (2001). Platelet-mediated clumping of Plasmodium falciparum-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proceedings of the National Academy of Sciences, 98*(4), 1805-1810.

Parra, M. E., Evans, C. B., & Taylor, D. W. (1991). Identification of Plasmodium falciparum histidine-rich protein 2 in the plasma of humans with malaria. *Journal of Clinical Microbiology, 29*(8), 1629-1634.

Perkins, D. J., Were, T., Davenport, G. C., Kempaiah, P., Hittner, J. B., & Ong'echa, J. M. (2011). Severe malarial anemia: innate immunity and pathogenesis. *Int J Biol Sci, 7*(9), 1427-1442.

Petel, U., Gandhi, G., & Friedman, S. (2004). Thrombocytopenia in plasmodium malaria. *Am J Trop Med Hyg, 59*, 859-865.

Pierrot, C., Adam, E., Hot, D., Lafitte, S., Capron, M., George, J. D., & Khalife, J. (2007). Contribution of T cells and neutrophils in protection of young susceptible rats from fatal experimental malaria. *The Journal of Immunology, 178*(3), 1713-1722.

Pongponratn, E., Riganti, M., Punpoowong, B., & Aikawa, M. (1991). Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. *The American Journal of Tropical Medicine and Hygiene, 44*(2), 168-175.

Pongponratn, E., Turner, G. D., Day, N. P., Phu, N. H., Simpson, J. A., Stepniewska, K., Mai, N. T. H., Viriyavejakul, P., Looareesuwan, S., & Hien, T. T. (2003). An ultrastructural study of the brain in fatal Plasmodium falciparum malaria. *The American journal of Tropical Medicine and Hygiene, 69*(4), 345-359.

Porta, J., Carota, A., Pizzolato, G., Wildi, E., Widmer, M.-C., Margairaz, C., & Grau, G. (1992). Immunopathological changes in human cerebral malaria. *Clinical Neuropathology, 12*(3), 142-146.

Price, R. N., Simpson, J. A., Nosten, F., Luxemburger, C., Hkirjaroen, L., ter Kuile, F., Chongsuphajaisiddhi, T., & White, N. J. (2001). Factors contributing to anemia after uncomplicated falciparum malaria. *The American Journal of Tropical Medicine and Hygiene, 65*(5), 614-622.

Rénia, L., Potter, S. M., Mauduit, M., Rosa, D. S., Kayibanda, M., Deschemin, J.-C., Snounou, G., & Grüner, A. C. (2006). Pathogenic T cells in cerebral malaria. *International Journal for Parasitology, 36*(5), 547-554.

Reyburn, H., Mbatia, R., Drakeley, C., Carneiro, I., Mwakasungula, E., Mwerinde, O., Saganda, K., Shao, J., Kitua, A., & Olomi, R. (2004). Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study. *Bmj, 329*(7476), 1212.

Riganti, M., Pongponratn, E., Tegoshi, T., Looareesuwan, S., Punpoowong, B., & Aikawa, M. (1990). Human cerebral malaria in Thailand: a clinico-pathological correlation. *Immunology letters, 25*(1-3), 199-205.

Robinson, L. J., D'Ombrain, M. C., Stanisic, D. I., Taraika, J., Bernard, N., Richards, J. S., Beeson, J. G., Tavul, L., Michon, P., & Mueller, I. (2009). Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of

clinical Plasmodium falciparum malaria in children from Papua New Guinea. *Infection and Immunity, 77*(7), 3033-3043.

Safeukui, I., Gomez, N. D., Adelani, A. A., Burte, F., Afolabi, N. K., Akondy, R., Velazquez, P., Holder, A., Tewari, R., & Buffet, P. (2015). Malaria induces anemia through CD8+ T cell-dependent parasite clearance and erythrocyte removal in the spleen. *MBio, 6*(1), e02493-02414.

Sahu, P. K., Satpathi, S., Behera, P. K., Mishra, S. K., Mohanty, S., & Wassmer, S. C. (2015). Pathogenesis of cerebral malaria: new diagnostic tools, biomarkers, and therapeutic approaches. *Frontiers in Cellular and Infection Microbiology, 5*.

Schmidt, N. W., Butler, N. S., & Harty, J. T. (2011). Plasmodium–host interactions directly influence the threshold of memory CD8 T cells required for protective immunity. *The Journal of Immunology, 186*(10), 5873-5884.

Shantsila, E., Watson, T., & Lip, G. Y. (2007). Endothelial progenitor cells in cardiovascular disorders. *Journal of the American College of Cardiology, 49*(7), 741-752.

Sherman, I. W. (1998). *Malaria: parasite biology, pathogenesis, and protection*: Zondervan.

Sicuri, E., Vieta, A., Lindner, L., Constenla, D., & Sauboin, C. (2013). The economic costs of malaria in children in three sub-Saharan countries: Ghana, Tanzania and Kenya. *Malaria Journal, 12*(1), 307.

Silamut, K., & White, N. (1993). Relation of the stage of parasite development in the peripheral blood to prognosis in severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 87*(4), 436-443.

Smith, T., Schellenberg, J. A., & Hayes, R. (1994). Attributable fraction estimates and case definitions for malaria in endemic. *Statistics in Medicine, 13*(22), 2345-2358.

Snow, R. W., & Marsh, K. (2002). The consequences of reducing transmission of Plasmodium falciparum in Africa. *Advances in Parasitology, 52,* 235-264.

Souza, M. C., Padua, T. A., & Henriques, M. G. (2015). Endothelial-leukocyte interaction in severe malaria: beyond the brain. *Mediators of Inflammation, 2015*.

Staalsoe, T., & Hviid, L. (1998). The role of variant-specific immunity in asymptomatic malaria infections: maintaining a fine balance. *Parasitology Today, 14*(5), 177-178.

Stevenson, M. M., & Riley, E. M. (2004). Innate immunity to malaria. *Nature Reviews Immunology, 4*(3), 169-180.

Stevenson, M. M., Tam, M. F., Wolf, S. F., & Sher, A. (1995). IL-12-induced protection against blood-stage Plasmodium chabaudi AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. *The Journal of Immunology, 155*(5), 2545-2556.

Storm, J., & Craig, A. G. (2014). Pathogenesis of cerebral malaria—inflammation and cytoadherence. *Frontiers in Cellular and Infection Microbiology, 4*.

Svenson, J. E., MacLean, J. D., Gyorkos, T. W., & Keystone, J. (1995). Imported malaria: clinical presentation and examination of symptomatic travelers. *Archives of Internal Medicine, 155*(8), 861-868.

Ta, T. H., Hisam, S., Lanza, M., Jiram, A. I., Ismail, N., & Rubio, J. M. (2014). First case of a naturally acquired human infection with Plasmodium cynomolgi. *Malaria Journal, 13*(1), 68.

Taylor-Robinson, A. W. (2010). Regulation of immunity to Plasmodium: implications from mouse models for blood stage malaria vaccine design. *Experimental Parasitology, 126*(3), 406-414.

Taylor, Fu, W. J., Carr, R. A., Whitten, R. O., Mueller, J. G., Fosiko, N. G., Lewallen, S., Liomba, N. G., & Molyneux, M. E. (2004a). Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nature Medicine, 10*(2), 143-145.

Taylor, D. W., & Voller, A. (1993). The development and validation of a simple antigen detection ELISA for Plasmodium falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 87*(1), 29-31.

Taylor, T. E., Fu, W. J., Carr, R. A., Whitten, R. O., Mueller, J. G., Fosiko, N. G., Lewallen, S., Liomba, N. G., & Molyneux, M. E. (2004b). Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nature Medicine, 10*(2), 143-145.

Taylor, W. R., Widjaja, H., Basri, H., Ohrt, C., Taufik, T., Tjitra, E., Baso, S., Fryauff, D., Hoffman, S. L., & Richie, T. L. (2008). Changes in the total leukocyte and platelet counts in Papuan and non Papuan adults from northeast Papua infected with acute Plasmodium vivax or uncomplicated Plasmodium falciparum malaria. *Malaria Journal, 7*(1), 259.

Tetteh, H. (2014). *Endothelial Biomarkers and the Pathogenesis of Cerebral Malaria.* University of Ghana.

Trampuz, A., Jereb, M., Muzlovic, I., & Prabhu, R. M. (2003). Clinical review: Severe malaria. *Critical Care, 7*(4), 315.

Tsuji, M., & Zavala, F. (2003). T cells as mediators of protective immunity against liver stages of Plasmodium. *Trends in Parasitology, 19*(2), 88-93.

Turner, G. (1997). Cerebral malaria. *Brain Pathology, 7*(1), 569-582.

Tuteja, R. (2007). Malaria− an overview. *FEBS Journal, 274*(18), 4670-4679.

UNICEF. (2007). Factseet on Malaria in Ghana.

Vampouille, A. A. D., Lafitte, S., Dive, D., Khalife, J., & Pierrot, C. (2010). A role for CD4+ and CD8+ cells and not for CD25+ cells in the control of Plasmodium berghei Anka blood stage parasites in rats. *Parasite, 17*(1), 53-60.

van Wolfswinkel, M. E., Vliegenthart-Jongbloed, K., de Mendonça Melo, M., Wever, P. C., McCall, M. B., Koelewijn, R., van Hellemond, J. J., & van Genderen, P. J. (2013). Predictive value of lymphocytopenia and the neutrophil-lymphocyte count ratio for severe imported malaria. *Malaria Journal, 12*(1), 101.

Verma, P., Biswas, S., Mohan, T., Ali, S., & Rao, D. (2013). Detection of histidine rich protein & lactate dehydrogenase of Plasmodium falciparum in malaria patients by sandwich ELISA using in-house reagents. *Indian Journal of Medical Research, 138*(6), 977.

Vinetz, J., Kumar, S., Good, M., Fowlkes, B., Berzofsky, J., & Miller, L. (1990). Adoptive transfer of CD8+ T cells from immune animals does not transfer immunity to blood stage Plasmodium yoelii malaria. *The Journal of Immunology*, *144*(3), 1069-1074.

von Seidlein, L., Olaosebikan, R., Hendriksen, I. C., Lee, S. J., Adedoyin, O. T., Agbenyega, T., Nguah, S. B., Bojang, K., Deen, J. L., & Evans, J. (2012). Predicting the clinical outcome of severe falciparum malaria in african children: findings from a large randomized trial. *Clinical Infectious Diseases, 54*(8), 1080-1090.

Wah, S. T., Hananantachai, H., Kerdpin, U., Plabplueng, C., Prachayasittikul, V., & Nuchnoi, P. (2016). Molecular basis of human cerebral malaria development. *Tropical Medicine and Health, 44*(1), 33.

Waitumbi, J. N., Opollo, M. O., Muga, R. O., Misore, A. O., & Stoute, J. A. (2000). Red cell surface changes and erythrophagocytosis in children with severe Plasmodium falciparum anemia. *Blood, 95*(4), 1481-1486.

Weiss, W., Sedegah, M., Berzofsky, J., & Hoffman, S. (1993). The role of CD4+ T cells in immunity to malaria sporozoites. *The Journal of Immunology, 151*(5), 2690-2698.

White, N. J., & Ho, M. (1992). The pathophysiology of malaria. *Advances in Parasitology, 31*, 83-173.

WHO. (2013). World Malaria Report 2013. Geneva.

WHO. (2014). World Malaria Report 2014. Geneva.

WHO. (2015). World Malaria Report 2015. Geneva.

WHO. (2016). World Malaria Report 2016. Geneva.

Wickramasinghe, S. N., & Abdalla, S. H. (2000). Blood and bone marrow changes in malaria. *Best Practice & Research Clinical Haematology, 13*(2), 277-299.

Wu, L., van den Hoogen, L. L., Slater, H., Walker, P. G., Ghani, A. C., Drakeley, C. J., & Okell, L. C. (2015). Comparison of diagnostics for the detection of asymptomatic Plasmodium falciparum infections to inform control and elimination strategies. *Nature, 528*(7580), S86-S93.

Yanez, D. M., Manning, D. D., Cooley, A. J., Weidanz, W. P., & Van Der Heyde, H. (1996). Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *The Journal of Immunology, 157*(4), 1620-1624.

Yetişkinlerinde, C. K. F. S. A., & Parametreler, H. (2013). Hematological parameters in severe complicated Plasmodium falciparum malaria among adults in Aden.

Zhou, J., Ludlow, L. E., Hasang, W., Rogerson, S. J., & Jaworowski, A. (2012). Opsonization of malaria-infected erythrocytes activates the inflammasome and enhances inflammatory cytokine secretion by human macrophages. *Malaria Journal, 11*(1), 343.

Zuilkowski, S. S., & Jukes, M. C. (2014). Early childhood malaria prevention and children's patterns of school leaving in the Gambia. *British Journal of Educational Psychology, 84*(3), 483-501.

APPENDICES

Appendix 1A: Consent for children with malaria

Title: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben Gyan, PhD

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

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

"Dear Volunteer,

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

Why this study is planned

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

and compare them to children who have less severe malaria, and to other children who are feeling well. The purpose of the study is to find out what factors they already have in their blood that may make them severely sick when they have malaria. If we can find the answer to this question, we hope to be able to suggest new ways of controlling such severe sicknesses in malaria.

General Information and your part in the study

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

Possible Benefits

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

Possible Risks

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

Withdrawal from study

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

Visits

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

Confidentiality

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

questions about being in the research, but you do not have to answer them. A court of law could order medical records shown to other people, but that is unlikely.

Contacts: If you ever have any questions about the research study or study-related problems, you may contact Dr. Maame Yaa Nyarko at Prince Marie Louise Hospital (Tel: 0244 018888) or Dr.Ben Gyan of the Noguchi Memorial Institute for Medical Research (0244 726016) at any time. For questions about the ethical aspects of this study or your rights as a volunteer, you may contact Dr. Samuel Ayete-Nyampong, Chairman, Institutional Review Board, NMIMR, University of Ghana (021 501178/9) or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 681109)

Your rights as a participant

This research has been reviewed and approved by the NMIMR IRB and Ghana Health Service Ethical Committee. An IRB or Ethical Committee is a committee that reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant you may contact [Dr. Samuel Ayete-Nyampong, Tel 21-501-178/179 or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 681109)

VOLUNTEER AGREEMENT

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

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

Date Signature or Thumbprint of volunteer

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

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

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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" (Tetteh, 2014)

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Date Signature Person who obtained Consent

Appendix 1B: Consent for healthy controls

Title: Circulating endothelial cells and the pathogenesis of malaria

Principal Investigator: Ben Gyan, PhD

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

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

"Dear Volunteer,

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

Why this study is planned

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

feeling well. The purpose of the study is to find out what factors they already have in their blood that may make them severely sick when they have malaria. If we can find the answer to this question, we hope to be able to suggest new ways of controlling such severe sicknesses in malaria.

General Information and your part in the study

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

Possible Benefits

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

Possible Risks

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

Withdrawal from study

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

Visits

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

Confidentiality

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

questions about being in the research, but you do not have to answer them. A court of law could order medical records shown to other people, but that is unlikely.

Contacts: If you ever have any questions about the research study or study-related problems, you may contact Dr. Maame Yaa Nyarko at Prince Marie Louise Hospital (Tel: 0244 018888) or Dr.Ben Gyan of the Noguchi Memorial Institute for Medical Research (0244 726016) at any time. For questions about the ethical aspects of this study or your rights as a volunteer, you may contact Dr. Samuel Ayete-Nyampong, Chairman, Institutional Review Board, NMIMR, University of Ghana (021 501178/9) or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 681109)

Your rights as a participant

This research has been reviewed and approved by the NMIMR IRB and Ghana Health Service Ethical Committee. An IRB or Ethical Committee is a committee that reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant you may contact [Dr. Samuel Ayete-Nyampong, Tel 21-501-178/179 or Chairman of the Ghana Health Service Ethical Committee (Tel. 021 681109)

VOLUNTEER AGREEMENT

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

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

Date Signature or Thumbprint of volunteer

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

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

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

Date Signature or Thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual" (Tetteh, 2014)

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

Date Signature Person who obtained Consent

Appendix 2: Buffers and Reagents

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

I.	Washing buffer	0.05% Tween 20 in PBS.
II.	Blocking buffer	1% BSA plus 0.05% Tween 20 in PBS.
III.	Reagent diluent	1% BSA in PBS.

B. Giemsa Buffer for parasite staining

Na_2HPO_4	1.0g
KH_2PO_4	0.7g
Distilled water	1 litre

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

C. Sickling test Buffer

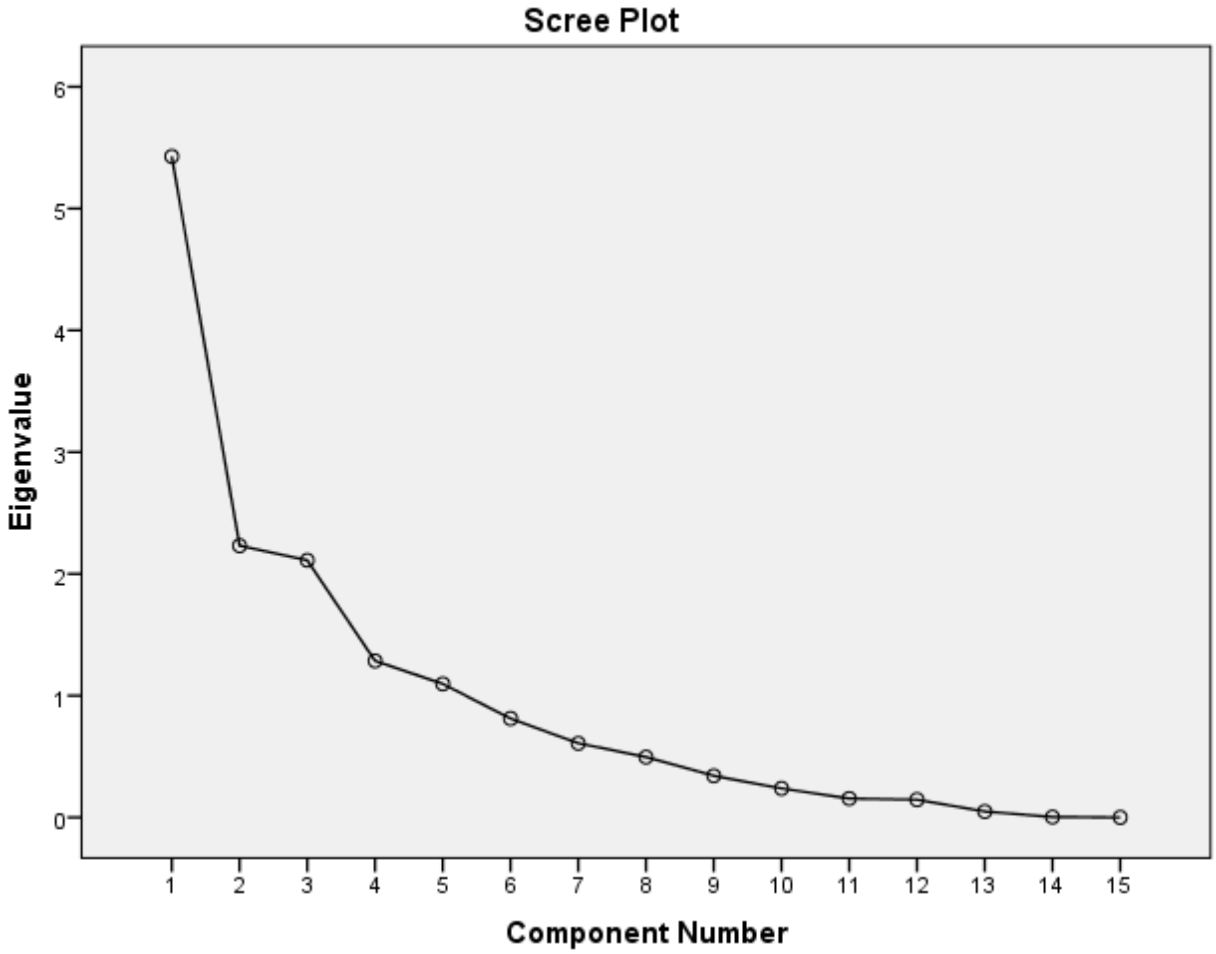
$Na_2S_2O_5$	2% of $Na_2S_2O_5$ in distilled water.

Appendix 3A: Descriptive statistics of variables used for PCA

Variable	N	Mean	Std. Dev	Skewness	Kurtosis	Range
Hb_day0	172	7.7340	2.75828	-.767	-.218	12.60
WBC_day0	171	10.3257	5.52521	2.191	5.852	30.60
RBC_0	169	4.5677	4.00892	11.529	144.102	53.29
HCT_0	171	21.943	8.3926	-.705	-.269	36.1
MCV_day0	172	71.357	8.9900	-1.068	5.718	72.7
MCHC_day0	171	34.886	3.0135	-.050	.230	16.4
PLT_day0	172	110.514	147.4919	1.262	2.767	920.0
LYM%_day0	169	35.377	17.2037	-.052	-.700	80.2
MXD%_day0	139	9.049	4.9638	2.190	9.434	38.7
NEUT%_day0	152	55.477	18.1323	.237	-.494	97.1
RDW-SD_day0	142	43.100	7.1328	-.632	9.216	66.9
RDW-CV_day0	160	17.720	4.6414	5.622	49.514	48.6
PDW_day0	121	14.540	2.9522	.168	-.317	15.1
MPV_day0	138	9.1629	1.20974	.100	-.005	6.39
P.BURDEN 7.3 ($X10^9$)	90	752.103	2122.114	3.583	14.194	12174.869
	Valid N (listwise)	35				

Appendix 3B: KMO and Barlett's Test of Sphericity

KMO	Bartlett's Test of Sphericity		
	Chi-Square	df	P value
0.553	623.419	105	< 0.0001



Appendix 3C: Screeplot of eigenvalues of factors

Appendix 3D: Pattern Matrix

Variable	Component				
	1	2	3	4	5
Hb_day0	-.855				
WBC_day0				.544	
RBC_0	-.625				
HCT_0	-.897				
MCV_day0		-.930			
MCHC_day0		.891			
PLT_day0				.874	
LYM%_day0	.768		.459		
MXD%_day0					.923
NEUT%_day0	-.655		-.463		
RDW-SD_day0		-.698		.439	
RDW-CV_day0				.701	
PDW_day0			.886		
MPV_day0			.820		
P.BURDEN 7.3				-.484	.714

Extraction Method: Principal Component Analysis.

Rotation Method: Oblimin with Kaiser Normalization.

a. Rotation converged in 13 iterations.

b. Showing coefficients higher than 0.4

Appendix 3D: Total Variance Explained

Component	Initial Eigen values			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings[a]
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total
1	5.428	36.186	36.186	5.428	36.186	36.186	4.220
2	2.231	14.876	51.063	2.231	14.876	51.063	3.461
3	2.111	14.075	65.138	2.111	14.075	65.138	2.044
4	1.285	8.567	73.706	1.285	8.567	73.706	2.885
5	1.097	7.311	81.016	1.097	7.311	81.016	1.841
6	.812	5.412	86.428				
7	.609	4.058	90.486				
8	.495	3.300	93.786				
9	.342	2.278	96.065				
10	.238	1.586	97.651				
11	.155	1.035	98.685				

12	.145	.969	99.654
13	.049	.325	99.979
14	.003	.020	99.999
15	.000	.001	100.000

Extraction Method: Principal Component Analysis.

a. When components are correlated, sums of squared loadings were not added to obtain a total variance.