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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JULY 2017
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.
ACKNOWLEDGEMENTS

All praise and thanks be to God for His faithfulness and the grace bestowed upon me in putting this work together. I am forever thankful to my supervisors, Professor Ben Gyan of the Immunology Department of Noguchi Memorial Institute for Medical Research (NMIMR) of the College of Health Sciences, University of Ghana, Dr. Bethel Kwansa-Bentum and Dr. Fred Aboagye-Antwi both at the Department of Animal Biology and Conservation Science of the College of Basic and Applied Sciences, University of Ghana for their encouragements, invaluable assistance and constructive criticism which have successfully contributed to the completion of this work.

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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>BCS</td>
<td>Blantyre Coma Scale</td>
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<tr>
<td>CECs</td>
<td>Circulating Endothelial Cells</td>
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<tr>
<td>CM</td>
<td>Cerebral Malaria</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<tr>
<td>DIC</td>
<td>Disseminated Intravascular Coagulation</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>EPCs</td>
<td>Endothelial Progenitor Cells</td>
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<tr>
<td>FBC</td>
<td>Full Blood Counts</td>
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<tr>
<td>GPD</td>
<td>Gross Domestic Products</td>
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<tr>
<td>Hb</td>
<td>Haemoglobin</td>
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<tr>
<td>HC</td>
<td>Healthy Controls</td>
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<tr>
<td>HCT</td>
<td>Haematocrit</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency Virus</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon-alpha</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IPTi</td>
<td>Intermittent Preventive Treatment in Infants</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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<td>IRS</td>
<td>Insecticide Residual Spraying</td>
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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
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>RDT</td>
<td>Rapid Diagnostic Test</td>
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<tr>
<td>RDW-CV</td>
<td>Red cell Distribution Width Coefficient of Variation</td>
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<tr>
<td>RDW-SD</td>
<td>Red cell Distribution Width Standard deviation</td>
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<td>RH</td>
<td>Ridge Hospital, Accra</td>
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<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic Curve</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WBC</td>
<td>White Blood Cells</td>
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<tr>
<td>SDGs</td>
<td>Sustainable Development Goals</td>
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<tr>
<td>SMA</td>
<td>Severe Malaria Anaemia</td>
</tr>
<tr>
<td>TGH</td>
<td>Tema General Hospital</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
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<tr>
<td>UM</td>
<td>Uncomplicated Malaria</td>
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<td>VSA</td>
<td>Variant Surface Antigens</td>
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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 malarriometric 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 malarriometric 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 malirometric 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 malirometric 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-$\gamma$), transforming growth factor alpha (IFN- $\alpha$) 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 malarriometric 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 malirometric 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 malirometry 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 malirometric 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 malirometric 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 malarial index and malaria phenotypes.
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 only 13 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. Arnosti* (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 for 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-inflect 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 parasiteemia, 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 specificitv owing to overlapping symptoms with other severe childhood diseases such severe pneumonia (English et al., 1996; Verma et al.,
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 necessarily 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 paesitaemia (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 parameters 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 Wolfrswinkel 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 malarial-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) ≤ 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°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 \textit{P. falciparum}.

3.13 \textbf{Estimation of malaria parasite biomass}

Individual total parasite biomass (\(P_{\text{tot}}\)) 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 \(P_{\text{tot}} = 7.3 \times \text{PfHRP2} \times (1-\text{Hct} \%) \times \text{body weight (Kg)} \times 10^{13}\), 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 (\(P_{\text{circ}}\)) from the peripheral blood was estimated using the formula: parasites/ \(\mu\)l \(\times 10^6\) \(\times\) blood volume, with blood volume defined as 0.08 \(\times\) body weight [kg] (Dondorp et al., 2005; Hendriksen et al., 2012). Sequestered parasite biomass (\(P_{\text{seq}}\)) was estimated as the difference between the total parasite biomass and the circulating parasite biomass.

3.14 \textbf{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™ 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°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

<table>
<thead>
<tr>
<th>Malaria phenotype</th>
<th>Study participants</th>
<th>Mean age</th>
<th>Sex</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM</td>
<td>46 (26.7%)</td>
<td>5.902</td>
<td></td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>CM</td>
<td>46 (26.7%)</td>
<td>4.924</td>
<td></td>
<td>32</td>
<td>14</td>
</tr>
<tr>
<td>SMA</td>
<td>23 (13.4%)</td>
<td>3.060</td>
<td></td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>HC</td>
<td>57 (33.1%)</td>
<td>6.546</td>
<td></td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>172 (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ P value; (F); [\chi^2] \quad < 0.0001; (8.294) \quad 0.140, [5.437] \]

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Malaria Phenotype</th>
<th>Unadjusted P values</th>
<th>Bonferroni adjusted P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UM</td>
<td>CM</td>
<td>SMA</td>
</tr>
<tr>
<td>P Density</td>
<td>22190.000</td>
<td>1880.000</td>
<td>22200.000</td>
</tr>
<tr>
<td>Ptot (X 10^8)</td>
<td>345.100</td>
<td>10075.000</td>
<td>9021.571</td>
</tr>
<tr>
<td>Pseq (X 10^8)</td>
<td>345.100</td>
<td>10075.000</td>
<td>9028.000</td>
</tr>
<tr>
<td>Pcirc</td>
<td>150803.670</td>
<td>7499.752</td>
<td>11834.873</td>
</tr>
</tbody>
</table>

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 CD4 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

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

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Malaria Phenotype</th>
<th>X²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC</td>
<td>UM</td>
<td>CM</td>
</tr>
<tr>
<td>HB</td>
<td>Normal</td>
<td>(56)98.2%</td>
<td>(40)87.0%</td>
</tr>
<tr>
<td></td>
<td>Anaemic</td>
<td>(1)1.8%</td>
<td>(6)13.0%</td>
</tr>
<tr>
<td>MCV</td>
<td>Normal</td>
<td>(56)98.2%</td>
<td>(38)82.6%</td>
</tr>
<tr>
<td></td>
<td>Microcytosis</td>
<td>(1)6.2%</td>
<td>(8)17.4%</td>
</tr>
<tr>
<td>MCHC</td>
<td>Normal</td>
<td>(42)73.7%</td>
<td>(43)95.6%</td>
</tr>
<tr>
<td></td>
<td>Hypochromasia</td>
<td>(15)26.3%</td>
<td>(2)4.4%</td>
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<tr>
<td>WBC</td>
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<td>(53)93.0%</td>
<td>(39)84.4%</td>
</tr>
<tr>
<td></td>
<td>Leukopenia</td>
<td>(2)3.5%</td>
<td>(3)6.5%</td>
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<tr>
<td></td>
<td>Leucocytosis</td>
<td>(2)3.5%</td>
<td>(4)8.7%</td>
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<tr>
<td>PLT</td>
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<td>(22)47.8%</td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia</td>
<td>(0)0.0%</td>
<td>(24)52.2%</td>
</tr>
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</table>
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 malarialmetric 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
The 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 come 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 cases (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 ≥ 14.55% and MPV ≥ 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 maliometric analysis, the study employed the use of a Principal Component Analysis (PCA) approach in computing a composite maliometric 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 deemed 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


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


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.
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 volunteer

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 1B: Consent for healthy controls

Title: Circulating endothelial cells and the pathogenesis of malaria


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.
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 volunteer

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$_2$HPO$_4$ 1.0g
   
   KH$_2$PO$_4$ 0.7g
   
   Distilled water 1 litre

   (Adjusted pH, 7.25; Temp. 30.5\degree C)

C. Sickling test Buffer

   Na$_2$S$_2$O$_5$ 2\% of Na$_2$S$_2$O$_5$ in distilled water.
### Appendix 3A: Descriptive statistics of variables used for PCA

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<td>LYM%_day0</td>
<td>169</td>
<td>35.377</td>
<td>17.2037</td>
<td>-.052</td>
<td>-.700</td>
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<td>139</td>
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<td>4.9638</td>
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<tr>
<td>RDW_SD_day0</td>
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<td>RDW_CV_day0</td>
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<td>4.6414</td>
<td>5.622</td>
<td>49.514</td>
<td>48.6</td>
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<td>-.005</td>
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<tr>
<td>P.BURDEN 7.3 (X10^9)</td>
<td>90</td>
<td>752.103</td>
<td>2122.114</td>
<td>3.583</td>
<td>14.194</td>
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**Valid N** 35 (listwise)
Appendix 3B: KMO and Bartlett’s Test of Sphericity

<table>
<thead>
<tr>
<th>KMO</th>
<th>Bartlett’s Test of Sphericity</th>
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<tr>
<td>Chi-Square</td>
<td>df</td>
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<tr>
<td>0.553</td>
<td>623.419</td>
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</table>

Appendix 3C: Screeplot of eigenvalues of factors
## Appendix 3D: Pattern Matrix

<table>
<thead>
<tr>
<th>Variable</th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
<th>Component 4</th>
<th>Component 5</th>
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</thead>
<tbody>
<tr>
<td>Hb_day0</td>
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<tr>
<td>WBC_day0</td>
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<td>.544</td>
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<td>RBC_0</td>
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<td>HCT_0</td>
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<td>MCV_day0</td>
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<td>MCHC_day0</td>
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<td>MXD%_day0</td>
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<td>-.463</td>
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<tr>
<td>SD_day0</td>
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<tr>
<td>RDW-</td>
<td></td>
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</table>
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

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial Eigen values</th>
<th>Extraction Sums of Squared Loadings</th>
<th>Rotation Sums of Squared Loadings&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% of Variance</td>
<td>Cumulative</td>
<td>%</td>
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<tr>
<td>1</td>
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<td>36.186</td>
<td>36.186</td>
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<td>2</td>
<td>2.231</td>
<td>14.876</td>
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<tr>
<td>3</td>
<td>2.111</td>
<td>14.075</td>
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<tr>
<td>4</td>
<td>1.285</td>
<td>8.567</td>
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<tr>
<td>5</td>
<td>1.097</td>
<td>7.311</td>
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<tr>
<td>15</td>
<td>.000</td>
<td>.001</td>
<td>100.000</td>
</tr>
</tbody>
</table>

Extraction Method: Principal Component Analysis.

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