ANGIOGENIC AND ANGIOSTATIC FACTORS IN THE SALIVA OF MALARIA PATIENTS

BY

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DECLARATION

The laboratory experiments described in the thesis was performed by me at Noguchi Memorial Institute for Medical Research of the College of Health Sciences, University of Ghana. The work was done under the supervision of Dr Nana Otoo Wilson and Dr Kwadwo Asamoah Kusi.

All references cited in this thesis have been fully acknowledged in the reference section.

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DEDICATION

This work is dedicated to the Almighty God for His protection, insight and grace to complete this work. I also dedicate it to my children and husband for their immense support throughout my study.

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LIST OF ABBREVIATIONS

ACT	Artemisinin-Combination Therapy
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
BSA	Bovine Serum Albumin
BBB	Blood Brain Barrier
CD	Cluster of Differentiation
ECs	Endothelial Cells
CM	Cerebral Malaria
EDTA	Ethylene diamine Tetra acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
Hb	Haemoglobin
HC	Healthy Control
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
ICAM-1	Intercellular Adhesion Molecule-1
IFN-ɣ	Interferon-ɣ
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
IP-10	Interferon Gamma-Induced Protein 10
Ibid	*Ibidem* (Latin: meaning the same place)
LLINs	Long-Lasting Insecticidal Nets
Mab	Monoclonal Antibody
ml	Milliliter
OPD	Out Patients Department
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PfEMP-1 1	*Plasmodium falciparum* Erythrocyte Membrane Protein

PfHRP	*Plasmodium falciparum* Histidine-Rich Protein
PLDH	Plasmodium Lactate Dehydrogenase
pRBC	Parasitized Red Blood Cell
RBC	Red Blood Cell
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
SMA	Severe Malaria Anaemia
Tek	Tyrosine Kinase
TNF- α	Tumor Necrosis Factor-α
UM	Uncomplicated Malaria
UNICEF Fund	United Nations International Children's Emergency
USAID	United States Agency for International Development
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF2	Vascular Endothelial Growth Factor 2
WBC	White Blood Cell
WHO	World Health Organization

ABSTRACT

Background: The mortality associated with severe malaria remains high even after appropriate treatment with effective anti-malarial drugs. Malaria death is associated with deregulation of host immune responses to inflammatory factors such as C-X-C motif chemokine 10 (CXCL10) and host angiogenic factors such as angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2). Current diagnosis of malaria relies on microscopic detection of the parasites in the blood film. This approach is invasive, increases accidental infections and uncomfortable for some patients. The aim of this study was to investigate biomarkers -CXCL10, Ang-1 and Ang-2 levels in the saliva of malaria patients and compare with plasma levels with regard to their potential use as biomarkers in malaria. This may be useful for further development of highly efficient non-invasive malaria detection methods using saliva.

Methods: This was a case control study involving 213 participants: 119 malaria patients and 94 healthy controls, aged 1 -16 years for children and > 16 years for adults. The study was conducted in the Shai-Osudoku District Hospital in Dodowa, Southeastern part of Ghana in the Greater Accra Region. Four millilitres (4ml) of blood and 1.5ml of saliva were collected from each participant into cell preparation tube (CPT) and OMNIgene-Oral tube respectively. The plasma and saliva levels of CXCL10, Ang-1 and Ang-2 of the study participants were measured using enzyme-linked immunoassay (ELISA) technique. Complete blood count (CBC) was measured with a Haematology auto analyzer. *Plasmodium* Lactate Dehydrogenase/Histidine-Rich Protein-2 (pLDH/HRP-2) Antigen Combo Card rapid diagnostic test (RDT) was performed to determine the presence of *P. falciparum* HRP-2 in participant's blood.

Thick and thin blood film slides were prepared, stained with Giemsa solution and examine for the presence of *P. falciparum* under the microscope.

Data were presented as mean ± standard error or median and interquartile range (IQR) where appropriate. Spearman's rank test was used to find the correlation between plasma and saliva biomarkers in malaria patients. A p-value < 0.05 was considered significant.

Results: Of the 213 participants, 119 malaria patients, 44 (37.0%) were male and 75 (63.0%) were female and 94 non- malaria controls, 27 (29%) were male and 67 (71%) were females with ages ranging from 1-78 years, with a median ages of 29 (IQR 22-35) and 23 (IQR 17-31) years respectively between non-malaria and malaria participants (Median age, $p<0.001$).There was decreased plasma levels of Ang-1 ($p<0.009$) and increased plasma levels of CXCL10 ($p<0.001$) and Ang-2 ($p<0.001$) in individuals with malaria compared to those without malaria. Similar trends were observed in the saliva samples from study participants. Saliva biomarkers CXCL10, Ang-1 and Ang-2 levels correlated significantly with plasma levels of malaria patients. Finally, Ang-2 was informative when combined with CXCL10 to predict the risk of malaria and could be useful in clinical decision-making.

Conclusions: These results provide insight into the use of saliva as a non-invasive diagnostic method and demonstrate that Ang-2 combined with CXCL10 is a promising predictive biomarker in malaria.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria remains a major health problem with an estimated 216 million cases and 445 000 deaths globally (World Malaria Report, 2016). Its greatest effect is experienced in sub-Saharan Africa with most cases described in children less than five years of age (Black *et al*., 2008). There are five main *Plasmodium* species that cause malaria in humans, these are *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax and Plasmodium knowlesi* with *Plasmodium falciparum* causing most of the malarial associated death (de Jong, Slager, Verbon, van Hellemond, & van Genderen, 2016). The main transmission vector of malaria is the female *Anopheles* mosquito. The local environment within many rural areas favours mosquito vector development and high parasite transmission (Buffet *et al*., 2011). In urban regions, transmission of malaria is low to moderate and usually a seasonal occurrence (White *et al*., 2014). Most individuals living in this endemic transmission zones progressively acquire immunity after exposure to several infections. Thus, this immunity protects against severe, life-threatening cases of malaria but does not confer sterile protection (Doolan, Dobaño, & Baird, 2009).

In Ghana, the infection accounts for about 13.2% of all mortalities and is rank fifth as the most common cause of death in children from birth to 4 years (WHO, 2011). A number of children who even survive suffer the consequences of severe malaria such as seizures or neurological dysfunction which can hinder long-term development (World Malaria Report, 2015). Malaria is a significant disease in Ghana and still

accounts for about 40% of all out-patients attendance in 2016 with the huge majority of the infection caused by *P. falciparum* (Awine, Malm, Bart-Plange, & Silal, 2017).

Symptoms of *P. falciparum* can progress from mild to severe within a matter of hours and life-threatening complications of *Plasmodium falciparum* may manifest as cerebral malaria (CM) and severe malarial anaemia (SMA) with death occurring within forty-eight hours of hospitalization mostly in children under age five years (Buffet *et al*., 2011).

However, the mechanism underlying the pathogenesis of severe malaria manifestations such as cerebral malaria (CM) is not clear and there are limited predictive tools available to ascertain which individual progresses to malaria complications (A L Conroy, Lafferty, & al., 2009; Lovegrove *et al*., 2009).

An accurate diagnosis of severe malaria is vital, especially since earlier studies have suggested that in some cases, malaria manifestations share similar indications to severe infections such as sepsis (T. E. Taylor *et al*., 2004). T. E. Taylor *et al*. (2004) observed that autopsy of children earlier diagnosed as CM cases actually died of non-CM causes, which highlight the need for accurate and reliable diagnostic tools. Identification of usable biomarkers which allows reliable detection of malaria would provide a tool that would be useful for the prompt detection, diagnosis and prognosis of the disease. A key factor to controlling this is the early and accurate diagnosis as it is essential to manage infected individuals effectively, to avoid unnecessary presumptive treatment and progression to severe malaria.

Presently, the World Health Organization (WHO) recommends treatment of malaria cases to be guided by detection of *Plasmodium* antigens or parasites in febrile patients

and asymptomatic carriers (World Malaria Report, 2015). These parasites detection necessitates the drawing of blood and also requires trained personnel who are not always available in remote malaria-endemic areas. This approach increases the risks of needle injuries and accidental infection from diseases such as HIV/AIDs (Nantavisai, 2014). The cultural objection of considering blood withdrawal as taboo, the fear of young children and some adults from needle pricks, the difficulty in taking blood samples from critically ill anaemic children and the requirement of repeated sampling during post-treatment follow-up may lead to poor compliance of patients (N. O Wilson, Adjei, Anderson W, Baidoo S, & Stiles, 2008). Thus, there is the need for the development of a non-invasive, cost-effective diagnostic tool that reduces the need for blood collection.

Biomarkers have become useful for the diagnosis and prediction of many chronic illnesses such as cancer, diabetes, autoimmune diseases and HIV/AIDS (C.-Z. Zhang *et al*., 2016). A couple of these markers have been used to assess patterns of inflammatory response to malaria and their association with the severity of the infection (Bruno B Andrade *et al*., 2010). Angiogenic factors such as Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are markers released during activation and dysfunction of the vascular endothelium in non-infectious as well as infectious diseases such as malaria (Gomes *et al*., 2014). Ang-1 and 2 are key regulators of endothelial activation and integrity and their levels have been defined as reliable biomarkers of severe malaria (Lovegrove *et al*., 2009).

Levels of Ang-1 and -2 from serum or plasma have been shown to accurately discriminate cerebral malaria from uncomplicated malaria in African patients and also

reported in Thai patients to discriminate cerebral malaria, severe non-cerebral malaria from uncomplicated malaria (A L Conroy *et al*., 2009; Lovegrove *et al*., 2009). Several studies have elaborated on the association between disease severity and plasma levels of Angiopoietins in *P. falciparum* infection as well as their relationship and the occurrence of cerebral malaria, placental malaria, retinopathy, and death (Andrea L Conroy & Kain, 2012; Andrea L Conroy, Liles, Molyneux, Rogerson, & Kain, 2011; Silver, Zhong, Leke, Taylor, & Kain, 2010).

In healthy individuals, Angiopoietin-1 levels are higher than the levels of Angiopoietin-2, as a result, Ang-1 promote endothelium stability and prevent the induction of a pro-inflammatory response (Fukuhara *et al*., 2010). However, during cellular stress, upregulation of Ang-1 stimulates the release of Ang-2 and increases the binding of Tie-2 receptors with Ang-2 which induces the generation of pro-inflammatory and pro-thrombotic responses (Gomes *et al*., 2014). This may imply that Ang-1 is needed to reverse the harmful effect of endothelial activation in severe malaria, an important event in preventing the loss of life (Samuel C Wassmer *et al*., 2015). Furthermore, other research that focused on clinical outcome has described high Ang-2 levels to predict mortality as well as, or even better than other blood markers in both children and adults with malaria complications in Asia and Africa (Prapansilp *et al*., 2013; Yeo *et al*., 2008). These results strongly suggest the important role of Ang-1 and 2 levels as reliable biomarkers for malaria infection.

CXCL10, also known as IP-10 is a chemokine induced by IFN-γ, TNF-α and other factors and has chemotactic activity for activated Th1 lymphocytes which have been demonstrated in addition to CXCL4, CXCL9 and CXCL11 as a set of prognostic

biomarkers in malaria (Wilson *et al*., 2011). Its role has also been discovered in various infectious diseases including malaria. Reports from recent studies in both Indian and Ghanaian patients with malaria, demonstrated that CXCL10 levels were elevated in serum and cerebrospinal fluid samples and was found to be associated with increased risk of fatal *P. falciparum*-mediated severe malaria (Armah, Wilson, Sarfo, Powell, & Bond, 2007; V Jain, Armah, Tongren, Ned, & Wilson, 2008; N. O. Wilson *et al*., 2011). The use of these biomarkers in parasitic infectious diseases is limited (Lucchi *et al*., 2011) and its use has not been adequately evaluated in other body fluids such as saliva. A recent study has detected levels of anti-malarial IgG in both saliva and plasma sample (Estévez *et al*., 2011). Saliva has been known as a non-invasive and a safe source that could be substituted for blood in the diagnosis and prognosis of diseases (C.-Z. Zhang *et al*., 2016).

N. O Wilson *et al*. (2008) detected *Pf* HRP2 in whole saliva with a sensitivity of 43% using a microplate enzyme-linked immunosorbent assay (ELISA) and Fung *et al*. (2012) achieved 100% sensitivity although the accuracy of these assays in saliva was found to be much lower than in plasma. These qualitative investigations revealed the potential of saliva-based malaria diagnostics, thus the need to evaluate these host biomarkers using saliva. A comprehensive correlation between the presentation of the disease and the production of a biomarker may assist in clinical decision-making and could reduce the risk of disease complications for patients (Bruno Bezerril Andrade & Barral-Netto, 2011).

This present study evaluates the predictive potential of CXCL10, Ang-1 and Ang-2 in malaria. Levels of circulating forms of these proteins (biomarkers) were examined in plasma and saliva in order to correlate their expression levels in malaria.

1.2 Problem statement

Although there have been several years of malaria control campaigns, the disease is still the cause of high mortality due to improper diagnosis resulting in unnecessary presumptive treatment, over-use or abuse of anti-malaria drugs and poor disease monitoring (WHO, 2012). Discriminating complicated malaria from other causes of serious illness in African children is difficult due to an inaccurate description of the incidence and high distribution of malaria which requires identification. Additionally, the lack of diagnostics to detect asymptomatic or subclinical infections and the lack of accurate data on the burden of asymptomatic infections are a hindrance to economic development and the ultimate goal of elimination of malaria in endemic countries. Thus, it is clear that early diagnosis and early treatment seeking behaviour has a major role in preventing malaria-associated mortality. Microscopy technique needs skilful technician when screening for parasites at low density or during mixed malarial infection.

In some settings, increasing levels of histidine-rich protein 2 genes (HRP2) deletions threaten the ability to diagnose and appropriately treat people infected with *falciparum* malaria. An absence of the HRP2 gene enables parasites to evade detection by HRP2-based RDTs, resulting in a false-negative test results (Cheng *et al*., 2014).

Further, malaria parasite is known to constantly develop resistance against antimalarial drugs making it more difficult to cure the disease (Na-Bangchang, Muhamad, Ruaengweerayut, Chaijaroenkul, & Karbwang, 2013).

In Ghana, malaria is the number cause of morbidity. It accounts for about 40% of all outpatient clinic attendance in health institutions and is ranked third in terms of the cause of death (6.9%) (Awine *et al*., 2017; Mba & Aboh, 2012).

1.3 Justification

Accurate malaria diagnosis aids in effective malaria control, by eliminating malaria-associated illness and death (Hopkins, Talisuna, Whitty, & Staedke, 2007). However, current malaria diagnosis relies on the detection of parasite antigens in blood or serum of symptomatic patients which requires the drawing of blood. This approach increases the risks of needle injuries and accidental infection from diseases such as HIV/AIDs (Nantavisai, 2014).

Meanwhile the cultural objection of considering blood withdrawal as taboo and the fear of young children as well as some adults of needle pricks is worrisome. Also, the difficulty of taking blood samples from critically ill anaemic children when repeated sampling is required during post-treatment follow-up may lead to poor compliance of patients (N. O Wilson *et al*., 2008). In view of this, saliva presents a reduced biohazard and can painlessly be collected in relatively large quantities by health personnel with less training. Research into the detection of CXCL10, Ang-1 and Ang-2 biomarkers in malaria patients using saliva has not be explored, hence the need to detect these markers that could provide a non-invasive, cost-effective diagnostic tool that reduces the need for blood collection. Thus, once these markers are detected, validated, and incorporated

into rapid diagnostic tests, it will be beneficial in the accurate and early identification of patients with malaria especially children for their subsequent clinical management in Ghana.

With the future aim of developing a non-invasive and cost-effective saliva-based malaria detection method that integrate relevant malaria species and these biomarkers, the advance detection of markers of malaria in saliva to save lives of individuals travelling to and from malaria-endemic countries is significant.

1.4 Hypothesis

Plasmodium falciparum infection induces host biomarkers (CXCL10, Ang-1 and Ang-2) that can be detected in saliva and may help in predicting the risk of malaria infection.

1.5 General Objectives

The aim of this study was to measure host biomarkers – CXCL10, Ang-1 and Ang-2 – in the saliva and plasma of individuals with malaria and non-malaria.

1.6 Specific objectives

- To determine the biomarkers-CXCL10, Ang-1 and Ang-2 levels in the saliva of malaria patients and non-malaria controls.
- To determine the biomarkers-CXCL10, Ang-1 and Ang-2 levels in plasma of patients with malaria and non-malaria controls.
- To correlate the saliva levels of the biomarkers with the plasma levels in the malaria cases.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria

Malaria is responsible for more than a million deaths each year and represents the most significant human parasite infection. The causative agent is a protozoan parasite of genus *Plasmodium*. *Plasmodium falciparum,* which is endemic in most of sub-Saharan Africa and throughout the tropics, may cause severe pathology and mortality (P. Jain, Chakma, Patra, & Goswami, 2014). *Plasmodium vivax* causes most common severe, acute febrile illness, which is most largely found in Asia and South America (Valecha *et al*., 2009). The severity of the disease depends on the infectious parasite species and the level of immune response of the host and its genetic deposition (WHO, 2014). Unlike *P. falciparum,* hypnozoites forms persist in the hepatocytes in *P. vivax* and therefore may relapse months to years after apparent resolution of infection. *Plasmodium malariae* does not frequently cause clinical malaria and most cases are found in Africa. It may produce a long-lasting parasitaemia persisting for decades if not treated but rarely results in death. *Plasmodium ovale* is an uncommon cause of mild to moderate relapsing malaria and is often found in the context of mixed *Plasmodium* infections. Some researchers have recognized *Plasmodium knowlesi* as a significant agent of severe malaria in Southeast Asia (Cox-Singh & Davis, 2008).

The most common symptoms of malaria are chills, fever, nausea, general body weakness and anaemia. The major complications of malaria include severe malaria

anaemia (AMA) and cerebral malaria (CM). Severe malaria carries a mortality of about 20% in sub-Saharan Africa despite treatment with an effective antimalarial (Kyu & Fernandez, 2009). Individuals at increased risk of severe disease include young children and pregnant women living in endemic areas, as well as non-immune travellers to transmission areas.

In children, the most common severe manifestations of *falciparum* malaria which progresses to death are cerebral malaria, severe anaemia, and respiratory distress with metabolic acidosis (Samuel C Wassmer *et al*., 2015).

2.2 Global Burden of Malaria

The public health impact of malaria is most crucial in the tropics, especially in sub-Saharan Africa, where it remains the leading cause of morbidity and mortality especially in children and pregnant women. Malaria is estimated to have caused 445 000 deaths globally in 2015 with most deaths occurring in Africa (92%) (World Malaria Report, 2016). In sub-Saharan Africa, its burden on health is evident mostly in children under age five and repeated malaria infections contribute to the development of severe anaemia, making young children more vulnerable to other common childhood diseases, such as diarrhoea and respiratory infections, thus contributing to related death (WHO, 2011). The heavy burden of malaria in some endemic population has also been as a result of inefficient control of the mosquito vectors and resistance of the mosquito vectors as well as parasites to the major classes of insecticides and anti-malaria drugs respectively and the lack of an effective vaccine against the disease (Trape *et al*., 2014). Other factors may partly be as a result of misdiagnoses since many facilities lack

laboratory capacity and it is often difficult clinically to distinguish malaria from other infectious diseases (WHO, 2011).

2.2.1 Malaria Burden in Ghana

Malaria still remains one of the important health problems in Ghana and it is a significant cause of death and loss of work days due to illness (Awine *et al*., 2017). The estimated malaria cases in the country as of 2015 was 6% (World Malaria report, 2016). *P. falciparum* is the main *Plasmodium* species that cause malaria accounting for about 90% of all cases (Owusu, Brown, Grobusch, & Mens, 2017).

The effect of malaria is quite immense on people of all ages. When the infection is not properly treated in pregnant women, it could cause anaemia which may result in miscarriages, stillbirths, underweight babies and maternal deaths (WHO, 2011).

Malaria is also known to put a heavy toll on economic growth by reducing the efficiency of the labour force, thereby decreasing productivity with a resultant decrease in socio-economic income and causing a major constraint to economic development (Adu, 2010).

The challenging situations in Ghana include accessibility to proper healthcare facilities especially in the rural communities, improper diagnosis and effective treatment which are key to the eradication of the disease. Ghana and many West African countries have been classified to be in the control phase according to the global malaria elimination program (World Malaria Report, 2016).

Currently in Ghana, efforts and programs put in place to curb malaria situation is targeted towards control (WHO, 2013). Some of the policies and strategies adopted so far include free distribution and promotion of the use of insecticide-treated nets (ITNs)

or long lasting insecticide nets (LLINs) for all age groups by community-based volunteers to enhance usage, indoor residual spraying (IRS) (World Malaria Report, 2015). In spite of the high levels of various interventions, the level of malaria disease in Ghana is still relatively high, making elimination of local transmission of the disease before 2030 unlikely (Ameme *et al*., 2014).

2.2.2 Global Malaria Control and Challenges

From the time the mode of *Plasmodium* transmission by the anopheline mosquitoes was discovered, various intervention measures against malaria have focused primarily on environmental control of mosquito breeding sites (Konradsen, van der Hoek, Amerasinghe, Mutero, & Boelee, 2004).

Globally, this approach achieved much success in some parts of Zambia (Utzinger, Tozan, Doumani, & Singer, 2002), Egypt and Brazil (Killeen, Fillinger, Kiche, Gouagna, & Knols, 2002).

Currently, the World Health Organization's recommended package of core interventions to prevent infection and reduce morbidity and mortality comprises vector control, chemoprevention, diagnostic testing and treatment (World Malaria Report, 2016). The two mainly applicable vector control interventions are Indoor Residual Spraying (IRS) and Long-Lasting Insecticidal Nets (LLINs). The targets of these are designed to repel and kill indoor-resting mosquitoes, those that enter houses or other sleeping areas. The chemoprevention using Artemisinin-Combination Therapies (ACTs) and Intermittent Preventive Treatment (IPT) are strategies designed to reduce disease burden and transmission (World Malaria Report, 2015).

Diagnostic and treatment measures have also been put in place to ensures all patients suspected of having malaria are been diagnosed and confirm before administering antimalarial treatment and also ensuring that uncomplicated *P. falciparum* malaria is treated with quality-assured artemisinin-based combination therapy (Dondorp *et al*., 2010).

There have been worldwide efforts put in place by different organizations such as WHO, USAID, Bill and Melinda Gates Foundation and others to control malaria with the goal of eradication through training and education. Other strategies currently put in place to manage malaria include vaccine development, drug development and vector control (Zofou *et al*., 2014). Vaccine development for malaria is still ongoing, but the one that currently looks promising is the RTS, S vaccine which targets the blood stage of the *Plasmodium* parasite (Esen *et al*., 2012; RTS, 2012).

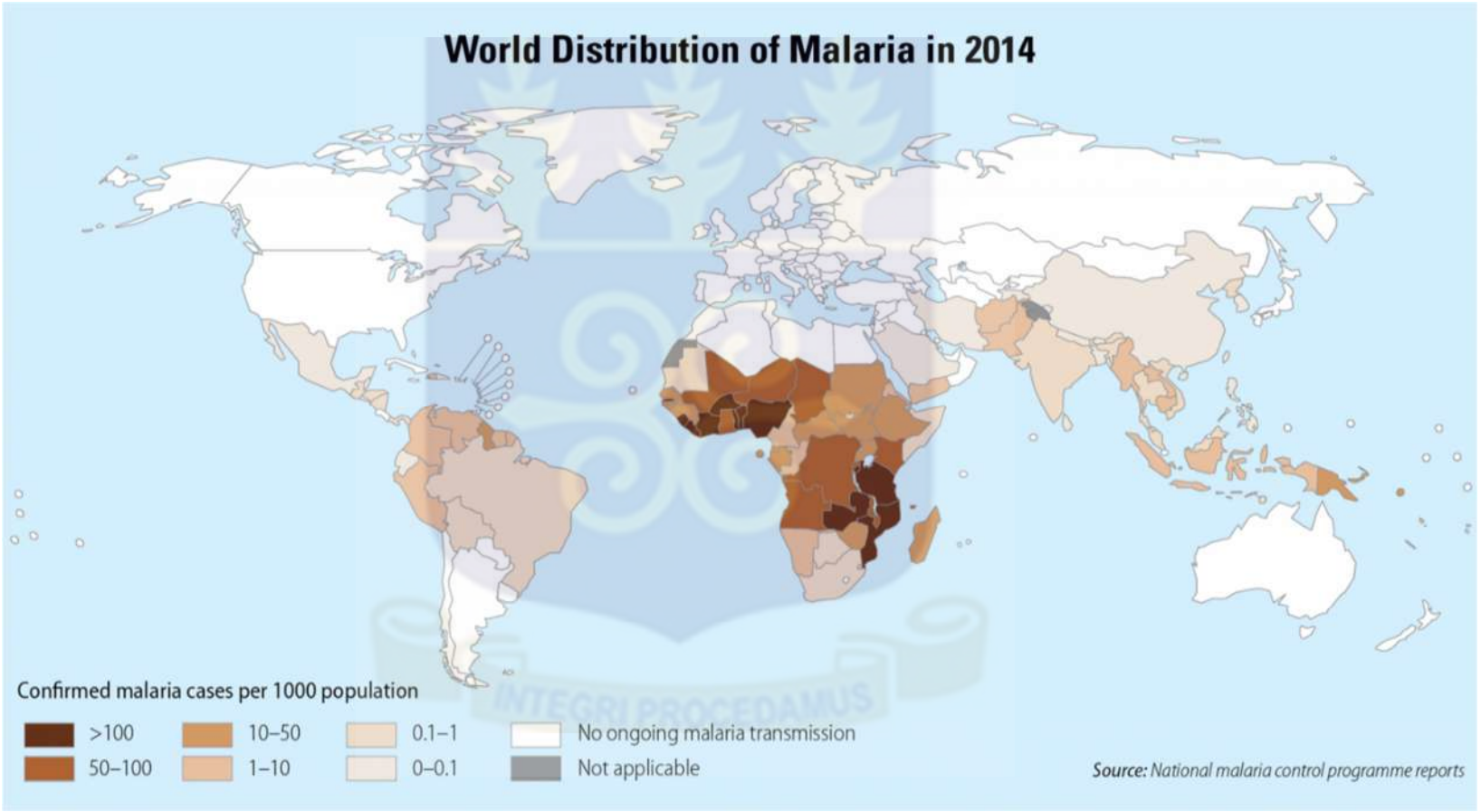
2.3 Malaria Distribution and Transmission

Malaria transmission is affected by local factors such as climate and weather, the proximity of mosquito breeding sites, and the mosquito species (WHO, 2013). In many rural areas where the local environment favours the development of the mosquito vector and its interactions with humans, malaria transmission is high and perennial (Trape *et al*., 2014). Transmission of malaria in other areas including urban zones is low to moderate and usually seasonal (White *et al*., 2014). Persons living in high and stable transmission areas progressively acquire immunity after experiencing and surviving to several infections and this immunity protects against severe, life-threatening cases of malaria but does not confer a sterile protection (Doolan *et al*., 2009). In endemic areas, clinical malaria is seen in young children while many healthy

adults who carry the parasites are asymptomatic. Adults who usually die of the disease are pregnant women and non-immune individuals from low transmission zones

In Ghana transmission of malaria is intense, perennial and quite stable in the rainforest regions. Environmental conditions throughout the year in the forest regions of Ghana is favourable for malaria transmission hence, malaria is endemic in the forest regions with the highest transmission being observed during the rainy season (Kasasa *et al*., 2013).

Figure 2. 1: A map showing global confirmed malaria cases by country

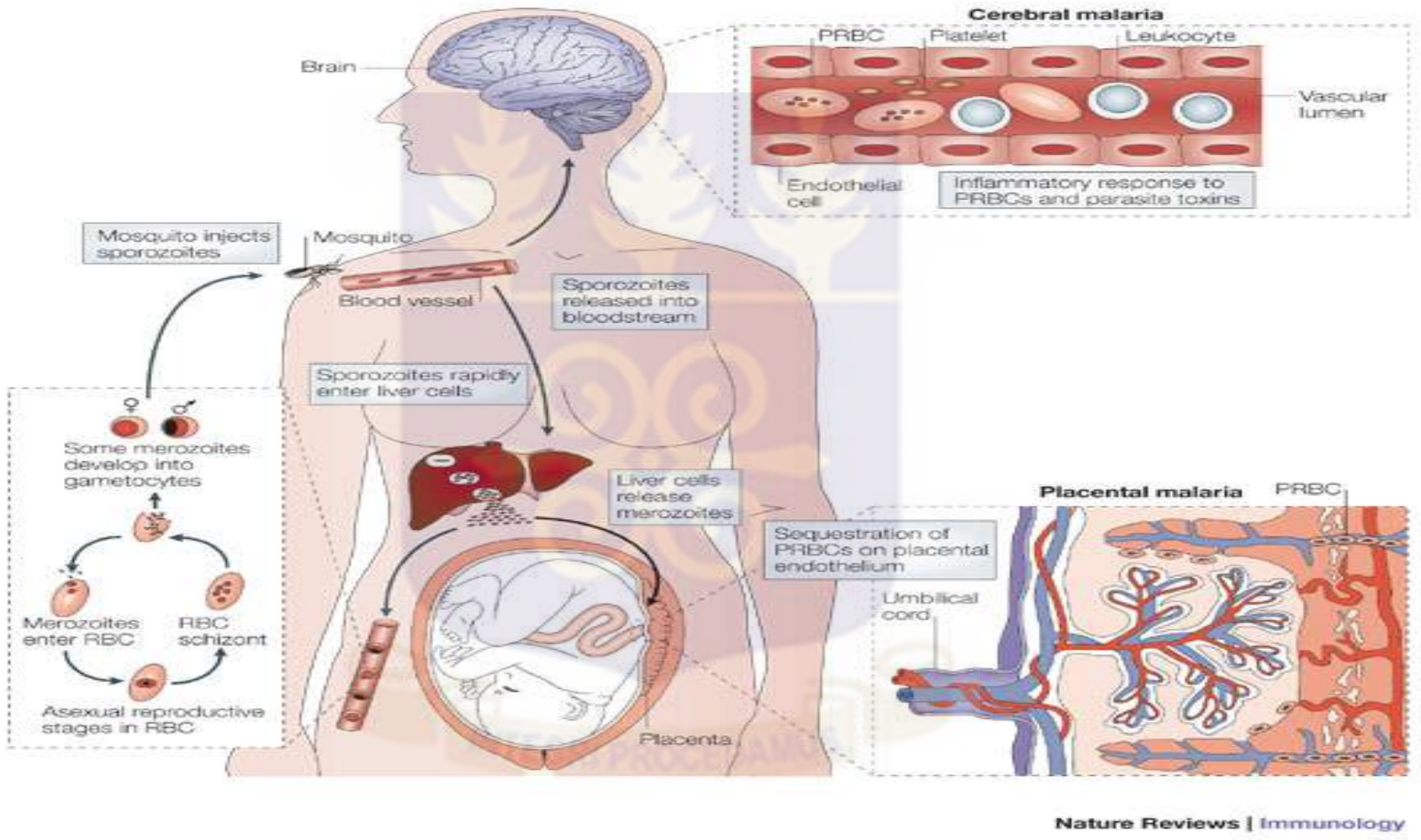


Confirmed malaria cases in 2014. Adapted from World Malaria Report (2014). Available at https://rollbackmalaria.com/ [cited-2017 December 19]

2.3.1 Life Cycle and pathogenesis of Malaria Parasite

Malaria is transmitted when an infected female Anopheles mosquito injects sporozoites into the skin while feeding. Sporozoites enter the bloodstream and are carried to the liver, where they infect liver cells. This initiates asexual reproduction (schizogony), an asymptomatic phase (fig. 2.2). (Doolan *et al*., 2009; Nkhoma *et al*., 2012).

Figure 2. 2: A diagram showing the life cycle of malaria parasite



Adapted from Miller *et al*., Nature, 2011.

Ruptured hepatocytes release merozoites from the liver into the bloodstream and quickly invade red blood cells initiating the blood stage infection. The merozoites in the red blood cell develop into trophozoite which then replicates to form schizonts (Greenwood *et al*., 2008). When the schizonts mature, the red blood cell ruptures and releases merozoites that infect new red blood cells. The characteristic episodic fevers that are associated with malaria are triggered by synchronous parasite development and ruptured red blood which releases new merozoites, malarial antigens and toxic metabolites. Some of the trophozoites develop into male and female sexual gametocytes. When the mosquito feeds, gametocytes are ingested into its stomach initiating the sexual reproduction (sporogony) in the mosquito's gut. The gametocytes emerge from the infected blood cells, becoming gametes. The male and female gametes fuse to produce a zygote. The zygotes elongate to form ookinetes which move through its stomach wall. The ookinetes develop into oocysts. The oocysts grow and rupture, releasing sporozoites. The sporozoites migrate to the salivary glands, ready to be transmitted to the human host to restart the cycle. The sporogonic phase may last between 8-15 days in the mosquito (Eichner *et al*., 2001).

In malaria caused by *P. vivax* and *P. ovale*, but not *P. falciparum*, some parasites stay behind in the liver and these dormant forms can cause a relapse of the disease long after treatment of the blood stage infection. The cycle of erythrocyte invasion and asexual multiplication is responsible for the pathogenesis of malaria.

2.4.1 Pre-patent and Incubation Period

There are a number of factors that contribute to the outcome of infection from the time of sporozoite inoculation to either the onset of detectable parasitaemia or malaria

symptoms. In some cases, the incubation period for malaria may be as short as seven days or as long as several years. Factors such as the level of acquired immunity of the host due to previous exposures, the type of *Plasmodium* species responsible for the infection and anti-malarial prophylaxis that are used to prevent transmission, may also increase the length of the malaria incubation period by weeks or months. Infection with *P. falciparum* can cause severe disease in the non-immune individual where parasitaemia can be detected between 5–10 days, with malaria symptoms occurring between the range of 6–14 days depending on the effectiveness of both the innate and acquired immune mechanisms as well as anti-malarial prophylaxis treatment used (Doolan *et al*., 2009). The incubation period for both *P. vivax* and *P. ovale* malaria is usually longer and could range from 15–16 days, and may relapse months or years after exposure, due to the presence of hypnozoites in the liver. However, *P. vivax* has the longest reported incubation period of 30 years (S.-J. Kim *et al*., 2013).

2.4.2 Clinical Features and Pathophysiology

Classically, symptoms are paroxysmal and periodic, recurring approximately every forty-eight hours in *P. falciparum, P. vivax* and *P. ovale* and every seventy-two hours for *P. malariae*. Signs and symptoms of malaria may present as early as seven days, but usually an average of 10-21 days after being bitten by an infected mosquito. The clinical manifestation of *P. falciparum* is associated with the life cycle of the parasite (Andrea L Conroy *et al*., 2012).

Infection with *P. falciparum* is potentially fatal and clinical manifestations are induced by the asexual stages of the parasite that develops inside red blood cells (RBCs) (Buffet *et al*., 2011). The features present in the form of fever which occurs following bursting

(lysis) of schizonts in the blood (Figure 2.1) to release merozoites that invade RBCs. The lysis of the infected RBCs (iRBCs) is believed to release a malarial toxin that induces macrophages to secrete tumour necrosis factor (TNF-α) and interleukin-1 which initiate a paroxysmal response in the human host (L. H. Miller, Good, & Milon, 1994). These toxins have been observed to initiate a toxic shock response where monocytes activate the production of cytokines such as the pro-inflammatory TNF-α or activate the production of reactive nitrogen intermediates necessary for the killing of asexual and sexual parasites with the exception of ring stages which are relatively heat-resistant (Langhorne, Ndungu, Sponaas, & Marsh, 2008). During malaria infection, the production of cytokines results in the release of endothelial activation biomarkers, Ang-1 and 2 (Gomes *et al*., 2014). The binding of parasitized red blood cells to the brain microvessel endothelial which results in their occlusion and subsequent dysregulation of angiogenic factors such as Ang-1 and 2 is thought to play a key role in the pathogenesis of disease severity (Canavese & Spaccapelo, 2014).

2.5 Malaria Diagnosis

Accurate diagnosis of malaria is a foundation of individual patient management and control efforts globally. However, in resource-poor endemic areas, misdiagnosis and continues overtreatment of fever with antimalarial is rampant such that, 32 to 96% of clinical cases are negative by laboratory testing (Amexo, Tolhurst, Barnish, & Bates, 2004). Globally, estimated many malaria-related deaths and over a million presumptive treatments could potentially be averted with sensitive and specific diagnostic tools (Rafael *et al*., 2006). In non-endemic areas, diagnosis of imported malaria cases poses different challenges, due to lack of experience with clinical malaria among

practitioners. Clinical diagnosis of malaria is extremely unreliable given the non-specific nature of signs and symptoms of the disease, which share common characteristics with other potentially life-threatening conditions such as, bacterial sepsis, or meningitis (Ohrt, Sutamihardja, Tang, & Kain, 2002).

2.5.1 Conventional Microscopy

Microscopic detection of parasites on thick and thin Giemsa-stained blood smears remains the gold standard for malaria diagnosis (Anthony Moody, Hunt-Cooke, Gabbett, & Chiodini, 2000). Giemsa stained slide microscopy is regarded as the most appropriate diagnostic instrument for malaria control as it is widely available and relatively cheap allowing not only the identification of malaria species but also the quantification of peripheral parasite density. However, the staining and interpretation process is laborious and requires considerable expertise, especially for accurate species identification at low parasitaemia and when mixed infections occur (Tangpukdee, Duangdee, Wilairatana, & Krudsood, 2009). Quality control of this highly technician-dependent method is problematic in resource-limited areas and the ability to maintain the required level of expertise is challenging especially in non-endemic areas where the disease is encountered infrequently (Hawkes & Kain, 2007). Thick smears and thin Giemsa-stained blood smears are used for the screening of *Plasmodium* parasites, with thick smear allowing detection of more parasites whilst thin smear allows species identification (AH Moody & Chiodini, 2002).

2.5.2 Alternative Diagnosis

Even though examination of the thick and thin blood smear remains the gold standard for diagnosing malaria, important advances have been made in diagnostic testing,

including fluorescence microscopy of parasite nuclei stained with acridine orange, rapid dipstick immunoassay, and polymerase chain reaction assays (Cordray & Richards-Kortum, 2012). Rapid dipstick immunoassays detect species-specific circulating parasite antigens targeting either the histidine-rich protein-2 of *P. falciparum* or a parasite-specific lactate dehydrogenase. The ability to detect both HRP-2 and a pan-*Plasmodium* antigen on a single test strip permits the identification of non-falciparum infections. HRP-2-based assays have excellent sensitivity and specificity across a variety of clinical and research settings.

Additional diagnostic modalities, including polymerase chain reaction, mass spectrometry and flow cytometry have also been applied to the diagnosis of malaria (Hawkes & Kain, 2007). Also, confirmation of *P. falciparum* antigen using a second molecular target reduces the probability of a false positive diagnostic test (*ibid*). However, whereas these more sophisticated molecular techniques demonstrate improved sensitivity and definite interpretation of clinical specimens, cost and the need for laboratory equipment limit their use in resource-limited settings. Beyond routine laboratory testing, biomarkers that reflect activation of key pathways in malaria pathogenesis may provide valuable prognostic information.

2.6 Treatment and Management of Malaria

Drugs targeting the parasite are currently the only specific therapy known to arrest the infection and remain the primary mode of treatment. As such, children suspected of having severe malaria must receive the best available antimalarial by the parenteral route as quickly as possible and specific therapy should not be delayed. The WHO recommends artemisinin combination therapy (ACT) as first-line therapy for

uncomplicated malaria (WHO, 2010) and parenteral Artesunate is the most potent agent for the treatment of severe malaria (Dondorp *et al*., 2005). The emergence of such resistant strains of *P. falciparum* has motivated a switch towards a new class of artemisinin-derived antimalarial compounds. Currently, the artemisinin-derived combination therapies (ACTs) which involve the use of artemisinin derivatives in combination with other traditional antimalarial drugs, is regarded as the best treatment option (Abiodun, Brun, & Wittlin, 2013). Thus, both uncomplicated and severe malaria are best treated with the artemisinin derivatives. For *P. vivax* and *P. ovale* which are known to possess dormant stages and can cause relapses, Primaquine is often administered in addition to ACTs (Fernando, Rodrigo, & Rajapakse, 2011). Unfortunately, any therapy is not always certain to work against the disease since several strains of the parasite resistant even to ACTs have been reported in different parts of the world (S. M. Taylor & Juliano, 2014). Although several strategies and multiple drug treatment protocols do exist to prevent drug-resistant malaria, detecting the disease at its early stage remains vital for the successful treatment. Hence, in this situation, the importance of biomarker-based technique for timely diagnosis of the disease becomes more essential.

In addition to specific therapy with anti-malarial medications, supportive therapy plays an important role in disease management including control of convulsions and fever, attention to fluid and electrolyte balance, transfusions for severe and symptomatic anaemia, maintenance of the airway, and good nursing care. Supportive treatment towards patient's specific organ dysfunctions is essential in this multi-system disease, ideally in an intensive care unit setting (Day & Dondorp, 2007).

2.7 Complications of *Plasmodium falciparum* Malaria

2.7.1 Cerebral Malaria

Cerebral malaria (CM) is a common presentation of severe *Plasmodium falciparum* that can cause the most severe and life-threatening form of malaria in man (Bartoloni & Zammarchi, 2012).

Pathogenesis of CM is complex and multifactorial (Sahu, Satpathy, Mishra, & Wassmer, 2015). The endothelium, which is the critical interface between the PRBC and brain parenchyma is also considered central to the pathogenesis of CM. During severe malaria infection, there is systemic activation of endothelial cells including the cerebral endothelial cells of the blood-brain-barrier. Activated endothelium has increased permeability through the disruption of the endothelial transmembrane proteins and cause upregulation of a variety of surface adhesion molecules such as ICAM-1, VCAM-1 and P- and E- selectin (Dejana, Tournier-Lasserve, & Weinstein, 2009; Fiedler & Augustin, 2006) which have been implicated to mediate cytoadhesion. Evasion mechanism for *P. falciparum* is cytoadherence of parasitized erythrocytes to endothelial cells which aid to evade clearance of PRBC by phagocytes such as macrophages in the spleen (Craig & Scherf, 2001). The infected red blood cells also bind to other uninfected red blood cells which eventually leads to microvascular occlusion, a contributing factor to the resultant coma in cerebral malaria (Dondorp *et al*., 2005). Again, activated endothelial cells rapidly exocytose pre-synthesized and stored molecules from Weibel-palade bodies (WPB) in response to changes in vascular microenvironment. Even though the main constituent of WPB is Von-Willebrand factor and its pro-peptide, other molecules such as P-selectin, CD63, interleukin-8 and angiopoietin (Ang)-2 are released. WPB release is the initial step in the transition from

a resting endothelial cell to a responsive endothelium (Fiedler *et al*., 2004). Angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2) are biomarkers produced during activation and dysfunction of the vascular endothelium in non-infectious as well as infectious diseases, including malaria (Eklund & Saharinen, 2013; Yeo *et al*., 2008). Recent human studies have implicated dysregulation of angiopoietins in the pathogenesis of severe and fatal malaria. A recent study in children with an increased plasma Ang-2 and Ang-2/1 ratio and reduced Ang-1 have been associated with disease severity and risk of death in CM (Andrea L Conroy *et al*., 2012; V. Jain *et al*., 2011).

2.7.2 Severe Malaria Anaemia (SMA)

Severe malaria anaemia is the main cause of illness and death in malaria infection particularly in pregnant women and in children (Nussenblatt & Semba, 2002). A haemoglobin level of less than 5.0g/L defines severe anaemia with patients normally affected being younger children (Thuma *et al*., 2011) than those with cerebral malaria or respiratory distress. Severe anaemia predominately occur in *P. falciparum* infections but different clinical manifestation may exist and vary in severity and outcome, depending on the parasite species , the organ involved and the access to care (Autino, Corbett, Castelli, & Taramelli, 2012).

Severe *falciparum* malaria involves a broad range of diseases, the development of which may be influenced by age, exposure and immune status (Samuel C Wassmer *et al*., 2015). However, a marked feature of SMA pathogenesis is the excessive lysis of both infected erythrocytes and non-infected erythrocytes by the spleen and phagocytic cells such as monocytes due to deposition of IgG on their surfaces (Waitumbi, Opollo, Muga, Misore, & Stoute, 2000). Pro-inflammatory cytokines such as TNF-α which

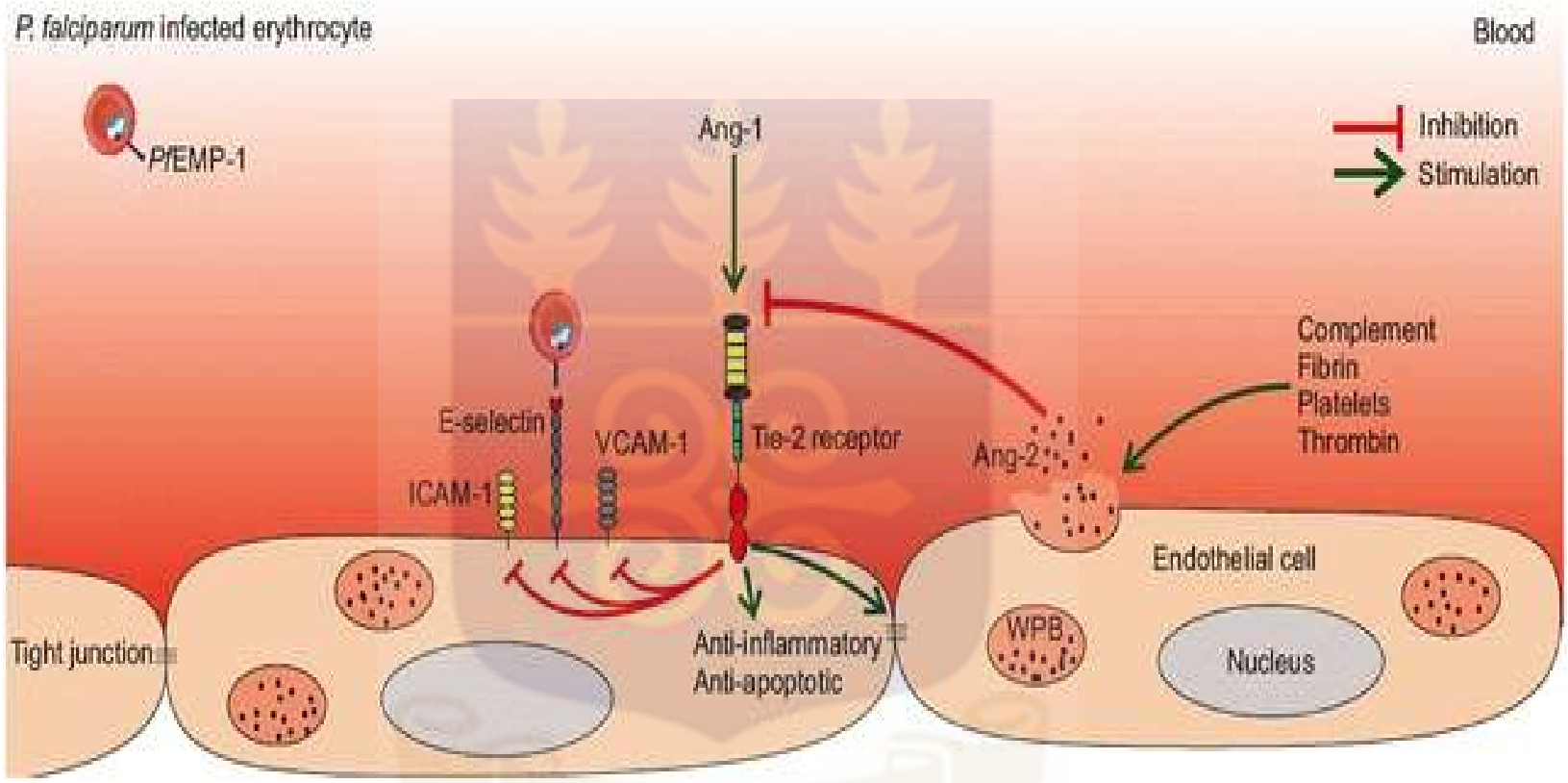
mediate cellular effector functions like phagocytosis have been implicated in the pathogenesis of SMA as they are thought to accelerate the hemolysis of damaged RBCs (Autino *et al*., 2012). Also, the destruction of parasitized and non-parasitized circulating red blood cells by the spleen may be a contributory factor to severe malaria anaemia (Buffet *et al*., 2011).

2.8 Angiopoietin-1, Angiopoietin-2 and Endothelial Cell Activation

Angiopoietins are critical regulators of endothelial cell activation and integrity. Ang-1 and Ang-2 are ligands of the Tie-2 receptor, which is expressed on endothelial cells and regulates endothelial quiescence during normal physiological conditions. The Tie-2 receptor (CD202B, Tek-receptor) is a receptor tyrosine kinase which is expressed mainly by endothelial cells and can also be expressed by haemopoietic progenitors and leukemic cells (Chittiboina *et al*., 2013). Ang-1 is constitutively produced and excreted into the blood by pericytes, smooth muscle cells and it is stored in platelets. Ang-2 is produced in endothelial cells and pre-stored in the Weibel–Palade bodies (WPB) together with vWF. Ang-2 is released from WPB in response to hypoxia or inflammatory stimuli where it competes with Ang-1 for Tie-2 receptor binding. Upon activation of endothelial cells, exocytosis of the WPB is induced and their content is released into the bloodstream (Yuan, Khankin, Karumanchi, & Parikh, 2009). In the presence of Ang-1, Ang-2 acts as a functional antagonist and the Ang-2-Tie-2 interaction results in the blocking of the protective, anti-inflammatory and anti-apoptotic effect of Ang-1 (fig 2.3). Therefore, Ang-2 release and binding to Tie-2 receptors further stimulate the binding inflammatory response of endothelial cells. Endothelial cells express several proteins in serum or plasma which plays significant

roles as indicators of endothelial activation and may be useful clinically as biomarkers of disease severity or diagnosis in systemic infectious diseases (Page & Liles, 2013).

Figure 2. 3: Schematic overview of function and localization of Ang-1, Ang-2 and the Tie-2 receptor.



Adapted from de Jong *et al*. (2016). Ang-2 is pre-stored in the Weibel–Palade bodies in endothelial cells and is released upon endothelial cell activation. Ang-2 replaces Ang-1 by binding the Tie-2 receptor, preventing its activation and thereby blocking the anti-inflammatory, anti-apoptotic and tight-junction supporting effects of Ang-1.

2.8.1 The functions of Angiopoietin-1and 2 in malaria

Angiopoietins (Ang) represent a distinct family of vascular growth factors, within which four molecules have been characterized: Ang-1, Ang-2, Ang-3, and Ang-4 with Ang-1 and 2 being the best-characterised members (Kesler *et al*., 2015). They play an important role in mediating inflammation and quiescence in mature vascular bed (Fiedler & Augustin, 2006; Novotny *et al*., 2009). Endothelial Ang-1 is expressed widely in normal adult tissues and is usually constitutively expressed in brain endothelium and binds to the Tie-2 receptor, thereby acting as an agonist. Under normal condition, the levels of Ang-1 are higher than those of Ang-2, thus, promoting the stability of the endothelium and preventing the activation of a pro-inflammatory response (Fukuhara *et al*., 2010). However, inflammation promotes liberation of Ang-2 and increases the binding of the Tie-2 receptors with Ang-2, principally in the endothelial cells and the smooth muscles, thus activating the vascular endothelium which induces the generation of pro-inflammatory and pro-thrombotic responses (Thurston & Daly, 2012).

As key regulators of endothelial activation, plasma levels of Ang-1 and Ang-2 may provide valuable information regarding the state of vascular endothelial dysfunction and their associated organ damage (Orfanos *et al*., 2007; van der Heijden *et al*., 2009). In malaria, endothelial activation leads to adherence of parasitized erythrocytes, microvascular obstruction, and tissue ischemia. Therefore, Ang-2 release and binding to Tie-2 receptors further stimulate the binding inflammatory response of endothelial cells. The protective roles of Ang-1 have become established in animal models and in vitro studies thus, a number of Ang-1/Tie2 target strategies are being investigated in pathologic processes (Chittiboina *et al*., 2013). Ang-2 has been reported to promote

leukocyte adhesion to the vascular endothelium and extravasation to inflammatory sites (Fiedler *et al*., 2006) thus, during endothelial activation, Ang-1 maintains vascular quiescence, while Ang-2 displaces Ang-1 and sensitizes the cells to become responsive to sub-threshold concentrations of tumor necrosis factor (TNF) (H. Kim, Higgins, Liles, & Kain, 2011).

2.8.2 Angiopoietins as Biomarker of Disease Severity

Angiogenic factors such as angiopoietin 1 (Ang-1) and angiopoietin 2 (Ang-2) are biomarkers produced during activation and dysfunction of the vascular endothelium in several infectious diseases. Several pilot studies have implicated circulating Ang-1 and Ang-2 in critically ill patients to provide valuable information on vascular barrier properties. A marked imbalance of the angiopoietin-Tie system in favour of Ang-2 has been consistently detected in critically ill patients (Gallagher *et al*., 2008; Ganter *et al*., 2008).

The clinical use of Ang-2 as an injury biomarker is being increasingly explored. In cancer, Angiopoietin-2 production is induced by tumour-derived vascular endothelial growth factor (VEGF) and increases VEGF-induced angiogenesis and tumour growth (Saharinen, Eklund, & Alitalo, 2017; L. Zhang *et al*., 2003).

In malaria, endothelial activation leads to adherence of parasitized erythrocytes, microvascular obstruction, and tissue ischemia. Ang-2 has been found to be elevated in severe malaria and a better predictor of disease outcome in adults and children of Asia and Africa with severe and cerebral malaria (Andrea L Conroy *et al*., 2012; Hemmer *et al*., 2006; Prapansilp *et al*., 2013; Yeo *et al*., 2008).

A number of studies have demonstrated the relationship of disease severity and plasma levels of angiopoietins in *P. falciparum* infections, as well as the relationship between the levels of these proteins and the occurrence of cerebral malaria, placental malaria, retinopathy, and death (Andrea L Conroy & Kain, 2012; Andrea L Conroy *et al*., 2011; Silver *et al*., 2010). Decreased levels of Ang-1 was associated with *Plasmodium falciparum* malaria in pregnancy, while an increased Ang-2:Ang-1 ratio was associated with both placental malaria and low birth weight infants (Silver *et al*., 2010).

Subsequent studies have all described Ang-1 to be a more reliable biomarker in malaria, discriminating between cerebral malaria and uncomplicated malaria in African children and also between cerebral, severe, and uncomplicated malaria in Thai adults (Lovegrove *et al*., 2009). Furthermore, decreased Ang-1 level and an increase in Ang-2 concentration have been observed during malaria (Page & Liles, 2013). This may imply that, in addition to antiparasitic drugs, Ang-1 is needed to reverse the deleterious endothelial activation in cerebral and prevent death (Samuel C Wassmer *et al*., 2015).

2.9 The CXCL10 Protein

CXCL10 is a chemokine that is induced by IFN-γ, TNF-α, and other factors and has chemotactic activity for activated Th1 lymphocytes (V Jain *et al*., 2008). The interferon-γ inducible protein 10kDa, a member of the CXC chemokine family binds to the CXCR3 receptor to exert its biological effects (Liu *et al*., 2011). The CXCL10 gene is located on chromosome 4 at band q21 and contains four conserved cysteine residues in the N-terminal region (Romagnani & Crescioli, 2012).CXCL10 is secreted by cells such as leukocytes, monocytes, neutrophils, endothelial cells, keratinocytes, fibroblasts in response to IFN-γ (Luster & Ravetch, 1987; Nana O Wilson *et al*., 2008).

2.9.1 Function of CXCL10

CXCL10 is secreted by leukocytes and tissue cells and functions as a chemoattractant, mainly for lymphocytes. After binding to its receptor CXCR3, CXCL10 evokes a range of inflammatory responses. CXCL10 is reported to act as an antiangiogenic/antitumor protein (Bodnar, Yates, Rodgers, Du, & Wells, 2009) and it is also believed to be involved in chemoattraction of macrophages, monocytes and activated T and NK cell (Park *et al*., 2001; Taub, Longo, & Murphy, 1996).

2.9.2 The Role of CXCL10 in Cerebral Malaria Pathogenesis

Several processes have been implicated in CM pathogenesis, including microvascular obstruction by *P. falciparum*-parasitized red blood cell, excessive pro-inflammatory cytokine production, loss of endothelial barrier function and endothelial dysregulation (Dorovini-Zis *et al*., 2011; van der Heyde, Nolan, Combes, Gramaglia, & Grau, 2006; Samuel Crocodile Wassmer *et al*., 2011). However, the way these pathological processes are connected and their influence by host and parasite factors are not clear. Chemotactic cytokines/chemokines recruit lymphocytes and monocytes to the site of pathogen encounter by binding to their respective chemokine receptor (Griffith, Sokol, & Luster, 2014). Cytokines mediate and generate inflammatory responses. CXCL10 chemokine is induced by IFN-γ and TNF-α and performs several roles, such as chemoattraction for monocytes and T cells, promoting T cell adhesion to endothelial cells, antitumor activity, inhibition of bone marrow colony formation and angiogenesis (Dufour *et al*., 2002). Altered CXCL10 levels have been implicated in various infectious diseases such as Rhinovirus (Schneider *et al*., 2010), *Helicobacter pylori (Eck et al., 2000)* and some protozoan infection. Studies by Hunt and Grau (2003) first reported CXCL10 to be a host-protective factor in murine experimental malaria (ECM)

and a similar study also revealed an expression of CXCL10 in resistant strains of mice to *Plasmodium berghei* ANKA infection (Chen & Sendo, 2001; Hanum, Hayano, & Kojima, 2003). Furthermore, other murine studies also confirm the role of CXCL10/CXR3 in recruiting CD8+T cell into the brain and activation of CD8+ T cells to murine CM pathogenesis (Campanella *et al*., 2008). Variations in CXCL10 mRNA and protein expression have been associated with the pathogenesis of various infectious diseases, chronic inflammatory and autoimmune diseases as well as cancer (Liu *et al*., 2011).

A study conducted in Ghanaian children and Indian adults demonstrated elevated levels of CXCL10 in serum and CSF samples and were associated with increased risk of fatal *P. falciparum*-mediated cerebral malaria (CM) in humans (Armah *et al*., 2007; Nana O Wilson *et al*., 2008). In another malaria study conducted in CM survivors (CMS) and non-survivors (CMNS), altered levels of CXCL4 and CXCL10 were suggested to play a prominent role in the pathogenesis of fatal CM associated death and may be used as functional or surrogate biomarkers for predicting CM severity (N. O. Wilson *et al*., 2011). These findings may imply that CXCL10, when combined with other biomarkers such as Ang-1 and Ang-2 could be a valuable target in predicting the presence of malaria in especially in asymptomatic individuals in endemic settings.

2.10 Innate and Adaptive immune responses to malaria

Immune responses are crucial factors in the pathophysiology of malaria, both in its initiation and progression. Parasite-triggered immune response and inflammation are considered to be a probable cause of death from CM associated complications. The important effector function of the innate immune response is by macrophages, DCs,

NK cells, NKT cells and possibly γδ T cells (Stevenson & Riley, 2004) while adaptive immunity is produced by CD4+, CD8+ T cells.

Macrophages and monocytes play a central role in both innate and adaptive responses to malaria. During acute infection especially in non-immune individuals, monocytes are able to phagocytose infected erythrocytes through non-opsonic phagocytosis. The innate immune response is needed in the clearance of *P. falciparum* from peripheral blood of the host especially during the early stages of the infection (Mohan, Moulin, & Stevenson, 1997).This mechanism of clearance involves an interaction between the class B scavenger receptor CD36 and the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) in the absence of malaria-specific antibody (Serghides *et al*., 2009).

Thus, control of acute infection requires a coordinated response including phagocytes, cytokines, and T-lymphocytes, culminating in a specific protective cytophilic antibody response that ends parasite replication. Cytophilic antibodies of the IgG1 and IgG3 subclasses are considered to be the most significant antibodies in protection against *P. falciparum* malaria in humans and in association with other effector immune cells, they may function through antibody-dependent cellular inhibition (ADCI) (Aucan, Traoré, Fumoux, & Rihet, 2001; Bouharoun-Tayoun, Oeuvray, Lunel, & Druilhe, 2014).

Specific, adaptive immunity to malaria remains incompletely understood, despite decades of research attempting to produce an effective vaccine. The adaptive immune response which involves both humoral and cell-mediated immunity is produced by B cells and T lymphocytes.

Natural immunity to malaria is generally slow to develop, requires continuous re-infection to be maintained and never reaches a sterile level (Serghides, Smith, Patel, &

Kain, 2003). Due to the complex life cycle of the *Plasmodium* parasite, antibody- and cell-mediated mechanisms may act at numerous points to limit parasite reproduction. Humoral immunity is mediated by antibodies such as Immunoglobulin-G (IgG) and Immunoglobulin-M (IgM). This may first interact with pre-erythrocytic stages (sporozoites) to prevent hepatocyte invasion or may bind merozoites to increase their clearance or block erythrocyte invasion. The importance of humoral immunity to pre-erythrocytic stages of the parasite which stems from successful trials of RTS, S malaria subunit vaccine using the circumsporozoite antigen of *P. falciparum* (Bejon *et al*., 2008; Bojang *et al*., 2001).

Cell-mediated immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages. This involves CD4+ and CD8+ T Cells. CD4+T cells are essential for immune protection against asexual blood stages in both murine and human malaria systems. CD8+T cells have important effector functions in pre-erythrocytic immunity and contribute to protection against severe malaria even though this is not clearly understood (Braeckel-Budimir & Harty, 2014).

2.11 Saliva as a non-invasive diagnostic tool for malaria detection

Saliva like serum is known to contain antibodies, hormones, growth factors, enzymes, microbes and their products (Haeckel & Hänecke, 1993; Pfaffe, Cooper-White, Beyerlein, Kostner, & Punyadeera, 2011). Salivary diagnostics is a dynamic and emerging field utilizing molecular diagnostics that aid in the diagnosis of oral and systemic diseases using salivary biomarkers for disease detection (Malamud, 2011). Studies have shown that blood and saliva are linked on a molecular level (Rehak, Cecco, & Csako, 2000). Although the evaluation of diseases mostly relies on blood and

it is effective, these procedures are invasive and expensive and often require extensive time to obtain any meaningful diagnostic results.

Molecular diagnostics have played an important role in the discovery of salivary biomarkers for the diagnosis of oral and systemic.

Current malaria detection and epidemiological surveys depend on the drawing of blood from patients and participants. This increases accidental transmission of infectious diseases, requires trained personnel and poses a lot of difficulties with patient compliance especially in young children who experience the highest malaria burden and a limitation when frequent blood collection is required (Nokes *et al*., 2001). Thus, a non-invasive approach provides an alternative method of sample collection which can rapidly enhance early detection of the disease. A lot of these saliva constituents can be seen in many cases as a reflection of the physiological state of the body. In recent time, saliva has become a better alternative sample source for many experiments due to its ease and non-invasive accessibility by healthcare staff with limited training and the need for special equipment (Nantavisai, 2014). Salivary biomarkers have been used in many systemic diseases such as cardiovascular diseases, diabetes, HIV, oral cancer, caries and periodontal diseases (Javaid, Ahmed, Durand, & Tran, 2016). Therefore it provides a new, non-invasive and simple way to help in the diagnosis of disease and it is expected to become a substitute for serum in disease diagnosis (C.-Z. Zhang *et al*., 2016). Although there have been concerns about the use of saliva for diagnostic purposes due to its low concentration of analytes compare to blood, this limitation has however been overcome with the introduction of highly sensitive detection methods (Chiappin, Antonelli, Gatti, & Elio, 2007; S. Miller, 1994). The use

of saliva for malaria diagnosis has focused on detection of host antibodies against malaria parasites, malarial antigens or malarial nucleic acid in patient saliva by using detection methods such as ELISA, RDTs and PCR (Nantavisai, 2014).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Design

This was a case control study that was carried out in malaria and non-malaria participants between March-June, 2018 in the Shai-Osudoku District (SOD) Hospital in Dodowa, Accra Ghana.

3.3 Informed Consent

Consented participants included in the study were those diagnosed with *P. falciparum* malaria and sought treatment at the healthcare facility solely for malaria symptoms. Written informed consent was given to participants to read or have it read to them in the case of those who could not read and translated. Consent from parents for children and assent from a child where appropriate was obtained at study entry and signed before the collection of epidemiologic, clinical data and sample at enrolment. Informed consent forms were signed by the patient, or in the case of children, by the parents. Should a volunteer, at any point during the study, decide that he/she did not wish to participate any further, their participation was terminated immediately.

3.4 Study Participants

This study involved a total of 213 participants with 119 malaria patients. Participant ages range from 1 year to 16 years for children and >16 years for adults spanning 2 consecutive malaria transmission seasons. A structured questionnaire was used to obtain relevant demographic and clinical data as well as knowledge of malaria from each study participant at enrolment after informed consent. Patients were eligible to participate in the study only if *P. falciparum* malaria infection was microscopically confirmed. Ninety-four healthy participants were included in the study as controls. The

control participants were voluntary individuals who use the facility where the case participants were enrolled. Control participants were eligible for the study if they had no *P. falciparum* parasitemia as defined by microscopy and absence of parasitaemia and had negative Lactate Dehydrogenase/Histidine-Rich Protein-2 (pLDH/HRP-2) Antigen malaria test. Enrollees in this group had no evidence of impaired consciousness, seizures, past history of mental illness, meningitis, head injury or malignancy.

3.5 Case Definition

Malaria was defined as reported thick and thin blood film positive and positive pLDH/HRP-2 RDT plus parasitaemia of 5 or more *P. falciparum* parasites per high power field (approx. ≥ 2500/µl) in the blood.

3.6 Hospital Based Cases

In this hospital-based study, participants with an acute febrile illness (history of fever within the previous 72 hours, or an axillary temperature ≥37.5°C at presentation); *Plasmodium* infection and the willingness of the accompanying parent/guardian to comply with the study were enrolled. The patient also had to be sickling negative, with no other obvious cause found for the fever and show no signs of any other disease.

3.4 Sample Size Calculation

The sample size was determined with an anticipated population proportion of 0.6 malaria cases and 0.4 non-malaria cases with an effect size of 0.2 and power of 0.8 at 0.05 significance level for the two-sided hypothesis test. The sample size was computed from EPIINFO 2005Software. These parameters require a minimum sample

size of 194 participants for the study with 97 participants in each group. Total sample recruited were 213 to account for possible attrition.

3.2 Study Site

The participants in this study were recruitment from Shai-Osudoku District (SOD) Hospital. Shai-Osudoku District (SOD) is located in the Southeastern part of Ghana in the Greater Accra Region. The Shai-Osudoku District Hospital is in Dodowa in the Dangme West District, 39km from Accra. The hospital is the referral point for many health clinics within the Dangme West District. The hospital has a referral unit for many clinical cases including malaria. This area is characterized by year-round transmission of *P. falciparum*. According to research conducted by Dodoo *et al*. (2008), the incidence of parasitaemia is between 50% and 73% representing the two seasonal peaks of malaria transmission in the area from March to August and also in the months of October to June coinciding with the major and minor rainy seasons respectively. The lowest transmission is however observed during the dry season. The people of Dodowa are mostly farmers and fishermen. Malaria is the number one cause of morbidity and mortality in the country, accounting for approximately 40% of all OPD attendance, 36% of all admissions, and 33.4% of all mortality in children less than five years of age (N. O. Wilson *et al*., 2011). *P. falciparum* is the most prevalent in the country with occasionally mixed infection with *P. malariae* (Unicef, 2012).

3.5 Ethical Approvals

The study was voluntary and participants were enrolled only after informed consent was obtained from parents and guardians of children and adults. All guardians of children and adults in the proposed studies received a complete explanation of the study

objectives in which blood and saliva samples were required. There were communications in the local language for those who needed translation and opportunity was given to individuals who refuse to participate or signed the written consent to withdraw. Informed consent and human subject research guidelines of the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana approved this study.

3.6 Inclusion Criteria

Only subjects with clinical malaria without any chronic disease or malignancy were included in the study. Enrolment criteria into the main study were;

3.6.1 Exclusion criteria

Subjects with other infectious diseases such as dengue, yellow fever, and types of viral hepatitis, leptospirosis, bacterial infections, pneumonia, heart failure, hepatic cirrhosis, and diabetes mellitus were excluded from the study. A child was not allowed to participate in the study if a parent or guardian refused to give informed consent and also adults who did not give their informed consent were not included in the study. Children and adults who have evidence of any chronic disease were not selected for this study.

3.7 Hospital Based Cases

In this hospital-based study, participants with an acute febrile illness (history of fever within the previous 72 hours, or an axillary temperature ≥37.5°C at presentation); *Plasmodium* infection and the willingness of the accompanying parent/guardian to comply with the study were enrolled. Malaria was defined as reported thick and thin blood film positive, RDT positive, plus parasitaemia ≥ 2500/µl in the blood.

3.8 Sample Collection and Storage

3.8.1 Collection of blood samples

A venous blood sample, (4ml for adult >16 years), (2ml for child 1-16 years) were collected from each study participant. A total of 4ml blood (2ml in the case of children) was taken into a cell preparation tube (CPT) and 1ml into Ethylene diamine tetraacetic acid (EDTA) tubes. Thick and thin film blood slides were prepared from blood samples. During enrolment, *Plasmodium* Lactate Dehydrogenase/Histidine-Rich Protein-2 (pLDH/HRP-2) Antigen Combo Card rapid diagnostic test (RDT; BestNet, London, UK) were performed. EDTA sample was used for complete blood count measurement. The complete blood count (CBC) parameters such as haemoglobin levels, total white blood cells (WBC) counts, total RBC counts, platelet counts, packed cell volume, MCH, MCHC and MCV were measured with an automated Haematological Analyser (Mindray BC5300, China). The CPT blood sample was centrifuged for 15 minutes at 1000g using the Forma 3L Gp 4500R centrifuge (Thermo electron corporation, MA, USA) to separate plasma, red cells and peripheral blood mononuclear cells (PBMCs) and these were stored at -80°C. Plasma samples were used for CXCL10, Ang-1 and Ang-2 estimations. Thick and thin blood film slides were prepared, Giemsa-stained and examined for parasitaemia.

3.8.2 Collection of saliva samples

Unstimulated oral fluid was collected from each participant. Participants rinsed their mouths with water and expectorated into the OMNIgene-Oral tube (DNA genoTek, Canada) containing preservatives. About 1.5ml of saliva sample was collected within 30 minutes and the samples were stored at 28-30 °C prior to analysis.

3. 9 Laboratory investigations

Laboratory investigations of biomarkers were carried out at the Noguchi Memorial Institute for Medical Research (NMIMR) Legon whilst parasitological and haematological examinations were at the Shai-Osudoku District Hospital, Dodowa.

3.9.1 Parasitological Examination

3.9.1.1 Giemsa staining technique for *Plasmodium falciparum* parasitaemia

The asexual and sexual form of the predominant *Plasmodium* species found in Ghana, *Plasmodium falciparum*, was tested. The malaria parasite test was carried out within 45 minutes of sample collection. Thick and thin films of peripheral blood were prepared for all 213 samples using clean and grease-free slides. Thick and thin films were then prepared from blood samples immediately after collection. Briefly, a drop of well-mixed EDTA anti-coagulated blood (10-15 µl) was placed near the frosted end of the labelled clean grease-free slide and spread in a circle to make a thick blood film. A thin blood film was made by placing 5 µl of well-mixed blood near the end of the thick smear and spread thinly by a spreader so that the red cells did not overlap and both smears were allowed to air dry. After fixing the thin smear in absolute methanol, both blood films were stained in 10% freshly prepared Giemsa stain (pH 7.2), allowed to air dry after thorough washing with phosphate buffer and examined under the microscope using X100 objective (oil immersion) for malaria parasites. The results were initially recorded as either positive (+ve) or negative (-ve). Parasite count was evaluated microscopically on the number of parasites counted against 200 WBCs and parasite densities were obtained when the total parasite counted within this range from the thick

blood film slide was multiplied by 8000 and divided by the number of 200 WBCs counted.

3.9.2 Haematological examination

The haematological parameters were measured using an automated haematological analyzer (Mindray BC5300, China). The automated haematological analyzer functions with the principle of hydrodynamic focusing in which 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 avoid the recirculation of cells. The number, volume and size of the cells are then detected by the machine. Parameters such as haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH), total red cell (RBC) counts, total white blood cell (WBC) counts, haematocrit (HCT) and mean cell haemoglobin content (MCHC).

3.9.3 Quantification of Plasma and Saliva Biomarker levels

Plasma concentrations of biomarkers Ang-1, Ang-2, and CXCL10 (DuoSets, R&D Systems, Minneapolis, MN) were measured by ELISA as follows:

3.9.3.1 Quantikine Human Angiopoietin

3.9.3.1.1 Principle of the Assays

This assay employs the quantitative sandwich immunoassay technique. A monoclonal antibody specific for human Angiopoietin-1 has pre-coated onto a microplate. Standards and samples are pipetted into wells and any Angiopoietin-1 or 2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Angiopoietin-1 is added to the wells. Following a washing to remove any unbound antibody-enzyme reagent, a

substrate solution is added to the wells and colour develops in proportion to the amount of Angiopoietin-1 bound in the initial step. The colour development is stopped using a stop solution and the intensity of the colour measured at 540 or 570nm.

3.9.3.1.2 ELISA Procedure for Angiopoietin-1

The levels of Angiopoietin-1 in the plasma of patients were determined by performing a sandwich ELISA technique using Quantikine ELISA using R&D Systems ELISA protocol.

Briefly, a 100 µl of Assay diluent RD1-20 (diluted 1:5) was added directly to each well in a 96-well microtiter ELISA plate pre-coated with a mouse monoclonal antibody against Angiopoietin -1 (mouse anti-human Ang-1 antibody (capture antigen), (R&D systems, USA). A broad range standard from angiopoietin-1 was prepared by serial dilution from angiopoietin-1 stock (Diluted to the eight-point). 50 µl of the standard, control and sample were added per well, covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker. Plates were then washed four times in wash buffer (buffered surfactant) with 30 seconds incubation between each wash using the Biotek ELx 405 automated ELISA plate washer (Biotek Instruments, Winooski, VT; USA).

The washed plates were blotted against the clean paper towel to adequately remove excess unbound capture antigen and 200µl of Angiopoietin-1 conjugate was added, covered with an adhesive strip and incubated at room temperature for 2 hours on a shaker. Plates were then washed four times in wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and 200µl of substrate solution was added to each well and incubated for 30 minutes at room temperatures in the dark. 50µl of stop

solution (2 N sulfuric acid) was added. The colour in the well changed from blue to yellow. Optical density (OD) was read at 540 nm with a reference at 570nm, using Spectra Max 190 fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA). Optical density (OD) values for the test samples were converted into concentrations with the standard reference curves generated for each ELISA plate.

3.9.3.2.0 Assay procedure for Angiopoietin-2

Human Angiopoietin-2 levels were measured by Quantikine ELISA using R&D Systems ELISA protocol. 100 µl of Assay diluent RD1-76 (buffered protein base) was added directly to each well in a 96-well microtitre ELISA plate coated with a mouse monoclonal antibody against Angiopoietin-2 (R&D systems, USA). A broad range standard from angiopoietin-2 was prepared by serial dilution from angiopoietin-2 stock (Diluted to the eight-point). 50 µl of the standard, control and sample were added per well, covered with an adhesive strip and incubated for two hours at room temperature on a horizontal orbital microplate shaker. Plates were then washed four times in wash buffer (buffered surfactant) with 30 seconds incubation between each wash using the Biotek ELx 405 automated ELISA plate washer (Biotek Instruments, Winooski, VT; USA). The washed plates were padded dry on a tissue paper and 200µl of Angiopoietin-2 conjugate was added, covered with an adhesive strip and incubated at room temperature for 2 hours on a shaker. Plates were then washed four times in wash buffer and 200µl of substrate solution was added to each well and incubated for 30 minutes at room temperatures in the dark. 50µl of stop solution (2 N sulfuric acid) was added. The colour in the well changed from blue to yellow. Optical density (OD) was read at 540 nm with a reference at 570nm, using Spectra Max 190 fluorescence microplate reader

(Molecular Devices Corp., Sunnyvale, CA). Optical density (OD) values for the test samples were converted into concentrations with the standard reference curves generated for each ELISA plate.

3.9.3.3 Quantikine Human CXCL10

3.9.3.3.1 Principle of the assay

This assay employs the quantitative sandwich immunoassay technique. A monoclonal antibody specific for CXCL10 has pre-coated onto a microplate. Standards and samples are pipetted into wells and any CXCL10 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for CXCL10 is added to the wells. Following a washing to remove any unbound antibody-enzyme reagent, the substrate solution is added to the wells and colour develops in proportion to the amount of CXCL10 bound in the initial step. The colour development is stopped using a stop solution and the intensity of the colour measured at 540 or 570nm.

3.9.3.3.2 ELISA Procedure

CXCL10 levels were measured by Quantikine ELISA using R&DSystems ELISA protocol. Briefly, 75 µl of Assay diluent RD1-56 (buffered protein base) was added directly to each well in a 96-well microtitre ELISA plate coated with a mouse monoclonal antibody against CXCL10 (R&Dsystems, USA). A broad range standard from CXCL10 was prepared by serial dilution from CXCL10 stock (Diluted to the eight-point). 75 µl of the standard, control and sample were added per well, covered with an adhesive strip and incubated for two hours at room temperature on a horizontal orbital microplate shaker. Plates were then washed four times in wash buffer (buffered surfactant) with 30 seconds incubation between each wash using the Biotek ELx 405

automated ELISA plate washer (Biotek Instruments, Winooski, VT; USA). The washed plates were padded dry on a tissue paper and 200µl of CXCL10 conjugate was added, covered with an adhesive strip and incubated at room temperature for 2 hours. Plates were then washed four times in wash buffer and 200µl of substrate solution was added to each well and incubated for two hours. To control for inter-assay and day-to-day variations in the ELISA procedure, each assay (ELISA plate) includes a calibration curve obtained by a 2-fold titration of the prepared calibrator. 200µl of the conjugate (stabilized hydrogen peroxide and chromogen tetra methyl benzidine) was added to each well covered with an adhesive strip and incubated for 30 minutes in the dark. 50µl of stop solution (2 N sulfuric acid) was added. The colour in the well changed from blue to green. Optical density (OD) was read at 540 nm with a reference at 570nm, using Spectra Max 190 fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA). Optical density (OD) values for the test samples were converted into concentrations with the standard reference curves generated for each ELISA plate.

3.10 Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0 SPSS Inc., Chicago, Illinois, USA). The frequencies of demographic factors and anaemia status were summarized as a percentage (%). Levels of full blood count components, CXCL10, Ang-1 and Ang-2 (non-normally distributed) data were expressed as median with the interquartile range [IQR]. Differences in data between groups were analyzed using the Chi-Square Test or Kruskal-Wallis Test. Pair-wise comparison of the statistically significant Kruskal-Wallis test was performed using the Mann-Whitney test and Fisher exact test.

Spearman's rank test was used to find the correlation between plasma and saliva biomarkers in malaria patients. Results were considered to be significant when the p-value was less than 0.05.

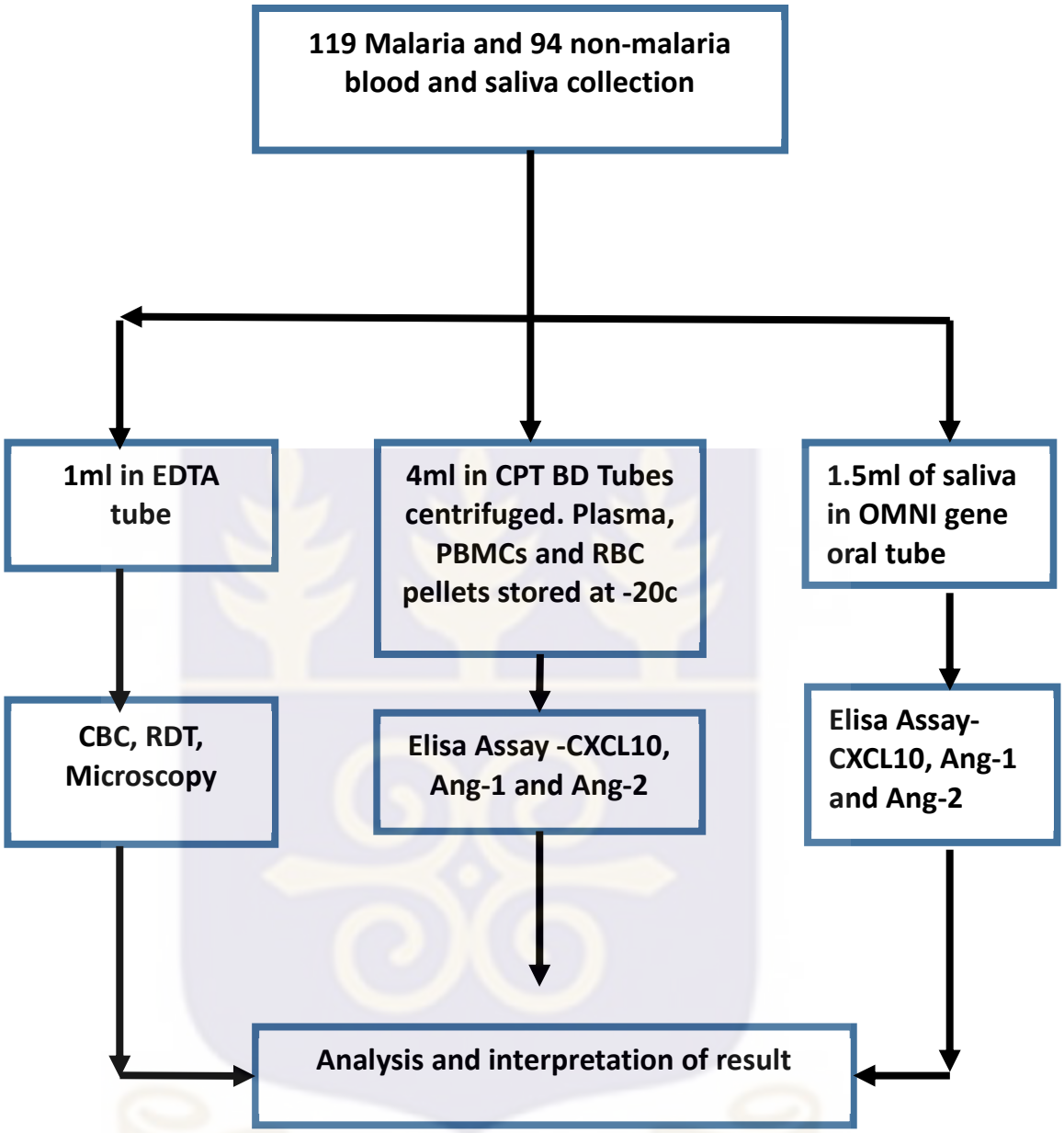


Figure3. 1: Flowchart for Method

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic and Haematological characteristics of Participants

A total of 213 participants were enrolled which was made up of 94 apparently healthy controls (27 males, 67 females) and 119 malaria subjects (44 males, 75 females). The participants were screened for *Plasmodium* species and determination of CXCL10, Ang-1 and Ang-2 levels were done. Table 4.1 shows the demographic and haematological characteristics of all participants. The malaria cases were significantly younger with a median age of 23years than the non-malaria controls with a median age of 29 years p=0.001). There was no significant difference in gender distribution between malaria (63%) and non-malaria (71.3%) participants (p = 0.205). The median platelets levels in malaria (152 x 10^9/L) were significantly lower than non-malaria controls (232.50x10^9/L, p<0.001). There was also a significant difference between lymphocytes counts in malaria compared to non-malaria (6.9x10^9/L and 5.8x10^9/L respectively), p<0.0001. Median values of RBCs and WBCs components count did not show any significant difference between patients with malaria and non-malaria participants. In terms of anaemia, there was a significant difference between malaria and non-malaria participants (31.1% and 16% respectively, p=0.011). There was, however, no significant difference in haemoglobin levels in malaria (12.20 g/dL) compare to non-malaria participants (11.92g/dl) (p =0.462, Table 4.1) and no significant difference either in white cell count between malaria and non-malaria participants (5.90 x 10^9/L and 6.25 x10^9/L respectively, p=0.396).

Table 4.1: Demographic and haematological characteristics of Subjects

Characteristics	Non-Malaria (N=94)	Malaria (N=119)	p- value
Sex			
Male	27 (38%)	44 (62%)	0.205
Female	67 (47.2%)	75 (52.8%)	
Age (Years)			
Median (IQR)	29 (22-35)	23 (17-31)	0.001
Blood count analysis - median (IQR)			
White blood cells ($x10^3$/uL)	6.25 (4.80-7.30)	5.90 (4.60-7.50)	0.396
Red blood cells ($x10^6$/uL)	4.66 (4.23-5.00)	4.52 (4.02-4.99)	0.125
Red cell distribution width – standarddeviation (fL)	42.90 (40.25-45.85)	43.50 (41.80-45.50)	0.39
Red cell distribution width - coefficient of variation (%)	14.20 (13.5-15.45)	14.70 (13.40-16.00)	0.238
Mean corpuscular volume (fL)	81.25 (76.00-85.20)	81.80 (76.00-85.40)	0.987
Hemoglobin (g/dL)	11.95 (11.2-13.5)	12.20 (10.50-13.50)	0.462
Mean corpuscular hemoglobin (pg)	27.10 (25.00-28.60)	27.70 (25.40-29.00)	0.211
Hematocrit (%)	36.85 (34.10-40.0)	36.40 (31.80-40.50)	0.169
Mean corpuscular hemoglobinconcentration (g/dL)	33.15 (31.80-34.00)	33.60 (32.40-34.70)	0.021
Platelets ($x10^3$/uL)	232.50 (181.50-279.25)	152.00 (109.00-213.00)	<0.001
Platelet distribution width (fL)	11.35 (10.50-13.48)	12.90 (11.70-14.10)	<0.001
Mean platelet volume (fL)	9.40 (8.83-10.50)	9.80 (9.10-10.50)	0.103
Platelet larger cell ratio (%)	20.55 (17.13-29.25)	25.50 (20.50 30.00)	0.006
Lymphocytes ($x10^3$/uL)	2.10 (1.70-2.50)	1.20 (0.80-1.83)	<0.001
Neutrophils ($x10^3$/uL)	3.15 (2.13-4.50)	3.85 (2.60-5.25)	0.040
Anaemia status			
Anemia	15 (16%)	37 (31.1%)	0.011
Severe anaemia	1 (1.1%)	3 (2.5%)	0.632

Table 4.1: shows categorical variables compared using chi-square and Fisher exact tests and continuous variables compared using Mann-Whitney tests. Values were reported as per cent (%) and number of observations for categorical variables or median and Interquartile Range (IQR) for continuous variables.

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

Plasma and saliva levels of Ang-1 was measured among the study participants using Quantikine Elisa kit (R&D Systems, Minneapolis, MN USA). Malaria participants were compared with those without malaria. Median plasma and saliva levels of Angiopoietin-1 (Ang-1) were significantly lower in malaria cases 2741.04 (IQR 1785.85-3582.68) pg/ml, and 720.27 (IQR 439.82-1086.74) pg/ml respectively compared to those without malaria 4571.65 (IQR 2704.57-5324.31) pg/ml and 1143.96 (IQR 720.27-1850.11) pg/ml respectively $p<0.001$ for all). The comparative plasma and saliva levels of Ang-1 are shown in Figure 4.1.

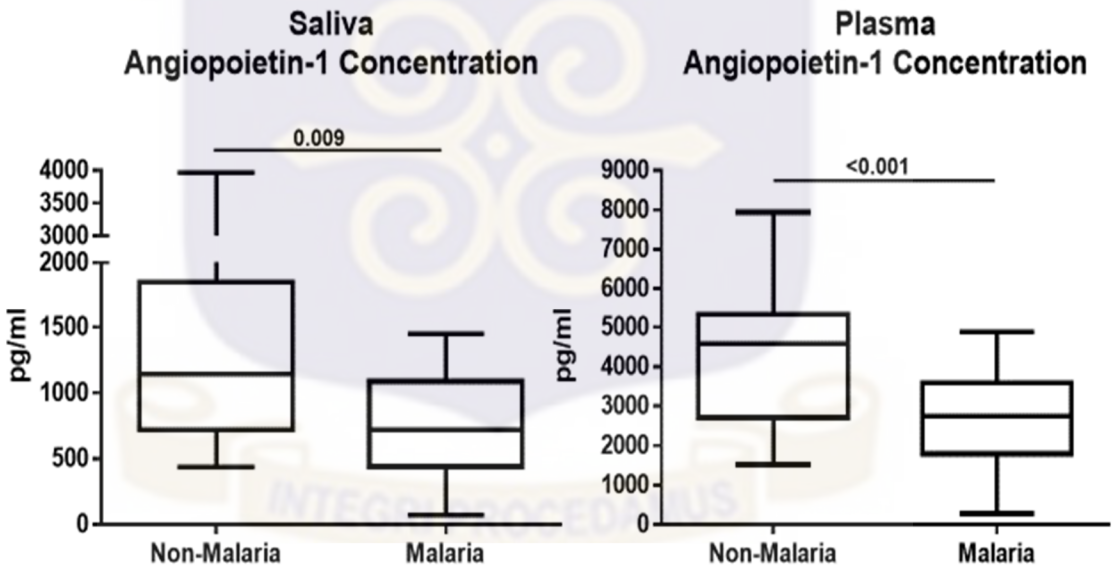


Figure 4. 1. Median levels of Angiopoietin-1 in plasma and saliva of the study participants.

Plasma and saliva Ang-1 were significantly lower in malaria patients compared to non-malaria controls. The middle lines in each box represent the median values of Ang-1.

The box plot of the upper boundaries represents the 75th percentile while the lower boundaries represent the 25th percentile. The upper bars represent the 90th percentile value and the lower bars represent the 10th percentile value.

4.3 Plasma and saliva Levels of Angiopoietin-2 (Ang-2) in the study participants

Plasma and saliva levels of Angiopoietin-2 (Ang-2) were significantly higher in malaria patients 3508.82 (IQR 2139.61-5091.63) pg/ml and 16.98 (IQR 10.08-33.26) pg/ml respectively compared to those without malaria 1916.89 (IQR 1166.52-3874.28) pg/ml and 9.91(IQR 5.19-14.65) pg/ml respectively, $p<0.001$ for all, Figure 4.2).

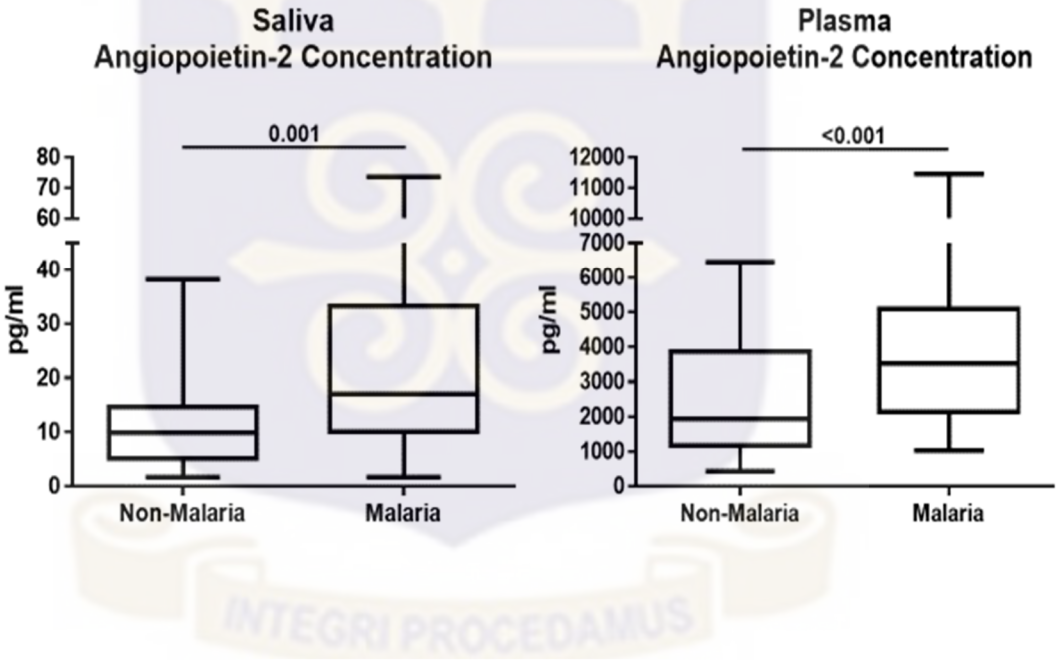


Figure 4.2: Median levels of Angiopoietin-2 in plasma and saliva of the study participants

There was a significant difference in plasma and saliva Ang-2 levels between malaria cases and non-malaria controls ($p < 0.001$). The middle lines in each box represent the median values of Ang-2. The upper boundaries represent the 75th percentile while the lower boundaries represent the 25th percentile. The upper bars represent the 90th value and the lower bars represent the 10th value.

4.4 Plasma and saliva levels of CXCL10 in malaria and non-malaria subjects

Plasma and saliva levels of CXCL10 were measured among the study participants using Quantikine Elisa kit (R&D Systems, Minneapolis, MN USA). Malaria patients express significantly higher plasma CXCL10 levels and increased in saliva CXCL10 levels than those without malaria ($p < 0.001$, $p< 0{,}004$). Median values for non-malaria controls in both plasma and saliva were 96.27 (IQR 47.70-233.50) pg/ml and 16.42 (IQR 11.94-17.91) pg/ml respectively. Median values for malaria patients in both plasma and saliva were 562.34 (IQR 425.28-833.07) pg/ml and 20.90 (IQR 16.42-31.34) pg/ml respectively (Figure 4.3)

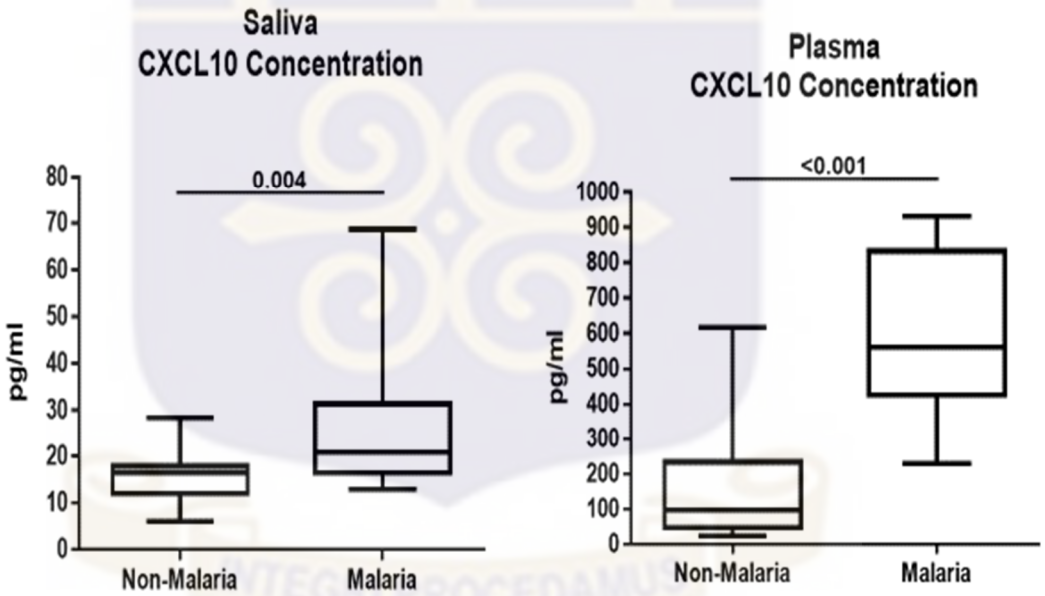


Figure 4.3: Plasma and saliva levels of CXCL10 in subjects with malaria and non-malaria

Malaria patients express significantly higher plasma and saliva CXCL10 levels than those without malaria ($p < 0.001$, $p<0.004$).The middle lines in each box represent the median values of CXCL10 levels. The upper boundaries represent the 75th percentile

while the lower boundaries represent the 25th percentile. The upper bars represent the 90th value and the lower bars represent the 10th value.

4.5 Correlation between plasma Ang-1 and Saliva Ang-1 in Malaria subjects

The association of plasma Ang-1 and saliva Ang-1 levels in malaria were determined using Spearman rank correlation tests. This demonstrated a significant positive correlation between plasma Ang-1 and saliva Ang-1 in malaria cases (R^2 =0.7, p<0.0001, Figure 4.4)

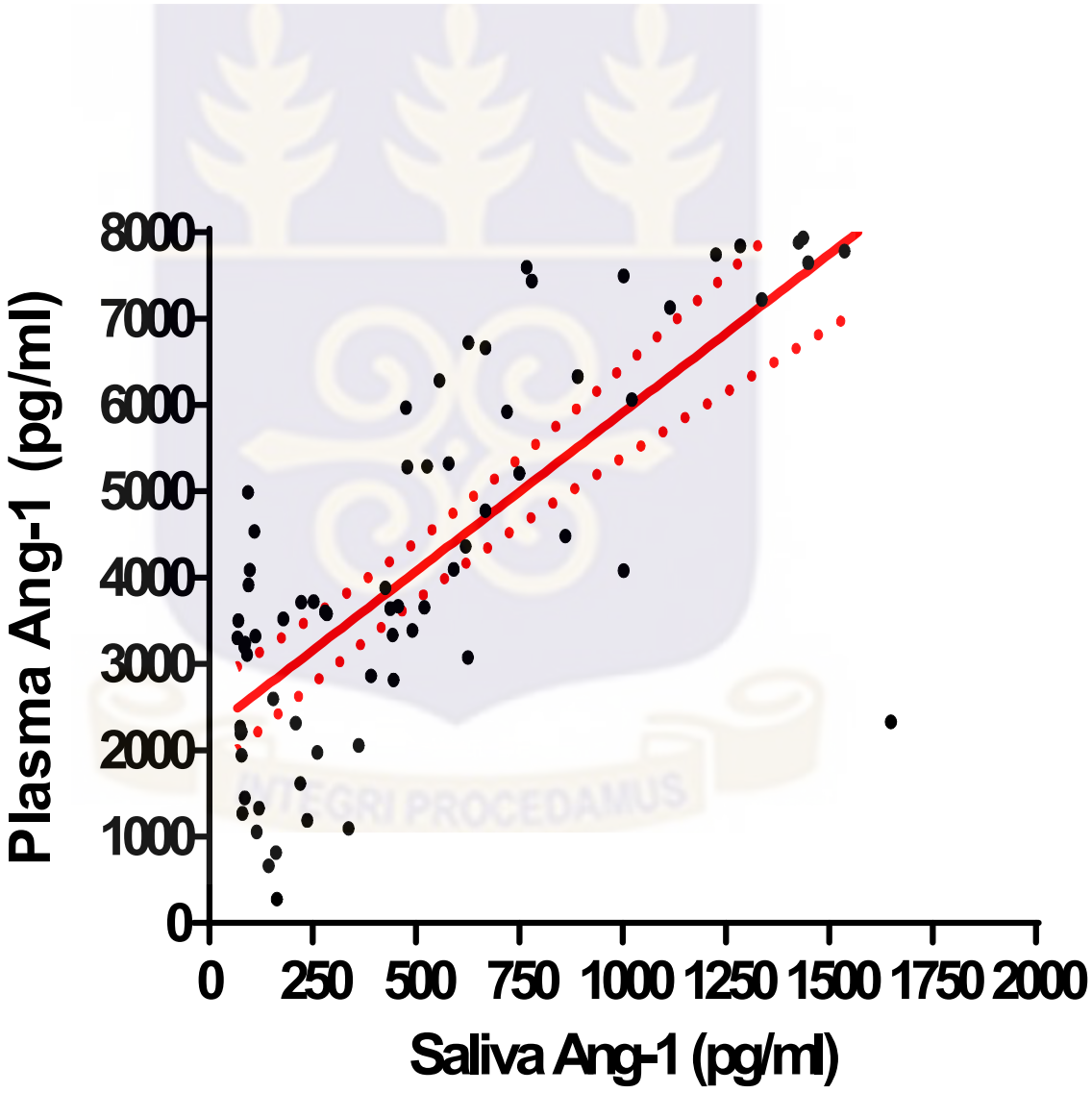


Figure 4. 4; Association of plasma Ang-1 and saliva Ang-1 levels in malaria subjects

4.6 Correlation between circulating plasma Ang-2 and Saliva Ang-2 in malaria subjects

The association of plasma Ang-2 and saliva Ang-2 levels in malaria were determined using Spearman rank correlation tests. This demonstrated a significant positive correlation between plasma Ang-2 and saliva Ang-2 in malaria participants (R^2 =0.4, p<0.0001, Figure 4.4)

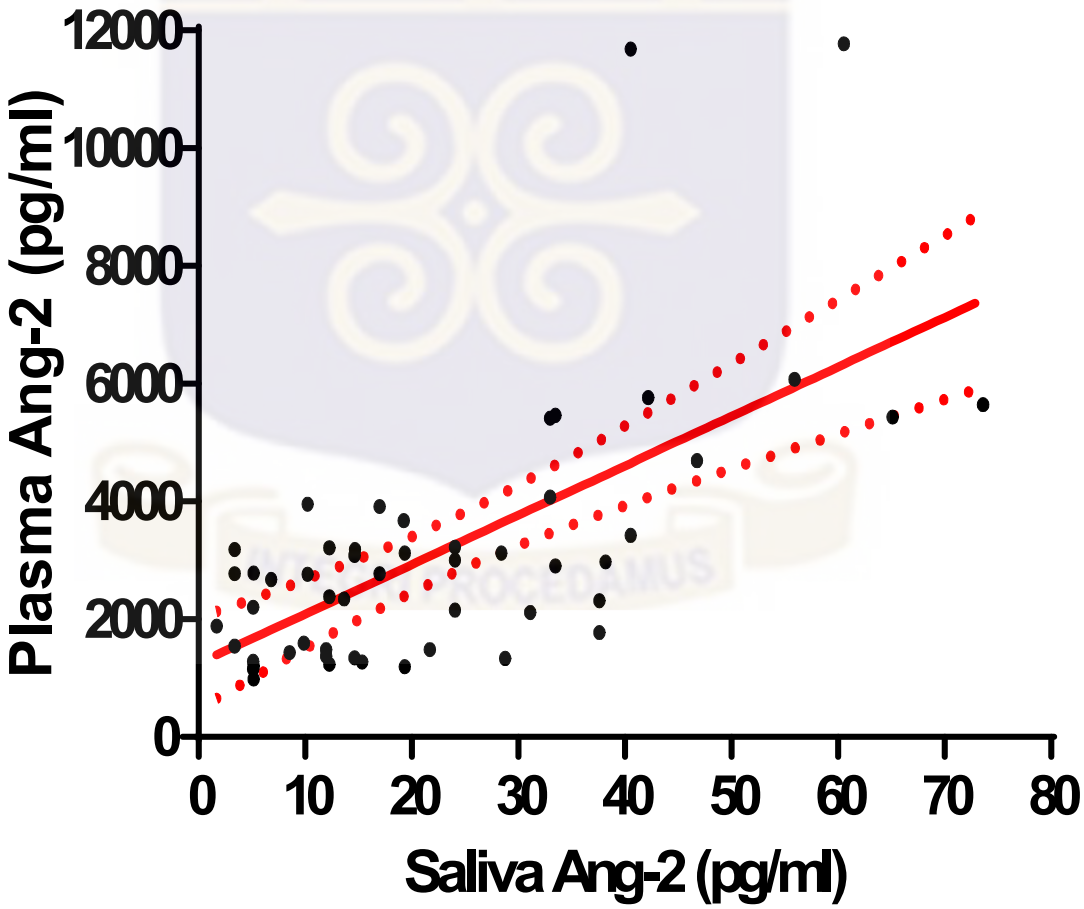


Figure 4. 5; Association of plasma Ang-2 and saliva Ang-2 concentrations in malaria subjects

4.7 Correlation between Plasma CXCL10 and saliva CXCL10 levels in malaria subjects

The association of plasma CXCL10 and saliva CXCL10 levels in malaria was determined by Spearman rank correlation tests. Plasma and saliva CXCL10 were determined by Elisa immunoassay. There was a strong linear relationship between plasma CXCL10 and saliva CXCL10 levels in malaria subjects (R^2 =0.7, p<0.0001, Figure 4.6).

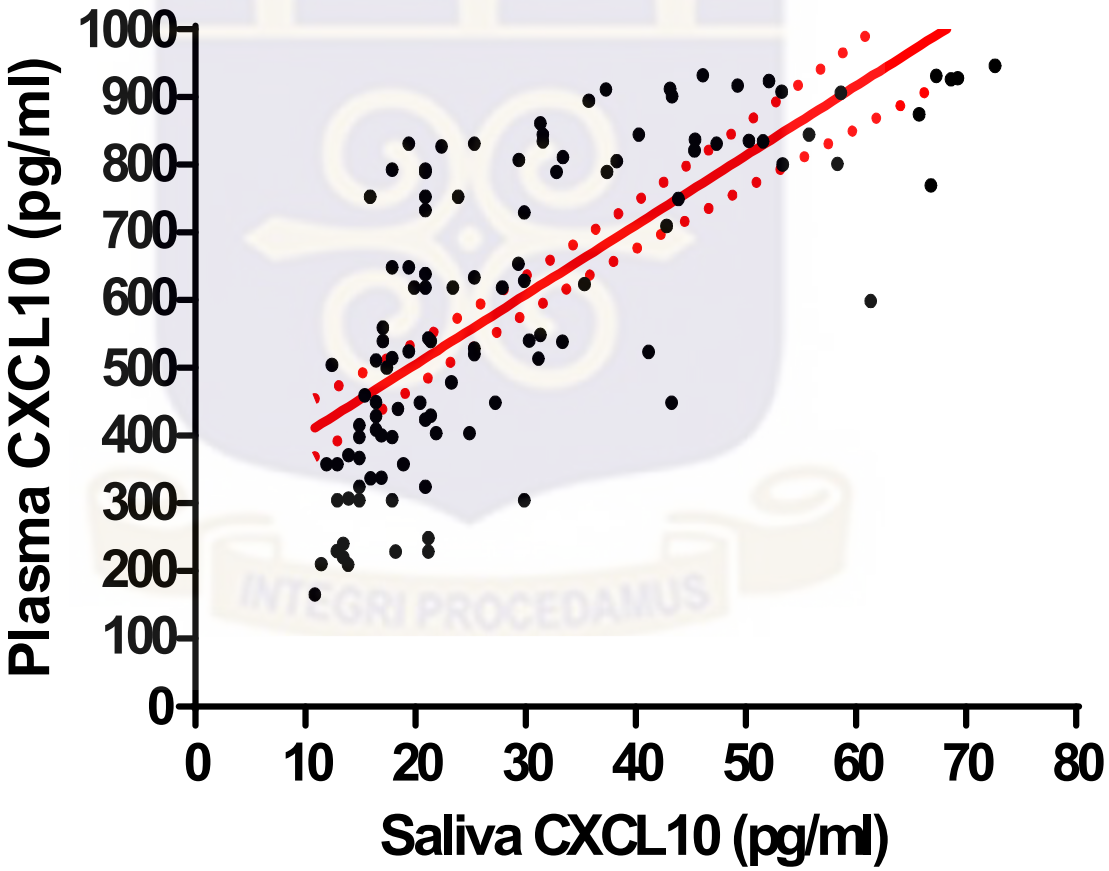


Figure 4. 6; shows Correlation between plasma CXCL10 with saliva CXCL10 concentration in malaria patients (n=119)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Study population and Haematological characteristics

The primary detection of any disease plays a significant role in its treatment. Early diagnosis can reduce the severity and possible complications of disease activity thus, helping in prompt clinical decision making. The aim of this study was to investigate the saliva levels of CXCL10, Ang-1 and Ang-2 and compare with plasma levels. Anaemia has been observed in most malaria-endemic areas with the incidence of severe anaemia and age-specific rates of anaemia which is strongly associated with the intensity of *P. falciparum* transmission (Koram *et al*., 2000). Patients with *P. falciparum* infection may develop rapid anaemia due to heavy parasite load, where at higher levels of parasitaemia, excessive hemolysis of parasitized erythrocytes may lead to anaemia (Kotepui *et al*., 2015). In this study, anaemia was prevalent in the malaria cases compare to non-malaria cases. Platelets levels were significantly lower in malaria patients compare to non-malaria cases.

Generally, during disease condition including malaria, most of the haematological factors are affected (Bakhubaira, 2013; Elbadawi, 2017). Haematological changes in the course of malaria infection are well documented (Kotepui *et al*., 2015) and there has been a well define association between malaria parasite and haemoglobin level (González *et al*., 2000). However, in this study, there was no significant difference in most of the haematological parameters such as white blood cells count, Mean corpuscular haemoglobin concentration, Mean corpuscular haemoglobin and Mean corpuscular volume (Table 4.1) between the cases and the control. These alterations

may vary with the level of malaria endemicity, background haemoglobinopathy, nutritional status, malaria immunity and others factors (Erhart *et al.*, 2004). This may also be due to the fact that participants may have lower parasites densities and so may not exhibit any significant change in haemoglobin concentration and the other factors during the infection. This could also be due to the fact that these malaria patients may have tried self-medication before presenting to the hospital. Dodowa is a semi-rural community with the moderate transmission. Malaria burden impacts are seen mainly in the younger population, therefore it is only the adult population that seems to better able to control their parasitaemia (Iqbal *et al*., 2016). This study had more adult participants than children.

5.2 Plasma and saliva Levels of Angiopoietin-1 and Angiopoietin-2 in the study population

This study assessed the relationship between plasma angiopoietin levels with saliva angiopoietin levels in malaria and non-malaria patients. The results show that plasma Ang-2 levels was significantly high in malaria patients than non-malaria, with similar results observed in saliva where Ang-2 levels was also high in malaria cases compare to non-malaria control. The results also revealed decrease levels of Ang-1 in malaria patients in both plasma and saliva samples. In this study, a positive correlation was observed between plasma Ang-2 and saliva Ang-2 in malaria patients. There was also a strong and positive correlation between plasma Ang-1 and saliva Ang-1 in malaria patients.

In infection with *P. falciparum*, the production of angiogenic factors has been found to be principally associated with an increase in the cytoadherence of infected erythrocytes to the vascular endothelium (Yeo *et al*., 2008). Previous studies have

demonstrated low plasma levels of Ang-1 and high values of Ang-2 to be associated with worst-case prognosis in patients with symptomatic *Plasmodium* infections (Gomes *et al*., 2014). Angiopoietin-1 and -2 (Ang-1 and -2) are main regulators of endothelial activation and integrity, and their levels are found to be altered during inflammatory conditions due to endothelial activation (Eklund & Saharinen, 2013; Page & Liles, 2013). Their levels have been described as reliable biomarkers in distinguishing uncomplicated malaria from severe malaria (Andrea L Conroy *et al*., 2010). This study only focused on determining the levels of these markers in saliva and compare to plasma levels in malaria and non-malaria participants. However, other studies have described their levels in relation to severe malaria where levels of Ang-1 was considered the best discriminator of uncomplicated malaria from severe malaria and was found to serves as a reliable diagnostic biomarker (A L Conroy *et al*., 2009; V. Jain *et al*., 2011). This study also found a significant increase in Ang -2 levels among malaria cases than those without malaria. The observation of higher plasma Ang-2 and lower plasma Ang-1 in malaria cases in this study supports the findings of other studies that revealed Ang-2 to be a better prognostic marker to signal any imminent severity in *P. falciparum* malaria than other laboratory marker of disease severity (Sahu *et al*., 2015) and Lovegrove *et al*. (2009) also reported an increase in plasma Ang-2 levels in adults and children with severe malaria and found Ang-2 to be a better predictor of death than other markers of disease severity. Elevated Ang-2 level has also been associated with endothelial damage and a sign for severe *falciparum* malaria in many studies (Andrea L Conroy *et al*., 2012; Yeo *et al*., 2008) and higher Ang-1 level has also served as a reliable diagnostic marker of malaria (V. Jain *et al*., 2011). These

observations may suggest that dysregulation of angiopoietins during malaria infection may eventually lead to the progression of severe malaria and that, the balance between Ang-2 and Ang-1 may provide prognostic value in relation to endothelial activation and disease severity. Other studies also revealed serum levels of Ang-1 and Ang-2 to have predictive value in a wide range of human pathologies including neoplastic, infectious pathologies, inflammatory, neuroendocrine tumours, rheumatoid arthritis, and malaria (Kurosaka *et al*., 2010; Lovegrove *et al*., 2009; Srirajaskanthan *et al*., 2009). Higher levels of Ang-1 promote quiescence within the vascular endothelium under normal condition. However, during inflammatory conditions, Ang-2 levels are higher mainly due to vascular endothelial activation. Higher levels of Ang-1 was detected in patients who were treated and recovered from *falciparum* malaria (Brouwers *et al*., 2013). To the best of my knowledge, this study provides the first evidence of angiogenic factors such as Ang-1 and Ang-2 in saliva which discriminated between malaria and non-malaria control cases.

From these observations, once these markers are validated and incorporated into routine hospital laboratory investigation, the systematic assay of angiopoietins could help isolate patients with the potential of developing severe malaria and may be useful in clinical decision making.

5.3 Plasma and saliva CXCL10 Levels in the study participants

This research finding revealed higher levels of CXCL10 in plasma and saliva of malaria cases compared to those without malaria which may suggest the role of host inflammatory response in the pathogenesis of severe malaria. Higher plasma levels observed in this study was in agreement with previous studies demonstrating an

association between CXCL10 and severe malaria in which CXCL10 was remarkably elevated in plasma and cerebrospinal fluid of patients who died from severe malaria compared to those who survive the disease after treatment (Armah *et al*., 2007; V Jain *et al*., 2008; N. O. Wilson *et al*., 2011). CXCL10 was also found to be an independent chemokine predictor of fatal severe malaria in Ghanaian children and in Indians who died of CM (V Jain *et al*., 2008; N. O. Wilson *et al*., 2011). Elevated levels of CXCL10 has been implicated in several diseases including cerebral malaria, hepatitis B virus (Deng *et al*., 2008), tuberculosis (Bihari *et al*., 2012; Oliver *et al*., 2012) and HIV (Sui *et al*., 2004).

5.4 CXCL10, Ang-1 and Ang-2 as biomarkers in the saliva of malaria patients

This study revealed a strong and positive correlation between plasma CXCL10, Ang-1 and Ang-2 and saliva CXCL10, Ang-1 and Ang-2 in malaria patients. A salivary biomarker is an informative body fluid containing an array of analytes (Protein and genetic material) that can be used as biomarkers for diagnosis and clinical applications (Shah *et al*., 2011). Saliva is a readily available specimen, which can be collected by non-invasive procedures, have reduced biohazard, simple, painless, does not clot and there is no need for special training or equipment thus, allowing multiple collections of specimens from the same individual at an ideal time for diagnostic information (Hofman, 2001; N. O Wilson *et al*., 2008).

Saliva obtained from patients can be tested for the presence of these markers and from the results, clinicians will be able to risk stratify the patients especially in endemic areas. In the absence of effective malaria vaccines, identification of host factors that contribute to differentiating severe forms of malaria is significant. With a future goal

of generating saliva biomarker for malaria detection, the discovery of these markers in saliva could allow the accurate and early identification of patients with malaria and their subsequent referral to tertiary healthcare facilities for immediate intervention to prevent malaria associated complications.

Furthermore, it will provide a cost-effective approach for the screening of large populations in epidemiological surveys while being affordable, rapid, non-invasive, and safe for patients and technicians in resource-limited endemic countries. The main limitation in this study was that the level of these biomarkers in saliva was not very high compared to plasma, a challenge due to the commercially available kit used, which is designed to detect higher levels of these markers in plasma compared to that in saliva. Also, the small number of patients examined restricted the possibilities of extrapolating the results to other populations.

This study, therefore, hypothesized that biomarkers of endothelial regulators Ang-1 and Ang-2, when combined with chemokine CXCL10, may have clinical utility as prognostic markers in determining *Plasmodium falciparum* infection and could be detected in saliva. The study aimed to detect the levels of Ang-1, Ang-2 and CXCL10 in plasma and compare to that in the saliva of malaria patients to determine if they could discriminate between malaria and non-malaria cases which may be useful in future development of cost-effective, non-invasive saliva biomarker detection method for malaria.

In summary, Ang-2 and Ang-2/Ang-1 have been considered as predictive biomarkers for the severity of various pathologies, both non-infectious (David *et al*., 2011; Goede *et al*., 2010) and infectious diseases including *falciparum* malaria (Andrea L Conroy

et al., 2012; V. Jain *et al*., 2011; Lovegrove *et al*., 2009; Page & Liles, 2013). This data indicates that lower levels of Ang-1 and higher levels of Ang-2 and CXCL10 may have clinical utility in the diagnosis of malaria.

CHAPTER SIX

6.0 CONCLUSION

This study has shown that biomarkers, CXCL10, Ang-1 and Ang-2 are detectable in appreciable levels in saliva and the levels correlate significantly and positively with the levels in plasma of malaria patients. The use of this non-invasive detection method may be useful in reducing the risks of drawing blood and improve patient compliance. Additionally, combining non-blood samples with molecular methods may be as beneficial as blood-based conventional microscopy in detecting parasite during post-treatment follow-up. This study has also shown that patients with *P. falciparum* malarial infections have lower plasma and saliva Ang-1, higher plasma and saliva Ang-2 and higher plasma and saliva CXCL10 levels than the non-malaria control group. Furthermore, elevated levels of Ang-2 and CXCL10 observed in the saliva of malarial patients and lower levels of Ang-1 was significant and their use as a prognostic tool could be explored. Further studies could be carried out to validate the current findings in other forms of malaria complications.

CHAPTER SEVEN

7.0 RECOMMENDATIONS

Further studies involving a larger number of patients and investigations of these biomarker levels in saliva of patients with severe malaria before and after therapy should be performed to validate these findings. With the levels of these detectable biomarkers in saliva, further studies can be carried out to explore their use as a prognostic tool.

Again further studies are needed in other malaria-endemic populations to confirm or replicate these findings especially in children. Furthermore, these combinations require validation across different ethnicities and malaria endemic areas as well as in individuals with cerebral malaria and severe malaria syndromes.

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APPENDIX I

CONSENT FORMS

A. Consent for participants with malaria

Title: **Angiogenic and Angiostatic factors in the saliva of malaria Patients**

Principal Investigator: **Cecilia Elorm Lekpor**

Address: Department of pathology, University of Ghana, 4236, Accra.

Information: (To be read or translated to participants/child's parent/guardians in their own mother tongue)

Dear Participant/Guardian,

This consent form contains information about the research entitled *Angiogenic and Angiostatic factors in the saliva of malaria patients*. In order to be sure that you are informed about being part of this research, you will be asked 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). A copy of the signed form will be given to you. Please ask me to explain anything you may not understand.

Why is this study planned?

Malaria is caused by a germ that is passed from one person to the other by the bite of an infected female anopheles mosquito.

Malaria causes a lot of deaths in Ghana as well as in many African countries. There is the need to detect the infection early. Early detection of the disease will prevent unnecessary presumptive treatment and reduce over-use of anti-malarial drugs. Also, detecting malaria usually requires the drawing of blood which is very painful, not easy

to get from children who are very sick and weak. To help with this problem, we need to study people who come to the hospital with malaria, take their blood and saliva and compare them to those without malaria to see if the factors produced during malarial infection can be found in the saliva. The purpose of the study is to find out if these factors are in the saliva of those who have malaria. If we can find the answer to this question, we hope to be able to suggest new ways of testing for malaria which is not painful.

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 16 years and adult should be between 16 and 78 years. If a participant agrees to be part of the study, venous blood and saliva sample will be collected from you for laboratory diagnosis.

Possible Benefits

There are no direct benefits to you from this study. However, your participation may help us to develop a better method for detecting malaria using saliva instead of blood. You will not be paid for participating in this study

Possible Risks

The amount of blood and saliva collected is harmless. There may be a slight pain and bruising at the bleeding site when taking the blood. Sterile techniques and disposable, single-use equipment will be used at all times.

Withdrawal from study

I would like to stress that, this study is strictly voluntary. Should any of you decide not to participate; it will have no consequences for him/her. Should you, at any point during the study, decide that you/ your children do not wish to participate any further, you are free to redraw with immediate effect. Any such decision will be respected without any further discussion. Your decision will not affect the care you would normally receive from the facility. You are free to contact me you have any concerns.

Confidentiality

All information gathered would be treated in strict confidentiality. I will protect information about your taking part in this research. You or your child will not be named in any reports.

Contacts: If you ever have any questions about the research study or study-related problems, you may contact Cecilia E. Lekpor at the Department of Pathology, Korle-Bu Teaching Hospital (0244780322).

Your rights as a participant

This research has been reviewed and approved by the Ethical Protocol and Review Committee of the college of Health Sciences (EPRC-CHS), University of Ghana. The EPRC 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

[Chairperson, Ethical protocol and Review Committee. College of Health Sciences, (Tel. +233(0)306665103/4 or email address:eprc@chs.edu.gh).

PARTICIPANT'S AGREEMENT

The above document describing the benefits, risks and procedures for the research title Angiogenic and Angiostatic factors in the saliva of malaria patients has been read and explained to me. Any questions and concerns about the research has been answered and addressed to my satisfaction. I/my child agree to participate in this research.

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

Date Signature or Thumbprint of participant

If a participant'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 participant. All questions were answered and the participant's Guardian/Parent has agreed to take part in the research.

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

Date Signature or Thumbprint of witness

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

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

Date Signature Person who obtained Consent

B. Consent for healthy controls

Title: **Angiogenic and Angiostatic factors in the saliva of malaria**:

Principal Investigator: **Cecilia Elorm Lekpor**

Address: Department of pathology, University of Ghana, 4236, Accra.

Information: (To be read or translated to participants/child's parent/guardians in their own mother tongue or their preferred language).

Dear Participant,

This consent form contains information about the research entitled *Angiogenic and Angiostatic factors in the saliva of malaria patients*. In order to be sure that you are informed about being part of this research, you will be asked 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). A copy of the signed form will be given to you. Please ask me to explain anything you may not understand.

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Malaria is caused by a germ that is passed from one person to the other by the bite of an infected female anopheles mosquito.

Malaria causes a lot of deaths in Ghana as well as in many African countries. There is the need to detect the infection early. Early detection of the disease will prevent unnecessary presumptive treatment and reduce over-use of anti-malarial drugs. Also, detecting malaria usually requires the drawing of blood which is very painful, not easy to get from children who are very sick and weak. To help with this problem, we need to study people who come to the hospital with malaria, take their blood and saliva and compare them to those without malaria to see if the factors produced during malarial infection can be found in the saliva. The purpose of the study is to find out if these factors are in the saliva of those who have malaria. If we can find the answer to this question, we hope to be able to suggest new ways of testing for malaria which is not painful.

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 16 years and adult should be between 16 and 78 years. If a participant agrees to be part of the study, venous blood and saliva sample will be collected from you for laboratory diagnosis.

Possible Benefits

There are no direct benefits to you from this study. However, your participation may help us to develop a better method for detecting malaria using saliva instead of blood. You will not be paid for participating in this study

Possible Risks

The amount of blood and saliva collected is harmless. There may be a slight pain and bruising at the bleeding site when taking the blood. Sterile techniques and disposable, single-use equipment will be used at all times.

Withdrawal from study

I would like to stress that, this study is strictly voluntary. Should any of you decide not to participate; it will have no consequences for him/her. Should you, at any point during the study, decide that you/ your children do not wish to participate any further, you are free to redraw with immediate effect. Any such decision will be respected without any further discussion. Your decision will not affect the care you would normally receive from the facility. You are free to contact me you have any concerns.

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Contacts: If you ever have any questions about the research study or study-related problems, you may contact Cecilia Lekpor at the Department of pathology, Korle-Bu Teaching Hospital (0244780322).

Your rights as a participant

This research has been reviewed and approved by the Ethical Protocol and Review Committee of the college of Health Sciences (EPRC-CHS), University of Ghana. The Ethical Protocol and Review Committee (EPRC) 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 [Chairperson, Ethical protocol and Review Committee, College of Health Sciences, (Tel. +233(0)306665103/4 or email address:eprc@chs.edu.gh).

PARTICIPANT'S AGREEMENT

The above document describing the benefits, risks and procedures for the research title Angiogenic and Angiostatic factors in the saliva of malaria patients has been read and explained to me. Any questions and concerns about the research has been answered and addressed to my satisfaction. I/my child agree to participate in this research.

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

Date Signature or Thumbprint of participant

If a participant'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 participant. All questions were answered and the participant's Guardian/Parent has agreed to take part in the research.

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

Date Signature or Thumbprint of witness

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

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

Date Signature Person who obtained Consent

APPENDIX II

QUESTIONNAIRE

SALIVA-BASED DIAGNOSTIC FOR TESTING, TREATING, & TRACKING (T3) MALARIA STUDY

T3 QUESTIONNAIRE

INTRODUCTION AND CONSENT

Hello. My name is Cecilia Lekpor_____. We are conducting a study called Saliva-Based Diagnostic for Testing, Treating, and Tracking (T3) Malaria. The purpose of this study is to be able to diagnose malaria using saliva instead of blood. We are asking if you will volunteer to participate in this study. If you choose not to participate it will not affect the services, you came in for today. If you choose to participate we will collect saliva and 4ml of venous blood from you. In addition, I would like to ask you some questions about you and your knowledge on malaria. The questions usually take about 10 to 15 minutes. All the answers you give will be confidential and will not be shared with anyone other than members of our study team. We hope you will agree to answer the questions since your views are important. If I ask you any question you don't want to answer, just let me know and I will go on to the next question or you can stop the interview at any time.

In case you need more information about the study you may call this number 0244533968 or 0244780322.

Do you have any questions?
May I begin the interview now?

SIGNATURE OF INTERVIEWER:_____ DATE:_____

RESPONDENT AGREES TO BE INTERVIEWED?	YES . . . . . 1 →	101
	NO . . . . . 2 →	END INTERVIEW

QUESTIONNAIRE NUMBER:_____

SECTION 1. RESPONDENT'S BACKGROUND

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	SKIP
101	Gender	MALE.....1 FEMALE 2	
102	In what month and year were you born?	MONTH . . . . . ☐☐ DON'T KNOW MONTH.....98 YEAR . . . . . ☐☐☐☐ DON'T KNOW YEAR 9998	
103	How old were you at your last birthday? COMPARE AND CORRECT 102 AND/OR 103 IF INCONSISTENT.	AGE IN COMPLETED YEARS ☐☐	
104	Have you ever attended or currently attending school?	YES .....1 NO . . . . . 2 →	106
105	What is the highest level of school you attended or currently attending?	PRIMARY .....1 JSS/JHS .....2 SSS/SHS .....3 HIGHER.....4	
106	Are you currently married or living together with a romantic partner as if married?	YES, CURRENTLY MARRIED. . . 1 → YES, LIVING WITH A ROMANTIC PARTNER.....2 → NO, NOT IN UNION.....3	201 201
107	What is your marital status now: are you single, widowed, divorced, or separated?	SINGLE .....1 WIDOWED.....2 DIVORCED.....3 SEPARATED 4	

SECTION 2. MALARIA

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	SKIP
201	Have you heard about malaria?	YES ..... 1 NO ..... 2 →	301
202	What transmits malaria?	RATS ..... 01 DOGS ..... 02 MOSQUITOES..... 03 HOUSEFLIES .....04 COCKROACHES..... 05 DON'T KNOW.....98 OTHER_____96	
203	Do you think malaria can kill if left untreated?	YES ..... 1 NO ..... 2 DON'T KNOW.....8	
204	What do you think are the most common signs and symptoms of Malaria infection? CHECK ALL THAT APPLY	FEVER.....01 ☐ LOSS OF ENERGY .....02 ☐ VOMITTING.....03 ☐ SWEATING..... 04 ☐ HEADACHE.....05 ☐ BODY PAINS.....06 ☐ ITCHING.....07 ☐ LOSS OF APPETITE. . . 08 ☐ CHILLS ..... 09 ☐ DIZZINESS .....10 ☐ DON'T KNOW.....98 OTHER_____96	
205	Do you think malaria can be prevented?	YES ..... 1 NO ..... 2 DON'T KNOW.....8	

NO.	QUESTIONS AND FILTERS	CODING CATEGORIES	SKIP
206	What personal protective measures do you use to guard against malaria infection? CHECK ALL THAT APPLY	REPELLENTS ..... 01 ☐ MOSQUITO COILS..... 02 ☐ SPRAY ..... 03 ☐ MOSQUITO NETS..... 04 ☐ CLOSE WINDOWS & DOORS ..... 05 ☐ WEAR PROTECTIVE CLOTHING ..... 06 ☐ GAUZE WIRE IN WINDOWS .....07 ☐ BURN COW DUNG/LEAVE..... 08 ☐ OTHER_____96	
207	At any time in the past 12 months, has anyone come into your dwelling to spray the interior walls against mosquitoes?	YES .1 NO ..... 2 DON'T KNOW.....8	
208	Does your household have any mosquito nets that can be used while sleeping?	YES ..... 1 NO . . . . . 2 →	212
209	How many months ago did your household get the mosquito net?	MONTHS AGO. .. . . . ☐☐ MORE THAN 36 MONTHS AGO ..... 95 DON'T KNOW.....98	
210	When you got the net, was it already treated with an insecticide to kill or repel mosquitoes?	YES ..... 1 NO ..... 2 DON'T KNOW.....8	
211	Did you sleep under this mosquito net last night?	YES ..... 1 NO 2	
212	Malaria is a serious and life-threatening disease	YES ..... 1 NO ..... 2 DON'T KNOW.....8	
213	Best way to prevent yourself from getting Malaria is to avoid getting mosquito bites	YES ..... 1 NO ..... 2 DON'T KNOW.....8	

APPENDIX III

ETHICAL CLEARANCE

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:.....

31st August, 2017.

Cecilia Elorm Lekpor
Department of Immunology
SBAHS
Korle-Bu, Accra

ETHICAL CLEARANCE

Protocol Identification Number: **CHS/2016-2017**

The Ethical and Protocol Review Committee of the College of Health Sciences approved your research proposal.

TITLE OF PROTOCOL: **"Angiogenic and Angiostatic Factors in the Saliva of Malaria patients"**

PRINCIPAL INVESTIGATOR: **Cecilia Elorm Lekpor**

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

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

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

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

This ethical clearance is valid till 31st August, 2018.

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

Signed: .....
PROFESSOR ANDREW A. ADJEI
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Dean, SBAHS
Head of Department

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APPENDIX IV

PREPARATION OF BUFFERS AND STANDARDS

I. Washing Buffer (1X wash buffer) For ELISA (Same for all biomarkers)

To prepare 2000.0ml of 1X washing buffer, 100ml of 20X wash buffer was added to a beaker containing 1900.0 ml deionized water and placed on a magnetic stirrer without heating and stirred until all is in solution.

II. Washing buffer 0.05% Tween 20 in PBS.

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

IV. Reagent diluent 1% BSA in PBS.

B. Giemsa Buffer for parasite staining

Na2HPO4 1.0g

KH2PO4 0.7g

Distilled water 1 litre

Colour Solution [TMB (3, 3', 5, 5'-Tetramethylbenzidine)]

The substrate solution, ready to use TMB (3, 3', 5, 5'-Tetramethylbenzidine) was obtained from the manufacturer (Kem-En-Tec Diagnosis A/S, Taastrup, Denmark) and added to plates at 100µl/well.

Stop Solution (0.2M H2SO4)

To prepare 1000.0ml of stop solution, 20.0ml of 10.0M H2SO4 was added to 980.0ml of deionized water and the solution shaken to mix. It was then cooled to room temperature and kept in the hood until required.