# IMMUNOLOGICAL CHARACTERIZATION OF A NOVEL MEROZOITE SURFACE PROTEIN (PFMSP11)


**BY**

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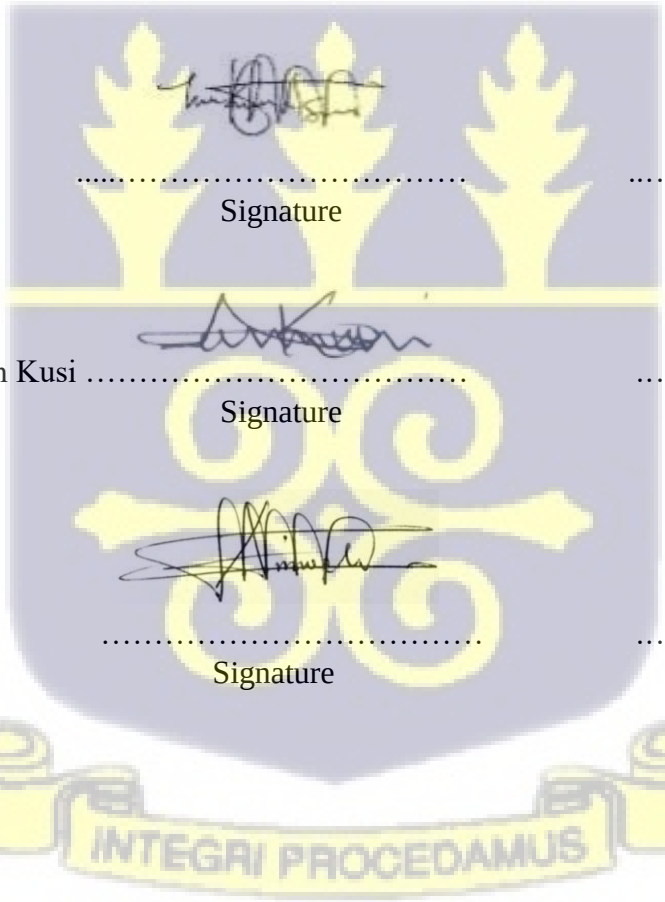
## DECLARATION

I, Kwame Asiedu, hereby declare that with the exception of references to other works, which have been duly acknowledged, this thesis is the result of my own research conducted at the Immunology

Department of Noguchi Memorial Institute for Medical Research, supervised by Dr. Kwadwo Asamoah Kusi at the Immunology Department, Noguchi Memorial Institute for Medical Research and Dr. Yaw Aniweh, at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana. Neither all nor parts of this thesis have been presented for another degree elsewhere.



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## DEDICATION

I dedicate this work to my parents for their unending support throughout my studies. I also dedicate this work to Prof. Gordon Awandare, Director of the West African Centre for Cell Biology of Infectious Pathogens, University of Ghana, and Pastor Enoch Boamah, of the Christ Embassy Youth Church, Airport City, for their support and mentorship over the years. I love you all. God bless you.

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## LIST OF ABBREVIATIONS

µl - microliters

aa - Amino acids

ACT – Artemisinin-based combination therapy

ALP - Alkaline phosphatase

AMA1 - Apical Membrane Antigen 1

APC - allophycocyanin

BD - Becton Dickinson

bp - base pair

CHMI - Controlled human malaria infection

CMI - Cell-mediated immunity

Con A - Concanavalin A

CSP - Circumsporozoites protein

CTL - Cytotoxic T cell

CVac - chemoprophylaxis vaccination

DBS - Dried Blood Spot

DCs – Dendritic cells

DDT - Dichlorodiphenyltrichloroethane

DMSO – Dimethyl sulfoxide

ELISpot - Enzyme linked immunospot

EtBr - Ethidium bromide

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

FITC - Fluorescein isothiocyanate

G6PD - Glucose 6 phosphate dehydrogenase

Hb - Haemoglobin

HBsAg - Hepatitis B virus surface antigen

HLA – Human leukocytes antigen

HVR - Hyper variable regions

IFN-γ - Interferon-γ

IgG - Immunoglobulin G antibodies

IL – Interleukin

iRBC – infected red blood cell

IRS - Indoor residual spraying

ITNs - Insecticide-treated nets

kDa - Kilo Dalton

LLINs - Long-lasting insecticidal nets

MACS - Magnetic-activated cell sorting

MHC - Major histocompatibility complex

min - minutes

ml - millilitres

MSP2 – Merozoite surface protein 2

NHS – Normal human serum

NK - Natural killer cell

PBMC - Peripheral Blood Mononuclear Cell

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline / Tween-20

Pf - *Plasmodium falciparum (P. falciparum*)

PfAMA1 - *Plasmodium falciparum* apical membrane antigen

PfRH5 - *Plasmodium falciparum* reticulocyte-binding Protein

RON - Rhoptry Neck Protein

RPMI - Roswell Park Memorial Institute

sfc/m – Spot forming cells/million

Tregs - Regulatory T cells

TCR – T cell receptor

Th1 - Type 1 helper

Th2 - Type 2 helper

TNF - Tumour necrotic factor

WHO - World Health Organization

## ABSTRACT

**Background:** *Plasmodium falciparum* merozoite surface protein 11 (PfMSP11) is a novel surface protein that plays a critical role during the parasite's blood stage development. This project aimed at immunologically characterizing the *Plasmodium falciparum* Merozoite Surface protein (PfMSP11).

**Method:** Antibody (IgG) responses from 283 child clinical malaria samples Accra, Kintampo and Navrongo which represents different transmission intensities, were measured against the 20 synthetic peptides of PfMSP1 using ELISA. Overall 27 synthetic peptides were tested against PBMCs from 10 apparently healthy subjects to measure IFN-γ responses using ELISpot.

**Results:** Peptides at the C-terminus of PfMSP11 gave higher antibody responses than peptides at the N-terminus. Peptide 2 to Peptide 12, and Peptide 17 recorded low antibody responses across all sites. Peptide 16 recorded the highest antibody responses across all sites. One subject (M14) showed a highest response across all pools in measuring IFN-γ responses . Subject M9 tested positive for pools 1 and 3 while subject M10 and M12 tested positive for pool 2Peptide 7 in pool 2 against subject M10 was the only peptide that tested positive.

In conclusion, this study has shown that there are more antibody epitopes at the C-terminus than the N-terminus and more immunogenic T cell epitopes at the N-terminus.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

*Plasmodium* parasites are the main cause of malaria which is a mosquito-borne disease of the tropics. *Plasmodium falciparum* is the most virulent among the five species that can affect humans. It causes most of the malaria-related deaths globally. According to the latest WHO Report, in 2019, there were still 229 million cases of malaria resulting to 409,000 deaths worldwide. Children below 5 years are the most susceptible to malaria and account for 67% of all malaria deaths (WHO, 2020). Current malaria intervension regimens includes drug treatment strategies and vector control. There is the need to improve on our attempts to eliminate this malaria infection as the current regimens is not sufficient to reduce malaria incidence rate (Ashley, Phyo, & Woodrow, 2018).

Sequencing of the *Plasmodium falciparum* genome has identified over 5,300 potential targets for drug and vaccine development (Gardner, et al., 2002). Prior vaccination strategies have been dissatisfactory even though a lot of work have been done in the elucidation of protective antigens (Miura, 2016). Opinions are diverse on how antibodies to blood stage antigens result in protection: inhibition of merozoite invasion into erythrocytes, triggering of the release of parasitostatic and parasitocidal substances by monocytes via antibody dependent cellular inhibition (ADCI) or inhibition of cyto-adherence of infected red blood cells to endothelial cells (Biryukov, et al., 2016).

Currently there are no effective malaria vaccines and as such new tools are required if we are to achieve levels of disease control and move toward elimination and eventual global eradication of malaria. The frequency of malaria infection can be reduced by vaccines that aim at targeting of sporzoite and erythrocytic stages via surface proteins. The RTS,S/AS01 vaccine based on *P. falciparum* circumsporozoite protein is the most studied vaccine. Merozoite-stage proteins are

used as vaccine targets because they are aimed to reduce asexual replication rate (RTS,S Clinical Trials Partnership, 2015). This protect the individual against the infection rather than produce sterile immunity. This leads to the acquiring of naturally immunity whiles protecting you from the severe forms of the disease.

### 1.2 Problem Statement

Some of the important targets of immune responses that play a role in protective immunity includes MSPs and invasion ligands . Antibodies specific to these targets are a vital element of protective immunity and have been an important research focus. Monocytes, macrophages, neutrophils are important components of cell-mediated immunity that are involved in antibody-mediated killing. The complexity associated with the vast array of antigens present on merozoite surface has made it complex to identify important targets of antibodies that provide protection or immunity. This complicacy has made it difficult to measure their relative importance. There is the need to understand more of the parasite biology, and this calls for more functional characterization of the genes with unknown function (Wilson, Flanagan, Prakash, & Plebanski, 2019).

### 1.3 Significance of The Study

The unsatisfactory results generated from past trials does not correspond to the huge efforts being invested in the various vaccine strategies.

*Plasmodium falciparum* merozoite surface protein 11 (PfMSP11) is a novel surface protein whose role in critical in the blood-stage development of the parasite. PfMSP11 consists of 476 amino acids and has a molecular weight of 58.35 kDa (Plasmodb). Polyclonal antibodies to PfMSP11 block merozoite invasion. PfMSP11 seems to be a junction marker and it is degraded after invasion. In this study, PfMSP11 was immunologically characterized using malaria clinical samples from children and healthy samples from adults. The immunogenicity of the protein was

evaluated using ELISA and ELISpot. ELISA and ELISpot was used to map out B cell and T cell epitopes respectively.

### 1.4 AIM

To immunologically characterize *Plasmodium falciparum* Merozoite Surface protein 11 (PfMSP11)

### 1.5 Hypothesis

1. *Plasmodium falciparum* Merozoite Surface Protein (PfMSP11) have B cell epitopes that can induce antibody responses
2. *Plasmodium falciparum* Merozoite Surface Protein (PfMSP11) have T Cell epitopes that can induce T cell responses

### 1.6 Specific Objectives

1. To evaluate the immunogenicity and map out B cell epitopes of PfMSP11
2. To evaluate the immunogenicity and map out T cell epitopes of PfMSP11

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Global Malaria Trends

Malaria is one of the leading causes of death, mainly in sub-Saharan Africa. Its causative agent is the plasmodium parasite. The female Anopheles mosquitoes act as the vectors of transmission of the disease. Among the five known species of parasites that infect humans, *P. falciparum* and *P. vivax* have been responsible for most malarial cases recorded (WHO, 2020).

In 2019, world malarial cases peaked at an estimated 229 million, with a death toll of 409, 000 in the same year (WHO, 2020). Statistics show that sub-Saharan Africa recorded the most cases, however, the Western Pacific, South-East Asia, Eastern Mediterranean and the Americas also are somewhat exposed to contracting the disease.

The risk levels vary among different populations, with children under 5 years being the most vulnerable. They also tend to be the most affected and account for 67% of global malarial deaths. Pregnant women, HIV/AIDS patients, immigrants from non-endemic countries, and other immune-deficient people are also highly susceptible to the severe forms of the disease. Measures have to be put in place to ensure these groups are well protected to reduce risk of contraction (WHO, 2020).

## 2.2 Clinical Presentations of Malaria

Symptoms associated with malaria usually vary slightly based on the stage of the disease. As the patient's condition progresses from mild to severe malaria, the symptoms of the conditions worsen and even causes death. Persons with mild malaria present with symptoms such as chills, sweats, fever, headaches, nausea and vomiting . It is important to know that all malarial symptoms are

associated with the bursting of erythrocytes and release of parasitic toxins at the blood stage of the parasite's life cycle. The release of these toxins such as haemozoin pigment into the blood causes the immune cells such as macrophages and neutrophils to release cytokines that trigger fever and other severe malarial symptoms . Usually, complicated or severe malaria is given the most attention due to the high mortality rates that has been associated with it. The major complications of severe malaria include cerebral malaria, pulmonary oedema, acute renal failure, severe anemia, and/or bleeding. The symptoms presented include abnormal behavior with loss of cognitive function, coma and seizures alongside other neurologic anomalies (Nanda, et al., 2012).

### 2.3 Malaria Prevention Methods

Malaria prevention is one of the top priorities of most developing countries. The impact that malaria, alongside other parasitic diseases exert on a nation's economy cannot be underrated. Aside loss of human lives, the economic costs include all the consultations and treatment processes that goes in hospitals for malaria patients (Erhun, Agbani, & Adesanya, 2005). As always, the best course of action which is the complete elimination of malaria is not only to develop effective vaccines but also to avoid contact with the vectors of transmission of the malaria parasite. Research conducted and experiences with malaria has proven that prevention of infection is a cheaper alternative to all the cost involved in its treatment (Tanner, et al., 2015). However, in places with low risks, preventive measures are often difficult to implement due to various beliefs of the inhabitants and the associated low risks (Winch, et al., 1994).

The use of insecticide-treated mosquito nets is one of the most effective methods in the quest for malaria elimination (Taremwa et al., 2017). In malaria-endemic areas, the use of treated mosquito nets has not been given a priority by the citizens (Taremwa et al., 2017). It prevents contact between the vectors of the disease transmission. A study conducted by the World Health

Organization in 2018 (World Health Organization, 2019) discovered that the use of insecticide mosquito nets reduced the risk of malaria infection by 50% of all the people in malaria-endemic areas (Pryce, Richardson, & Lengeler, 2018).

Malaria during pregnancy (MiP) is a major preventable cause of maternal and infant morbidity and mortality. The use of the combination medicine sulphadoxine-pyrimethamine (SP) during intermittent preventive treatment (IPTp), as well as appropriate case-by-case management based on malaria species, disease severity, age of gestation, drug availability, and local drug resistance patterns, have been suggested as prevention controls in pregnancy for malaria endemic areas. However, case management is hampered by a lack of available diagnostic tests, the use of artemisinin-based combination therapy in the second and third trimesters, and insufficient surveillance data on malaria in pregnancy across Sub-Saharan Africa (Sevene et al, 2010).

The use of insecticides can also play a role in the elimination of insect larvae (World Health Organization, 2019). Pools of water that are kept in malaria-endemic areas should be kept clean as they serve as breeding grounds for mosquito eggs and larvae. Spraying such places cuts short the mosquito's life cycle. The use of antimalarial is another prevention technique. People who take trips to places with high risks of malaria infection often take chemoprophylactic drugs that serve as a preventive measure against infection (World Health Organization, 2019).

**2.4 Immunity Against Malaria**

It is a relatively slower process of acquiring immunity to *P. falciparum* as compared to bacterial or viral pathogens. While children usually become immune to the most severe forms of disease after a few symptomatic infections, those living in malaria endemic areas develop immunity to clinical symptoms only after years of repeated infections (Huang et al., 2015). With time, the accumulative parasite exposure leads to immunity to clinical manifestations of the disease,

conferring the ability to maintain parasitaemia below a clinical threshold and preventing the onset of disease symptoms (Healer, Chiu, & Hansen, 2017).

Mechanisms elicited by the innate immune system of the human host to inhibit parasite growth are probably the reason for the low parasitaemia seen in acute *P. falciparum* infection (Perlmann & Troye-Blomberg, 2000). Innate immune responses to malaria infection contribute to the development of the disease and protective immunity. Early pro-inflammatory responses regulate anti-parasitic Th1 development and promote effector cell function for efficient clearance of the infection. Disease progression usually leads to the downregulation pro-inflammatory responses with a parallel upregulation of anti-inflammatory responses. This eventually results in a Th2 oriented response, leading to a downregulation of pro-/anti-inflammatory and Th1 response and resistance against pathogenesis. This however does not always occur as systemic and organ-related severe illnesses may be as a result of factors such as parasite sequestration and over upregulation of pro-inflammatory responses which dependent on the dynamics of host-parasite interaction (Huang et al., 2015).

The innate immune system is capable of detecting parasites proteins at the blood and liver stages, and this triggers the immune response by inducing the release of pro-inflammatory cytokines and chemokines. These cytokines prepare phagocytes to engulf and clear parasites. The chemokines facilitate recruitment of effector cells to sites, where parasites are sequestered or accumulated for effective infection clearance (Gowda & Wu, 2018).

Adaptive immune response against erythrocytic-stage *P. falciparum* infection is more sphisticated than other stages. The erythrocytic stage involves the release of merozoites from the liver cells to invade erythrocytes. At this stage, the targets are free merozoites and intraerythrocytic parasites and involves the production of specific immunoglobulins (IgG) to multiple antigens on the

parasite. The most abundant isotypes of IgG produced are IgG1 and IgG3, and these have been shown in some instances to give protection against the parasite. Humoral responses, which involves antibody production, target free merozoites, while T cell responses are important in controlling intraerythrocytic parasites. Merozoites released from liver cells can be opsinized for capture or to prevent invasion of RBCs by antibodies. Functions of antibody includes, facilitation cellular killing, blocking the adhesion of infected RBCs to endothelium. It also neutralizes parasite toxins to prevent excessive inflammation from occurring. Also antibody marks merozoites for lysis by complement. This stage can also be termed as proinflammatory cytokine response. This response stimulates macrophages. The involvement of cytotoxic T cells in the erythrocytic stage is of little consequence (Huang et al., 2015). CD4+ T helper cells are important in the production of pro-inflammatory cytokines that activate macrophages, and are also implicated in the activation of specific B cell clones. Others immune cells like NK cells and $\gamma\delta$T cells are also involved in the immune response as IFN-$\gamma$. Production of perforin and granzyme by NK cells play a role in killing of *P. falciparum* infected RBCs.

Host-derived antibodies from blood meals are also involved in the killing of gametocytes through complement. Gamete fusion in the anopheles mosquito can also be prevented by host-derived antibodies. Gametocytes can be killed by nitric oxide produced by macrophages (Belachew, 2018)

**2.5 T cell interaction with peptide-bound HLA**

The presence of intracellular and extracellular pathogens in the body determines the type of immune response elicited and the pathway used in that regard. Humoral and cell-mediated responses are the main adaptive response types. Whereas antibodies secreted by B cells bind to specific epitopes on the antigen's surface, for complete T cell activation, the TCRs need to bind to the antigens that have been complexed with Human Leucocyte Antigens (HLA), the human major

histocompatibility complex (MHC) molecules. T cells have the ability to recognize amino acids of antigenic peptides at specific positions that have been bound and presented by the HLA I and II molecules (Corse, Gottschalk, & Allison, 2011). Polymorphism is known to have a major effect on the type of T cell responses generated against antigenic peptides, and all potential vaccines based on these antigens may be affected (Nlinwe, Kusi, Adu, & Sedegah, 2018). HLA class I molecules are found in all nucleated cells and are used to present antigenic peptides emanating from infected host cells. These peptide-bound HLA types are recognized by CD8+ cytotoxic T cells which undergo proliferation, differentiation and clonal selection to produce effector cytotoxic T lymphocytes (CTLs) that are more effective at killing pathogen-infected cells (Wieczorek, et al., 2017). Extracellular pathogens that have been digested and processed by professional antigen-presenting cells (APCs) are complexed and presented on HLA II molecules. These complexes are recognized by CD4+ helper T cells that function through the activation of B cells, cytotoxic T cells and macrophages through the release of cytokines.

The products of T cell activation can cause harmful effects to the host's cells if not established at the appropriate times. This activation is enhanced through other co-stimulatory molecules that must also bind at the HLA-peptide-TCR complex sites in order to stabilize the binding and complete the activation of T cells.

**2.6 Cytokine responses to malaria**

Cytokines are a key component of the host's immune response to malaria parasites. Both the adaptive and innate immune systems are involved in immune regulation via the secretion of the polypeptides or glycoproteins known as cytokines. Upon early infection, the innate immune system releases cytokines that activate and signal other immune cells such as neutrophils and

macrophages to the site of the body with high parasitemia. This is soon followed by the adaptive immune system with more specialized cells and responses.

Most of the cytokines that are secreted in response to pathogens are produced by CD4+ helper T cells (Luckheeram, Zhou,, Verma, & Xia, 2012). The activation of a helper T through binding to MHC class II complexed with antigenic peptides causes the production of T helper (Th0) which further differentiates into T helper types 1 and 2. T helper type 1 cells are known to produce interleukin-12 (IL-12), lymphotoxin-α and IFN-α/γ. The cytokines produced by T helper type 1 cells help to signal CD8+ T cells and B cells to act against intracellular pathogens such as *plasmodium falciparum*. However, the type 2 helper T cells release IL-4, 5, 6, 9, 10, and IL-13. These tend to activate B cells to act against extracellular pathogens. In acute malaria cases, increased levels of IFN- γ in the blood is usually associated with a reduction in parasitemia (Winkler, et al., 1999).

**2.7 Human genetics and immune response**

Invasion of the RBC is a critical component of the life cycle of malaria parasites, and thus any disorder that affects RBC tend to have an impact on malaria. When the merozoites leave the hepatocytes, they are released into the blood where they invade the RBC and multiply, causing the production of more merozoites upon rapture. In genetic disorders such as G6PD deficiency, the body lacks the glucose 6 phosphate dehydrogenase enzyme that protects the RBC from oxidative stress and they are therefore destroyed more easily (Mombo, et al., 2003). In ovalocytosis, where an abnormally large number of the RBC have adopted an oval shape, the disease has been known to offer some resistance against cerebral falciparum malaria (Genton, et al., 1995). Hemoglobin variants cause diseases such as sickle cell anemia with abnormally-shaped RBC (Chotivanich, et al., 2002). Individuals with blood group O tend to have a reduction in rosetting and sequestration

of the infected RBC. This prevents malaria-infected RBCs from affecting more uninfected RBCs at an extremely faster rate (Rowe, et al., 2007). People with RBC disorders have some form of protection against malaria.

**2.8 Vaccines**

Vaccines are biological substances that are designed to increase the immune response of the body to foreign pathogens and effectively eliminate them. Vaccines provide active acquired immunity to a wide variety of infectious diseases caused by viruses, bacteria or even parasites. They contain biological agents that bear close resemblance to disease-causing microorganisms and are usually made from less-harmful or killed forms of the microbes, proteins found on their surfaces, or toxins they release. These less harmful forms tend to be good enough to stimulate an immune response but are too weak to cause any major havoc to the biological system it is made to invade. Vaccines are not used equally for everyone. The type of vaccine used depends on varying factors such as the type of pathogen and underlying health conditions that may have rendered the recipient immunodeficient.

According to the United States Centers for Disease Control and Prevention (A CDC framework for preventing infectious diseases, 2011), vaccine administration was one of the most effective ways that caused the successful worldwide elimination of smallpox and restricted the spread of tetanus, polio, measles and other life-threatening diseases. Aside lifestyle practices, help in vaccines are usually one of the foremost prevention methods of novel viral infections such as Ebola and the coronavirus. Major biotechnology companies all around the globe attempt different approaches to achieve the same purpose of developing vaccines for infectious diseases. Developing a malaria vaccine is one of the challenges researchers have battled with for some time. The complexity of the parasite's life cycle and the immune responses towards its toxins has not

been well understood and hence the difficulty of developing an effective malaria vaccine (Ouattara & Laurens, 2015).

There are several different approaches to vaccine design. These include making use of the viral nucleic acid (DNA or RNA), surface proteins of the pathogen or those they release during their invasion, or a dead or weakened form of the pathogen. All these approaches have their pros and cons, and reflect the different vaccine types based on the particular viable candidate used.

### 2.9 Vaccine Candidates

Live attenuated vaccines use live organisms that have gone through processes that renders it non-virulent. Some examples include pathogens that cause smallpox, chicken pox and yellow fever. This method is often preferred as it is one of the most efficient ways of providing immunity because of the use of live organisms. The attenuated pathogen replicates and exerts the similar effects on the host, eliciting a strong response and producing large quantities of immunogens from the pathogen (Mooney et al., 2015). This response, along with the stimulation and of production of T-cells and memory B cells, produce a stronger secondary response upon invasion by the actual deadly pathogen. However, the tendency of the weakened pathogen to undergo mutation and revert causing a real infection steers scientists away from employing this method in some cases (Sinha & Bhattacharya, 2014). The use of a killed or inactivated organism in place of live ones results in extra costs as adjuvants will have to be used to enhance of the immunogenicity of the vaccine. Such vaccines are used for viral infections such as hepatitis A, flu, polio and rabies. Radiation, heat or chemicals are used to kill pathogens in this method (The original, 2017).

In the production of toxoid vaccines, bacterial toxins (e.g. tetanus or diphtheria) or the toxins produced from other pathogens is made harmless and injected into the body to stimulate an immune response. However, this method is only effective if the disease is solely caused by the

exotoxin and also requires that that pathogen causative agent be managed separately (Yang et al., 2016). Other vaccine types include subunit, recombinant, polysaccharide and conjugate vaccines. Conjugate vaccines are created by linking the outer polysaccharide coats of the pathogen to a protein in order to boost its immunogenicity. Subunit vaccines use a fragment of the pathogen to stimulate an immune response and examples include vaccines against hepatitis B virus which uses the viral surface proteins (Yang et al., 2016). This class of vaccine makes use of specific parts of the pathogen to give a very strong immune response and hence makes a good vaccine. These vaccines can also be used on immunodeficient people as the biological substances used have non-virulent properties.

### 2.10 Pre-Erythrocytic Vaccines

The sporozoites released through an infective bite of the female anopheles' mosquito invade the hepatocytes and during their maturation, they release spores into the surrounding medium. (Nahrendorf, Scholzen, Sauerwein, & Langhorne, 2015) These spores cause the induction of specific immune cells eliminate the liver cells cells that have been infected or block the formation of merozoites. Pre-erythrocytic vaccines use parasite toxins to cause the production of a primary immune response in anticipation of the real parasite (Zheng, et al., 2019). However, most of the current research trends have been focused on the vaccine development targeting parasite proteins like *Pf* circumsporozoite protein (PfCSP), the liver stage antigens (LSAs) and the thrombospondin-related adhesion protein (TRAP) (Zheng *et al*., 2019).

The *Pf* circumsporozoite protein (PfCSP) is a surface protein that exists on mature sporozoites and helps in the invasion of the hepatocytes (Pringle, Carpi, Almagro, & al, 2018). DNA vaccines are usually not excellent immunogens. The DNA vaccine gp96NTD-CSP was designed and in order to boost its immunogenicity, the Heat Shock Protein (HSP) was also designed to stimulate the

maturation of dendritic cells and cause cross-antigen presentation. The DNA vaccine component gp96NTD-CSP complexed with HSP enables the vaccine to be effectively delivered to MHC class I proteins, thereby causing the activation and stimulation of cytotoxic $CD8^+$ T-cells. In a study conducted by Tan *et al.,* (Malaria DNA vaccine gp96NTD-CSP elicits both CSP-specific antiantibody., 2015) , they concluded that the gp96NTD exhibited high adjuvant properties and boosted its immunogenicity by causing a high production of specific antibodies against CSP and enhance $CD8^+$ T-cell response.

**2.11 Erythrocytic stage vaccines**

Global malaria eradication is one of the set targets of health science. The science aims to achieve increasing levels of disease control, elimination and eventually the total eradication of the malaria on a global scale. Vaccination is one of the key tools that has shown great potential towards the achievement of this goal. Varying approaches used in different disciplines tends to target specific stages of the parasite's life cycle.

The pre-erythrocytic (sporozoite and liver) stage of the parasite's development is one of the most explored areas in vaccine development. It possesses the potential to prevent invasion of the RBC and reduce parasitemia and spread of the parasite's merozoite form. Vaccines that target the sporozoite stages prevent initial invasion of the hepatocytes. However, the complication that exists with this approach is that if a single sporozoite escapes the immune system, the entire cycle can still proceed, as any immunity developed against the various forms is stage-specific (Felgner, et al., 2013). A fully functioning pre-erythrocytic malaria vaccine should be able to prevent the establishment of the blood stage forms, which are more challenging to eliminate due to their rapid multiplication, immune evasion capabilities and adaptations their environment (Nahrendorf et al., 2015).

Vaccines that target the blood stage have the tendency to prevent disease and death as malarial symptoms develop at this stage. The infected RBCs releases more merozoites in addition to other parasitic antigens that produce malarial symptoms such as increased body temperature and chills. The blood stage vaccine would prevent invasion of the RBC, maturation of infected RBC and prevents the release of more merozoites from the matured trophozoites. However, they are limited in their inability to prevent spread of the parasites due to the large amounts of merozoites released from each infected RBC (Das et al., 2015).

Vaccines that target the sexual stages (formation of gametocytes during the blood stage), or transmission blocking vaccines, have the potential of reducing or halting transmission, but has little or no effect on the parasitemia or disease symptoms.

Another vaccine approach that has showed the most promise is the combination of antigens expressed at different stages of the parasite's life cycle (Theisen, et al., 2014). The addition of the blood stage integrant to an anti-sporozoite vaccine decreases disease and death risks caused by high parasitemia and reduces transmission rates while providing some insurance against future outbreaks. The antigens that have been used in multi-stage vaccines include CSP from sporozoites (Cohen, Nussenzweig, Nussenzweig, Vekemans, & Leach, 2010), PfRh5 on the merozoites (Douglas, et al., 2015), Pfs25, 48/45, and 230 on the midgut stages in the life cycle and AMA-1, which has been shown to be expressed on the merozoite and sporozoite stages.

### 2.12 Advancements in Malaria Vaccine Development

As malaria continues to be one of the leading causes of death around the globe, the World Health Organization (WHO) in 2015 announced and endorsed a new global approach for eliminating malaria within a period of 15 years starting with at least 10 malaria-free countries by 2020 (WHO). Research towards malaria vaccine development usually involves whole organisms (P. *falciparum),*

subunit vaccines, live attenuated vaccines and genetically modified vaccines (Zheng *et al*., 2019). However, a widely accepted vaccine is yet to be produced. There have been some recent studies that tend to investigate the effects *Pf* sporozoite (SPZ) vaccines which have shown great promise but needs larger sample sizes for testing and validation (Mordm¨uller, Surat, & Lagler, 2017). *Plasmodium falciparum* sporozite (PfSPZ) Vaccine is a vaccine candidate that is metabolically active, non-replicating, whole malaria sporozoite vaccine that has been reported to be safe and offers protection gainst *P falciparum* controlled human malaria infection in malaria-naive individuals. In a study by Sissoko, *et al*., (2017) the PfSPZ Vaccine showed significant protection in African adults against *P falciparum* infection throughout an entire malaria season.

The most extensively tested vaccine candidate for prevention of *P. falciparum* malaria is RTS,S. This vaccine directs immune responses against the major circumsporozoite protein (PfCSP) covering the surface of the infecting sporozoite (RTS,S Clinical Trials Partnership, 2015). The protection associated with this vaccine is short-lived and diminishes after been vaccinated for the first year. Adverse events were also associated with the vaccine after vaccination (Olotu, Fegan, & Wambua, 2016).

### 2.13 Merozoite Surface Proteins

Proteins or ligands involved in invasion of erythrocytes are mostly located on the surface of merozoite. Rhoptries and micronemes are organelles that contain these ligands at the merozoite's apex. Merozoite surface proteins are attached to the merozoite cell membrane as either integral membrane proteins, glycophosphatidylinositol (GPI)-anchored proteins or as peripherally-associated (Das et al., 2015)

MSP1 is synthesized as a precursor of ~196 kDa and crucial to the blood-stage development of the parasite. It is processed into four subunits by a subtilisin-like protease shortly before the infected

erythrocyte ruptures at the end of the 48-hour replicative cycle to release merozoites (Child, Epp, Bujard, & Blackman, 2010). The MSP has a spectrin-binding function which is activated when processed. This leads of the rupture of the cell by destabilizing the membrane skeleton of the host erythrocytes (Das, S. et al., 2015). The GPI anchored p19 fragment attached to the invading parasite as most of the MSP1 is shed from the surface of merozoite (Blackman, Heidrich, Donachie, Mcbride, & Holder, 1990). MSP1 is also presented on the nascent merozoites during pre-erythrocytic liver stage development of P. *falciparum*.

MSP1 elicits a humoral immune response in natural infections and research have established a correlation between antibodies against MSP1 and protection from clinical malaria (Fowkes, Richards, Simpson, & Beeson, 2010). Blood stage parasitemia can be controlled by MSP1 antibody titres via blocking the invasion of RBCs by merozoites and/or intraerythrocytic development (Woehlbier, Epp, Hackett, Blackman, & Bujard, 2010) and activation of complement (Boyle, et al., 2015). CD8+ T-cell-mediated cellular immune responses can be stimulated against liver stage parasites by MSP1 (Joos, et al., 2015). MSP1 vaccine has shown efficacy in animal studies (Etlinger, et al., 1991), however, immunization of humans using MSP1 was unsatisfactory and could not illustrate protection against the malaria infection (Ogutu, et al., 2009).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Materials

Phosphate buffer saline (PBS), ELISA plates, Tween 20 (cat# P7949 lot# SZBB 2070V/ E0630V, France), sodium azide (S-8032 Lot 32K2525 SigmaUltra [26628-22-8]- Germany), KPL Affinity purified Antibody Peroxidase Labelled Goat anti-human IgG (H+L) (Cat# 5220-0330, Lot# 10266871 from SeraCare, USA), 1-Step™ Ultra TMB ELISA (ref: 34029 Lot# UG2815571 Thermo Scientific- USA), sulfuric acid (258105 Lot# BCBD2634V Sigma Aldrich-Germany), ELISA plates, sodium chloride (Batch# 11I260017 Lot# 27810 297 Prolabo- Belgium), potassium chloride (60128-1KG-F Lot# BCBM0336V Sigma Aldrich-Germany), sodium phosphate (S93901K Lot# SZBC3620V Sigma Aldrich-Japan), potassium phosphate (P9791 SLBL2497V, Sigma Aldrich-Japan).

Biotinylated anti-human IFN-gamma mAb-1 (3420-6-1000 Lot#472), foetal bovine serum (F9665 Lot# 039K3396, Sigma Aldrich-USA), streptavidin ALP (33108 Lot#41075-8), AP conjugate substrate kit was obtained from Bio Rad Laboratories (USA), distilled water, isolated PBMC, Penicillin streptomycin Solution (15140-122Lot#2108968), HEPES 1M (156301080 Lot# 2120919,gibco-Taiwan), L-glutamine 200Mm (25030081 Lot#624236) Tween 20 (P7949 Lot#SZBE3240V), Trypan blue, Dimethyl sulfoxide (DMSO), Concanavalin A (Con A), RPMI-1640, normal human serum (H4522 Lot#SLBD2992V), ficoll-plaqueTM Plus (17144003 Lot# 10282407, GE Helthcare-Sweden), ELIspot plate, sodium carbonate, sodium bicarbonate, Phosphate buffer saline tablets (18912-014 Lot#2165327-UK)

Balance, pH meter, mega mixer, vortex, cell counter (Countess II FL Automated Cell Counter, Invitrogen, USA), NALGENETMCryo1$^{0}$C (ThermoFisher Scientific, USA), automated ELISpot plate reader (AID GmbH, Germany) ELISA reader & Washer

### 3.2 Methods

#### 3.2.1 Study Sites and Population

The study was conducted with samples from Kintampo, Accra, Navrongo.

Kintampo is located in a forest region and also fairly known for its malaria holo-endemicity. The transmission rate of malaria in Kintampo is always recorded to be at its peak all-year round with entomological inoculation rates (EIRs) of >250 infective bites/person/year. Navrongo, which is located in the Savanna region with seasonal hyperendemic transmission that is dependent on rainfall patterns, with EIRs of <250 infective bites/person/year. The average annual rainfall for Kintampo and Navrongo are 1345 mm and 1093.8 mm respectively (Owusu-Agyei, Asante, & Adjuik, 2009).

The average annual rainfall for Accra is below 1000 mm. Malaria transmission follows rainfall patterns and most malaria admissions to health facilities occur between May and August. Malaria transmission is generally lower than that in Navrongo and Kintampo (EIR, <50 infective bites/person/year) (Klinkenberg, McCall, Wilson, Amerasinghe, & Donnelly, 2008) but has a peak during the early rainy season, from June to August.

### 3.2.2 Overlapping Peptide Sequences of PfMSP11

| Peptide | Peptide Pool Composition | Peptide Sequences |
|---|---|---|
| 1 | 1 | CYEFSSLKRIFLDNHKINNNNSNNNNNNNNN |
| 2 | | CKINNNNSNNNNNNNNNNNNNNINNDWHTIYK |
| 3 | | CNNNNINNDWHTIYKIQKEKQNGYSEKKNNN |
| 4 | | CIQKEKQNGYSEKKNNNIYTNIMNDKDGKIY |
| 5 | | CIYTNIMNDKDGKIYLNTKSIINNYKEYIKN |
| 6 | 2 | CLNTKSIINNYKEYIKNTYIYYNTFFKYVKY |
| 7 | | CTYIYYNTFFKYVKYFITKIKNYNVIYEPYI |
| 8 | | CFITKIKNYNVIYEPYIILEQKSKTHISINN |
| 9 | | CILEQKSKTHISINNKKLVIQFLPPINKGTH |
| 10 | | CKKLVIQFLPPINKGTHILHNKTNDSIGNVL |
| 11 | 3 | CILHNKTNDSIGNVLNDFHNKLVKRRVLLAL |
| 12 | | CNDFHNKLVKRRVLLALMKRKKKDKRKSKLA |
| 13 | | CMKRKKKDKRKSKLAKKKKLKDKKKKKKKKS |
| 14 | | CKKKKLKDKKKKKKKKSKKDKQKKKKKKKKK |
| 15 | | CKKDKQKKKKKKKKKKKKNKNIKKNKKNKLR |
| 16 | 4 | CKKKNKNIKKNKKNKLRKNKKDKYEDDEYDD |
| 17 | | CKNKKDKYEDDEYDDQVDVMYDENNERKRKR |
| 18 | | CQVDVMYDENNERKRKRNKRRKKNKYNTKND |
| 19 | | CNKRRKKNKYNTKNDKLIMEEDSLHEHDDYD |
| 20 | | CKLIMEEDSLHEHDDYDENMNYHINEDEEER |
| 21 | 5 | CENMNYHINEDEEERRNYDHDNHYDDDDDIN |
| 22 | | CRNYDHDNHYDDDDDINYKDHYYKKNKKNKQ |
| 23 | | CYKDHYYKKNKKNKQKKKKNNKRRRKRRKKG |
| 24 | | CKKKKNNKRRRKRRKKGNNNNYDEHEDYDDD |
| 25 | | CNNNNYDEHEDYDDDHKNHDTHHDDEAEELEEKKHELE |
| 26 | | CHKNHDTHHDDEAEELEEKKHELEEINDIVNELTSKKR |
| 27 | | CEINDIVNELTSKKRLLKNAEHHMLKLLKDIHSLETKR |

*Color coding shows how the peptides overlap

### 3.2.3 Enzyme Linked Immunosorbent Assay

ELISA plates were coated by adding peptides (100µl/well) at 1µg/ml using 1X PBS as a diluent prior to overnight incubation in a 4°C fridge. Plate washing was done using a wash buffer (PBS with 0.05% Tween 20). Each wash cycle consisted of 3 washes with a soaking step during the second wash. The plates were blocked for one hour and at room temperature using 200µl/ml of 3% milk and incubated for one hour at room temperature. After blocking, the plates underwent another washing cycle (3) and were then incubated with 100µl/well of plasma samples at a dilution of 1/400 and 100µl/well of the standard at an optimized concentration for one hour. The plates were washed once more, and detection was done by incubating Peroxidase labelled Goat anti-human IgG (H+L) at 1/5000 dilution in the plate. 100µl/well of the substrate (TMB) was added to the plates after another washing cycle and incubated for ten (10) minutes; the reaction was stopped by adding 0.2 N sulfuric acid. The plates were read using Multiscan FC microplate reader at a wavelength of 450 nanometers.

### 3.2.4 Isolation Of PBMCS

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin anticoagulated blood by gradient centrifugation using Ficoll-plaque ™ plus density gradient media (density 1.077). Each participant's heparin anticoagulated blood was pooled into 50ml Falcon tubes and diluted with RPMI 1640 (dilution 1:1) containing 1% penicillin-streptomycin. 25ml of the RPMI-diluted blood was used to overlay 15 ml of Ficoll-plaque™ in another 50ml Falcon tube without mixing. This was then centrifuged for 10min at 2000 rpm at $24^0$C without breaks. The uppermost layer, mostly made up of plasma and RPMI 1640, was removed using serological pipette, leaving the mononuclear cells layer, which was then carefully transferred into a new 50 ml falcon tube and

washed with RPMI 1640 supplemented with 1% Hepes, 1% L-glutamine, 1%pen-strep and 5%FBS, and spinning at 2000 rpm, for 10 minutes, the washing was done three (3) times, and the pellet was re-suspended with HR10 containing 1% Hepes, 1% L-glutamine, 1% pen-strep and 10% normal human serum. A 10 µl aliquot of the cell suspension was added to an equal volume of 0.4% Trypan blue. Cell viability and concentration were determined using an automated cell counter.

### 3.2.5 Cryo-preservation of PBMC

The isolated PBMCs were spun at 1500 rpm for 7 minutes. The supernatant was then removed, and the cell pellets were carefully loosened by tapping the bottom of the Falcon tube with the index finger. The cells were then mixed with 90%FBS and 10% DMSO (freezing mix). The addition of the freezing mix was gently done while swirling the tube, and the cells were stored at 20 million per vial using a NALGENE™Cryo1$^0$C freezing container in a -80 $^0$C freezer overnight before transfer into liquid nitrogen tanks where it is stored till needed.

### 3.2.6 Recovery of frozen PBMC from liquid nitrogen

PBMCs were retrieved from the liquid nitrogen tanks and rapidly thawed at 37 $^0$C in a water bath for 2 minutes. They were quickly transferred into 30ml of pre-warmed washing buffer in a 50 ml falcon tube and centrifuged at 2000rpm for 10 minutes. The pellet was loosened by tapping the bottom of the tube with the index finger after discarding the supernatant. The cells were then re-suspended into 10 ml of complete medium. A 10µl of 0.4% Trypan blue was mixed with 10µl of aliquoted cells in an Eppendorf tube (dilution 1:1), and cell viability and concentration were determined using an automated cell counter. The cells were rested in an incubator for 20 hours at 37 C in 5% $CO_2$. The following day, the cells were washed in 15 ml washing buffer, and viability and concentration were re-determined and adjusted to a final concentration of 400,000 cells/ml before ELIspot assay.

### 3.2.7 Ex-Vivo ELISpot IFN-γ Assay

ELISpot assay was performed as described by (Ganeshan, et al., 2016). The ELIspot plates were coated with anti-human IFN-gamma antibodies at 15µg/ml, 100µl/well diluted into 0.05M bicarbonate buffer (sodium bicarbonate and sodium carbonate) of pH 9.6. The plates were incubated at 4°C in a fridge overnight and washed three times (250µl/well) with plain PBS (sterile). The plates were blocked for two hours using 200µl/well of HR10 (complete medium and 10% normal human serum). duplicate wells for each individual containing 100µl/well of cells (400,000 cells/ wells for each individual) were tested against 100µl of pools/peptides (20 peptides were put together 5 pools of 5 peptides, 10 µg/ml of each peptide in all pools). Background responses were assessed by using negative controls (PBMCs incubated with culture medium (HR10)). The plates were then kept at 37°C, 5% $CO_2$ concentration for 40 to 42 hours in the incubator. The plates were washed three times with 250µl/well of PBST (PBS and 0.05% Tween 20) then incubated for three hours with 100µl/ml of 1µg/ml biotinylated anti-IFN-γ monoclonal antibody. Afterward, the plates were washed three times with PBST, and 100µl/well of 1µg/ml alkaline-phosphatase-conjugated streptavidin was added and incubated for an hour. The plates were washed once again: three times with PBST and three times with PBS before incubation with an enzyme-specific chromogenic substrate for fifteen minutes for color development. The number of IFN-gamma producing cells /well (as evidenced by spots) were estimated using an automated ELISpot plate reader. The acquired data was finally exported into Microsoft Excel for analysis.

A **positive assay** will be defined by; at least a doubling of sfc in test wells relative to control wells, and at least 10 spots difference between test and control wells

# CHAPTER FOUR

# RESULTS

## 4.1 Demographics Characteristics of Study Subjects

The study involved two sets of different samples for ELISA and ELISpot assays. Samples from children were used for ELISA whiles adult samples were used for ELISpot.

A total of 283 child samples from three sites were used for the ELISA assay (table 4.1). Out of the 283 study participants, 105 of them were from Accra, 97 from Kintampo and 81 from Navrongo. Out of the total number of participants, 127 were females and 156 were males. There was no statistical difference in the gender across the three sites ($p$ = 0.63). Parasite density in Kintampo was significantly higher than those in Accra ($p$ <0.0001) and Navrongo ($p$ <0.0001). Parasite density levels between Accra and Navrongo were however not significantly different. There was no statistical difference in the haemoglobin levels ($p$ = 0.16).

**Table 4.1 Demographical Characteristics of Study Participants**

| Characteristics | Accra (n=105) | Kintampo (n=97) | Navrongo (n=81) | Total (n=283) | P value |
|---|---|---|---|---|---|
| Age, mean ± SD | 6.2 ± 3.3 | 5.3 ± 3.2 | 4.8 ± 2.7 | 5.4 ± 2.9 | < 0.0001 |
| Female sex, no. (%) | 46 (43.8) | 40 (41.2) | 41 (50.6) | 127 (44.8) | |
| Male sex, no. (%) | 59 (56.2) | 57 (58.8) | 40 (49.4) | 156 (55.2) | 0.6342 |
| Parasitemia level, parasites/µL, mean ± SD | 40,776.4 ± 42,523 | 145,262.6 ± 216,074.1 | 1,020.1 ± 786.8 | 61,006.5 ± 129,158.6 | < 0.0001 |
| Hemoglobin level, g/dL, mean ± SD | 10.24 ± 1.78 | 9.832 ± 1.751 | 9.943 ± 1.803 | 10.005 ± 1.778 | 0.1635 |

SD = Standard deviation, n = number of participants

### 4.2 Antibody Responses against all peptides

A heat map of antibody responses against all peptides in samples from all the study sites is shown in Figure 4.1. Peptide 2 to Peptide 12, and Peptide 17 had low antibody responses across all sites. Peptide 16 recorded the highest antibody responses across all sites. Peptide 19 recorded the second highest response. Peptides against Navrongo samples recorded the least responses. However, Peptide responses were highest against Kintampo samples. Individual peptide responses across all sites were compared (fig 4.2). Kintampo recorded the highest response to every peptide except pep 19 where Accra recorded the highest response. There was significant difference in peptide levels between the sites ($p<0.0001$).

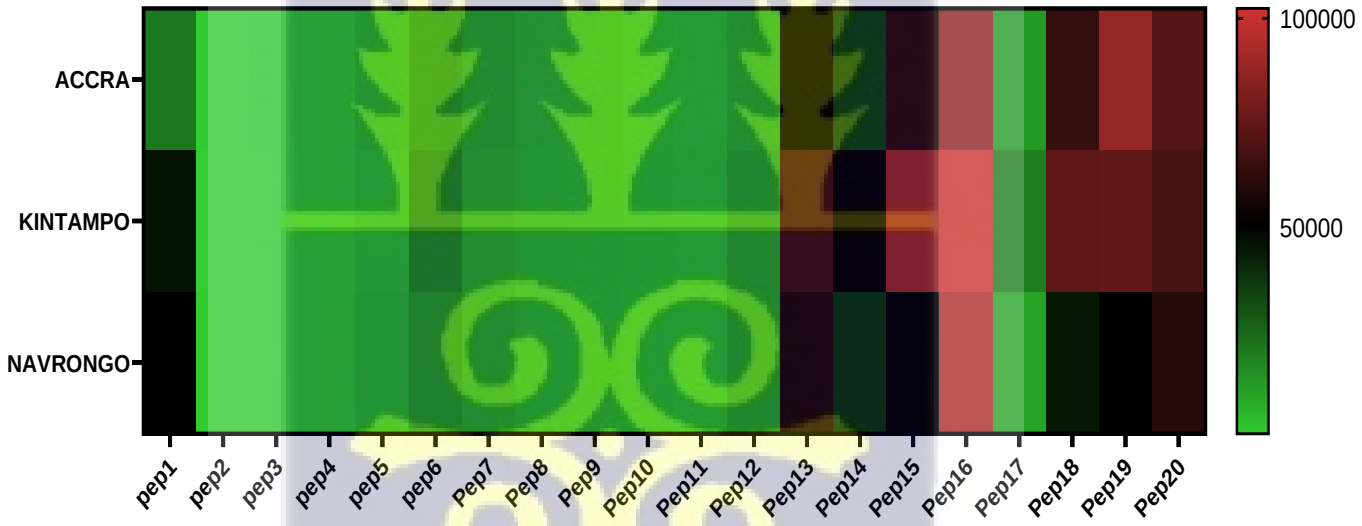



**Figure 4.1: Peptides responses across all sample sites.** This is a heatmap of values where antibody levels are depicted by color intensity. Sites indicated in the vertical axis (Accra, Kintampo and Navrongo) and individual peptides arranged on the horizontal axis.

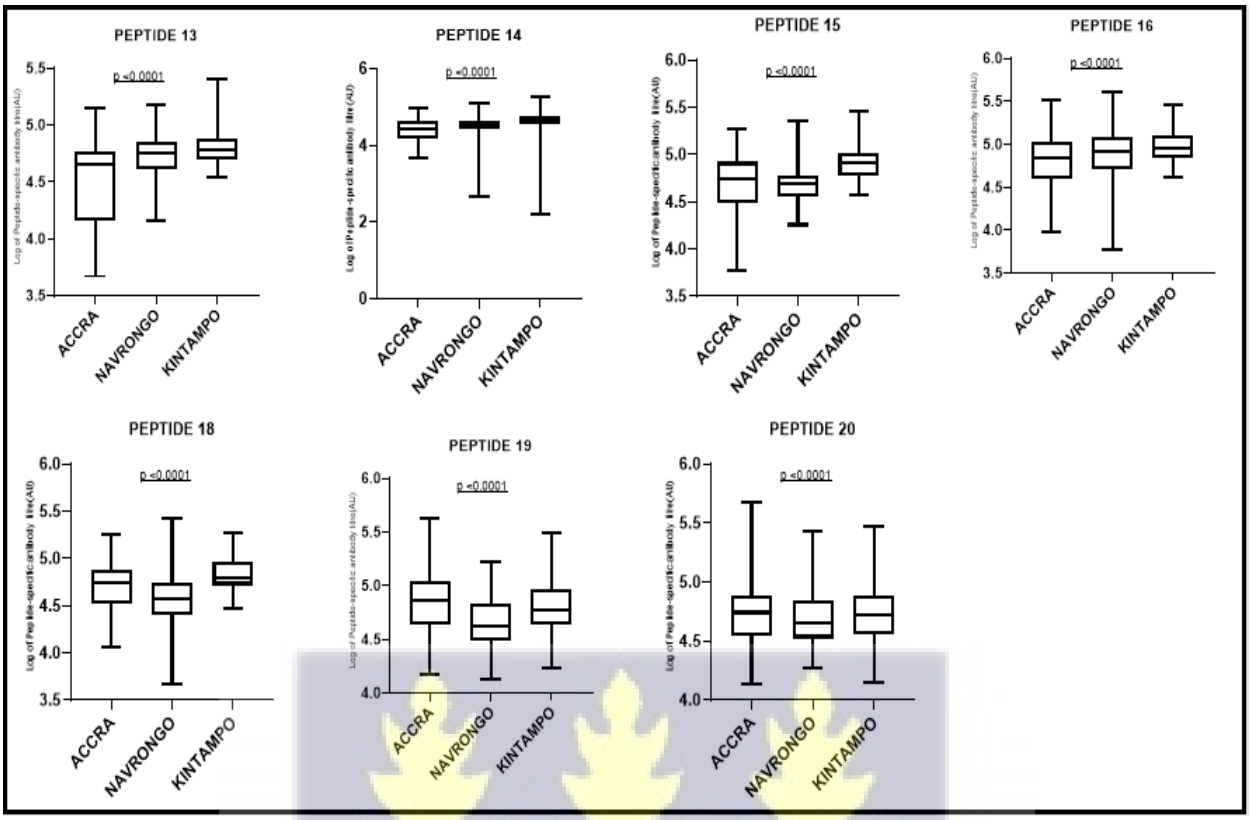



**Figure 4.2: Individual peptide responses across all sites.** Peptide 13 to 20 are peptides that recorded higher antibody levels as compared to remaining in the heatmap (fig 4.1)

### 4.3 Parasite Density

Parasite density between sites were compared (figure 4.4). Parasite density was highest in Kintampo and lowest in Navrongo.

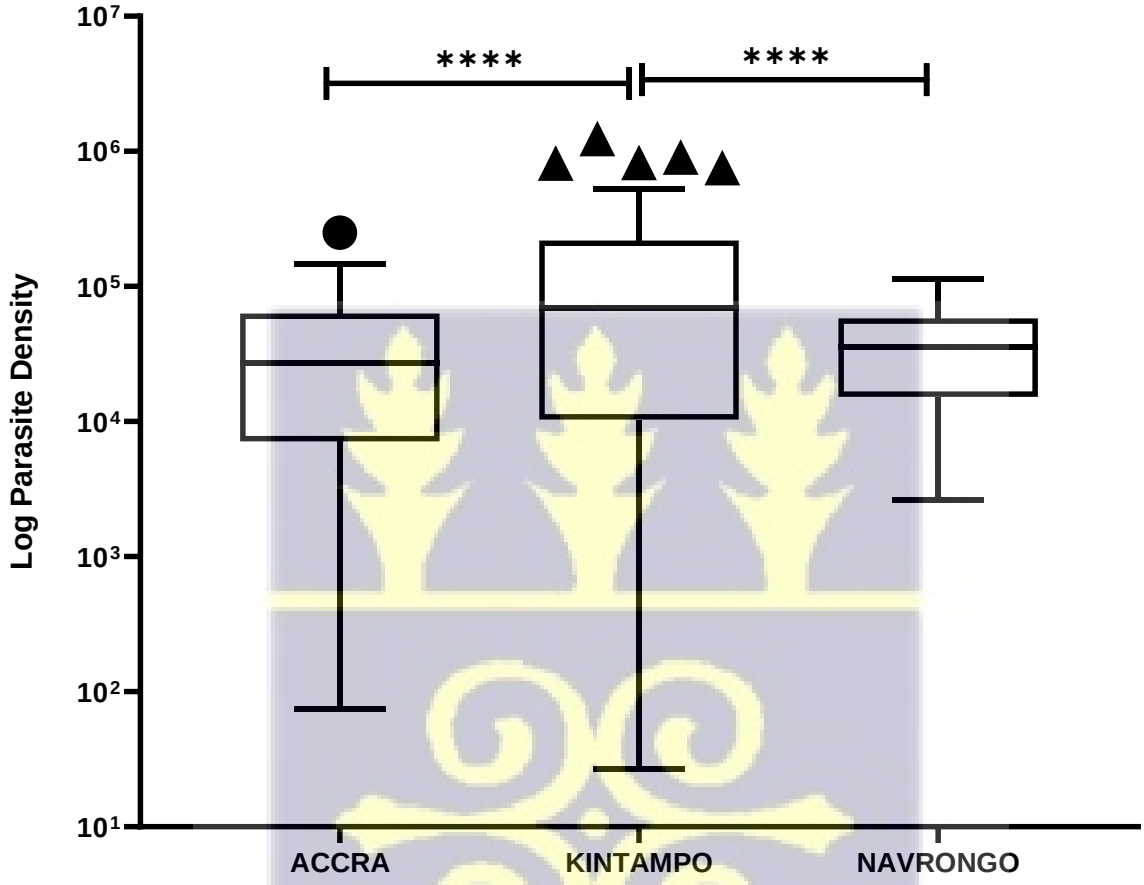
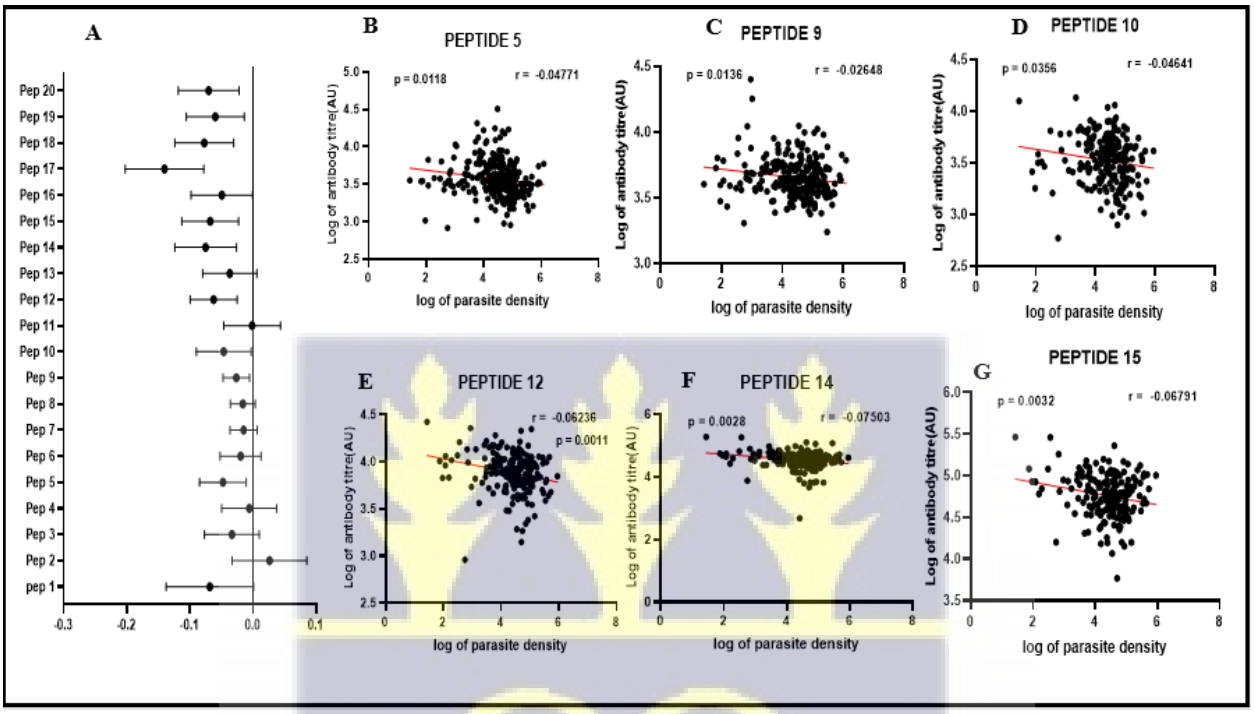



**Figure 4.3: Parasite density across all study sites**

### 4.4 Parasite Density and Antibody Levels Against Peptides

A linear regression between parasite density and peptide levels across all sites were performed. There was a negative correlation between parasite density and specific antibody levels for all peptides except peptide 2 that was positively correlated (fig 4.5M).



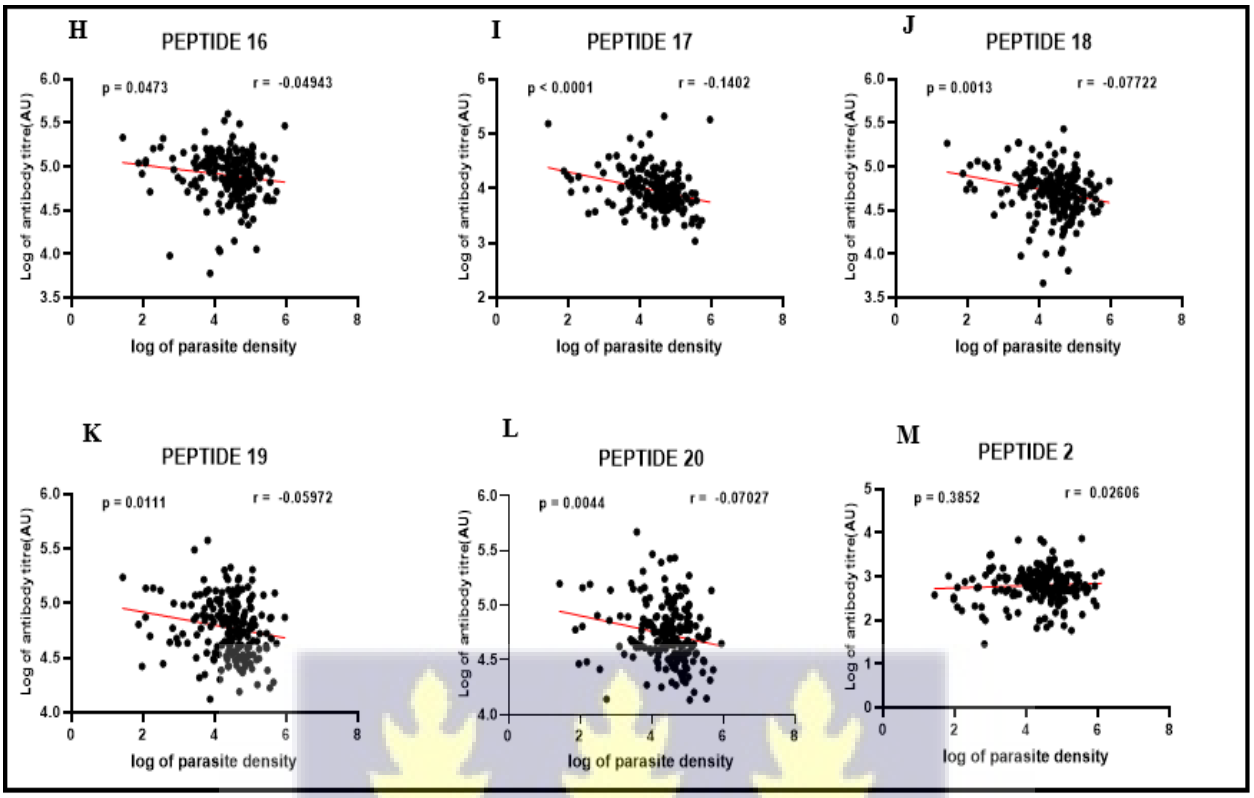



**Figure 4.4: A linear regression between parasite density and peptide levels across all sites**

### 4.5 Parasite Density and Antibody Levels Against Peptides at Each Study Site

A linear regression between parasite density and peptide levels at each site was analyzed. All peptides were negatively correlated among the Accra samples (fig 4.6A). However, Peptides 14, 15, 17, 18 and 20 (fig 4.6B-F) were negatively correlated and statistically significant.

For Navrongo site, peptide 2, 13 and 15 were positively correlated but not statistically significant. The remaining peptides were negatively correlated but not statistically significant except peptide 5, 6, 8, 9 and 17 which were however negatively correlated and statistically significant.

Kintampo had the highest spectrum of peptides that correlates with reduced parastaemia. Most of the peptides (peptide 1, 12, 13, 14, 15, 17 and 18) were negatively correlated and statistically

significant against Kintampo samples (fig 4.8B-H). Peptide 17 was the most negatively correlated across the different sites.

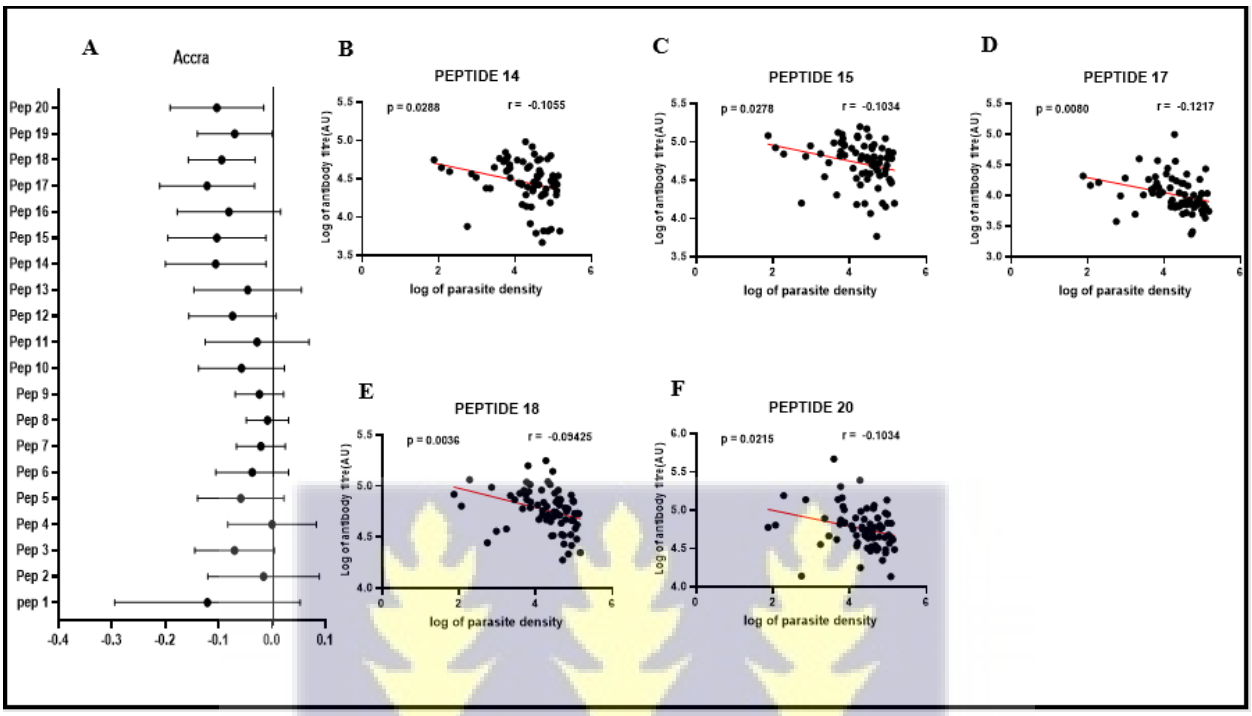



**Figure 4.5: Parasite density and antibody levels correlation for Accra samples**

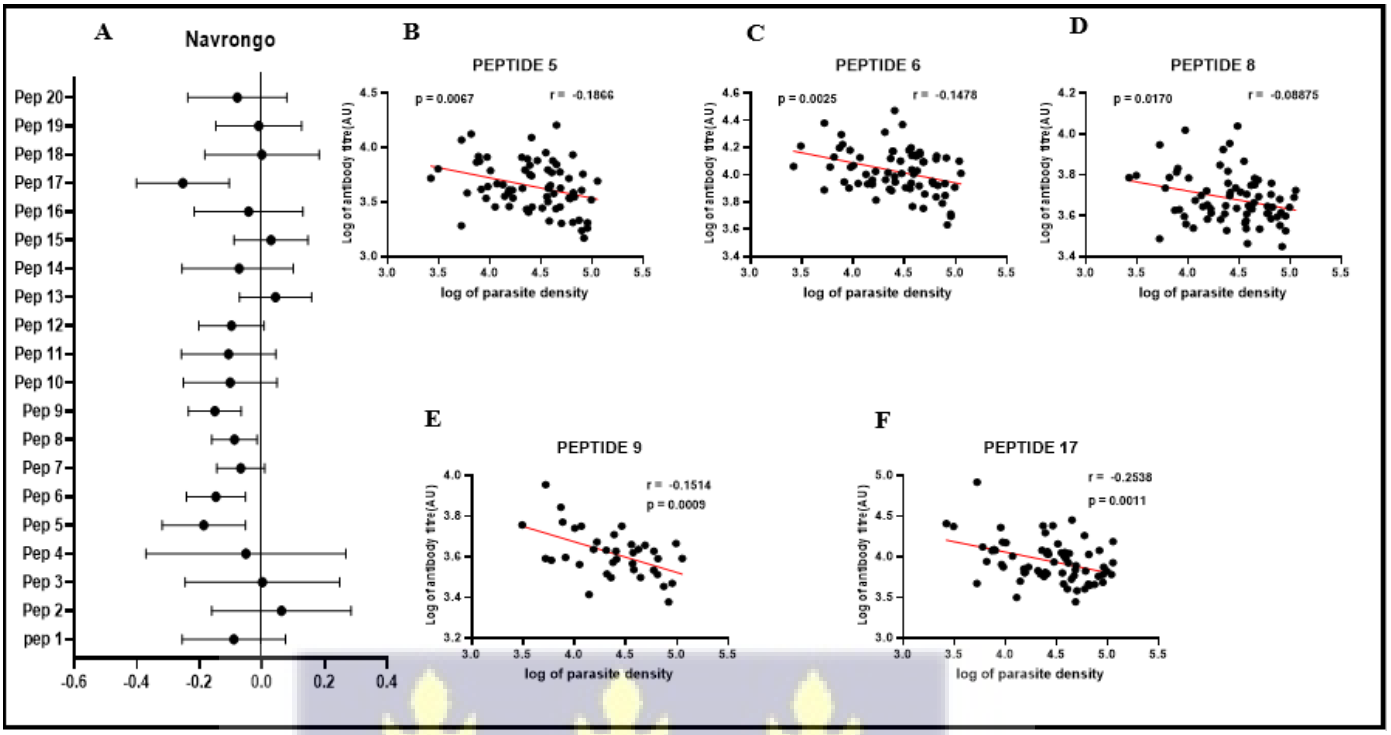



**Figure 4.6: Parasite density and antibody levels correlation for Navrongo samples**

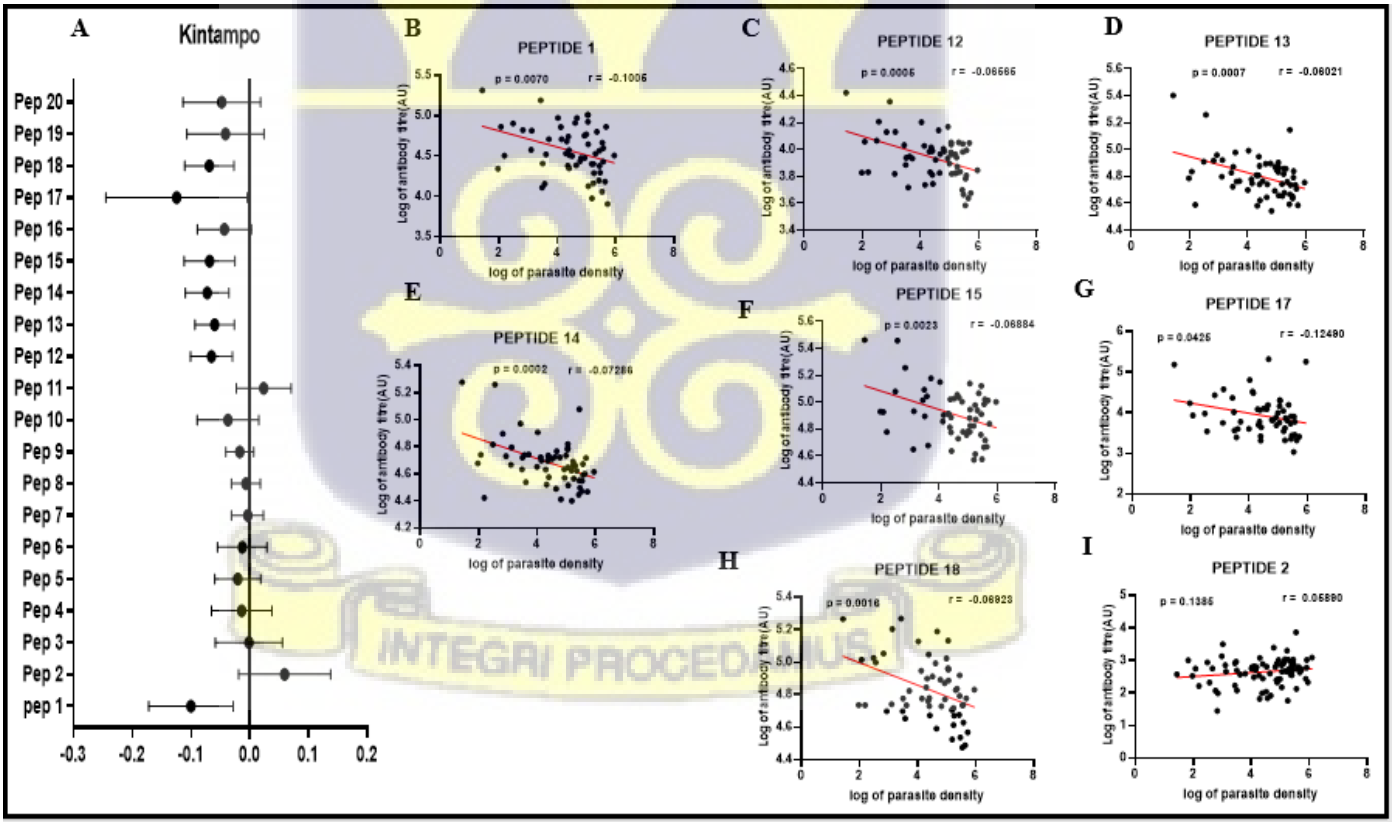



**Figure 4.7: Parasite density and antibody levels correlation for Kintampo samples**

### 4.6 Demographical Characteristics of Study Participants for ELISpot

For T cell immune response assessment,10 adult participants with ages ranging from 23 to 40 years were recruited from the Legon community. Out of the 10 participants, 9 (90%) were males and one female. The mean Hb levels and age were 15.31 ± 1.32 and 32.00 ± 9.018 respectively.

**Table 4.2 Demographical Characteristics of Study Participants in Legon**

| Characteristic | Study site<br>Legon (n = 10) |
|---|---|
| Gender | |
| Male no. (%) | 9 (90) |
| Female no. (%) | 1 (10) |
| Age mean ± SD | 32.00 ± 9.018 |
| Hemoglobin level, g/dL, mean ± SD | 15.31 ± 1.32 |

### 4.7 IFN-γ responses from PBMCs of subjects to PfMSP11 peptides pools

Peptide responses were first measured against overlapping peptide pools that were made from the PfMSP11 peptides used in ELISA. Each pool was made of 5 peptides except pool 5 which was made up of 7 peptides. There was no significance between peptide pools as IFN-γ responses from subjects against peptide pools were not different. M14 showed a highest response across all pools. Subject M10 showed the highest response against pool 2.

Peptides with positive sign (+) are those that made positive responses based on set positivity criteria. Subject M9 tested positive for pools 1 and 3 while subject M10 and M12 tested positive for pool 2. The remaining subjects made no responses to the peptide pools tested based on the positivity criteria. All subjects made positive IFN-γ responses to Con A.

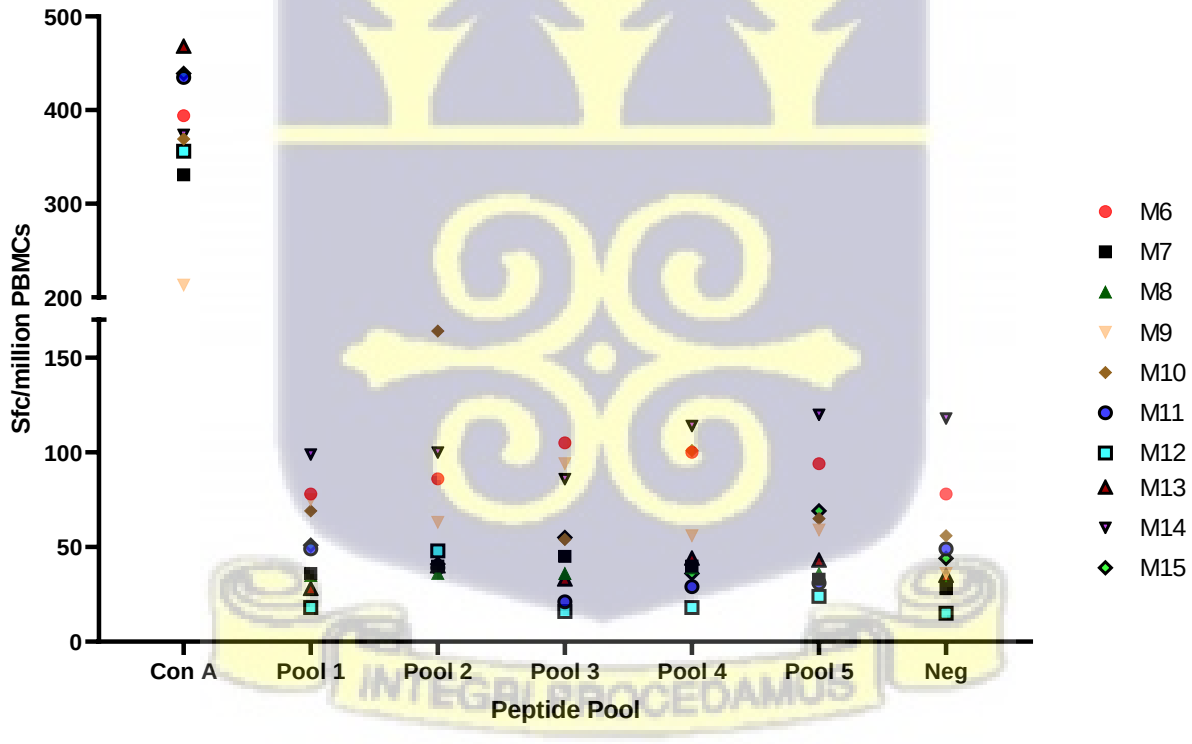



**Figure 4.8: IFN-γ responses to PfMSP 11 synthetic peptide pools of subjects.**

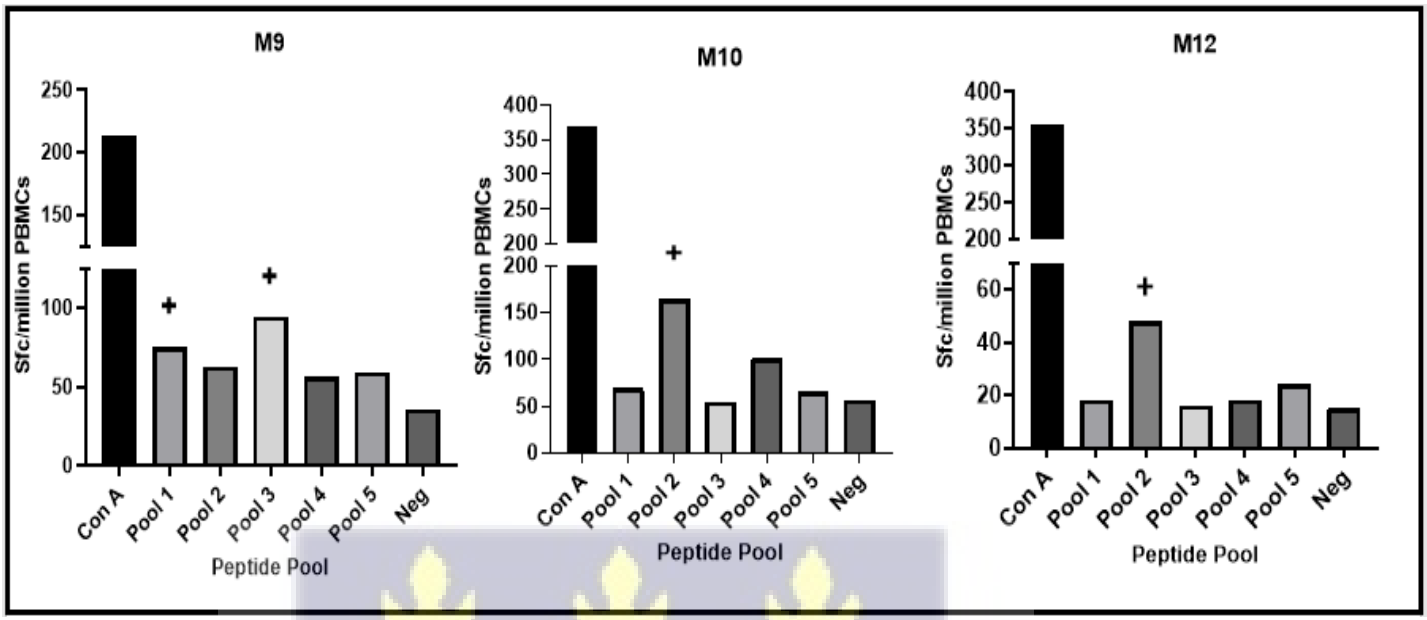



**Figure 4.9: IFN-γ responses to PfMSP 11 synthetic peptide pools of M9, M10 and M12.**

Peptides with positive sign (+) are those that made positive responses based on set positivity criteria

**Table 4.3 Analysis of Peptide Pools**

| Peptide Pool | Peptide Pool Composition | Responders | Mean SFU ± Standard Dev | Median SFU (Range) | SI |
|---|---|---|---|---|---|
| Pool 1 | Pep 1 to Pep 5 | 1 | 53.80 ± 25.73 | 50.00 (18-99) | 2.1 |
| Pool 2 | Pep 6 to Pep 10 | 2 | 65.70 ± 40.96 | 44.50 (36-164) | M10 (2.9)<br>M12 (3.2) |
| Pool 3 | Pep 11 to Pep 15 | 1 | 54.50 ± 30.92 | 49.50 (16-105) | 2.6 |
| Pool 4 | Pep 16 to Pep 20 | 0 | 57.70 ± 34.25 | 42.00 (18-114) | - |
| Pool 5 | Pep 21 to Pep 27 | 0 | 57.40 ± 30.78 | 51.00 (24-120) | - |

### 4.9 IFN-γ Responses from PBMCs of Subjects to PfMSP11 Peptides Pools That Tested Positive

Pools that tested positive against subjects were subsequently tested using the individual peptides making up that pool. Peptide 7 in pool 2 against M10 was the only peptide that tested positive. All other peptides of the various pools tested negative against their respective subjects. All subjects made positive IFN-γ responses to Con A.

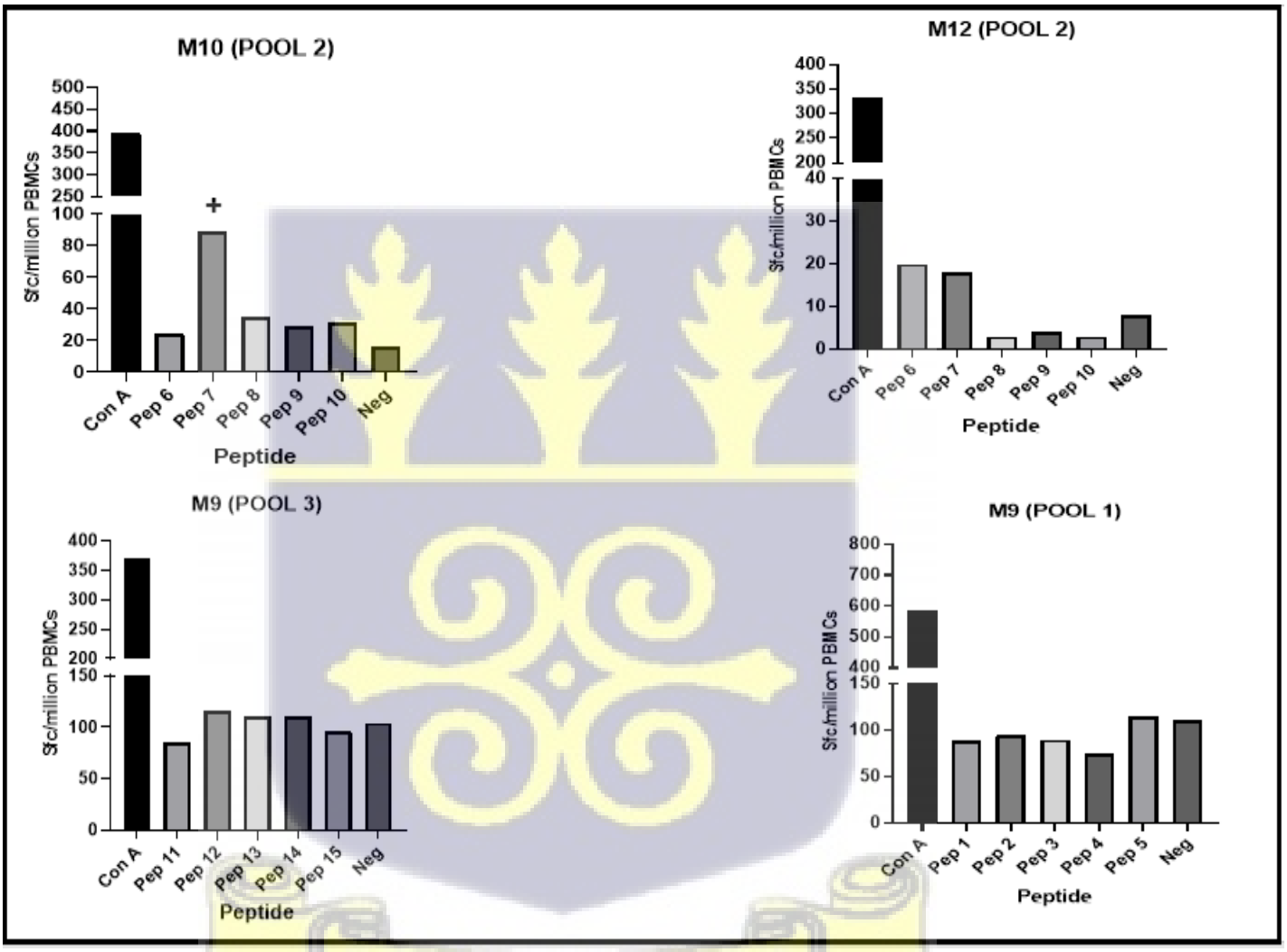



**Figure 4.10: IFN-γ responses to PfMSP 11 peptide pools that tested positive against their respective subjects (M9, M10 and M12).**

Peptides with positive sign (+) are those that made positive responses based on set positivity criteria**.**

# CHAPTER FIVE

# DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Discussion

Integrated disease control programmes have played a key role in the reduction of the global malaria disease burden. Merozoites invasion and replication within red blood cells are related to the major symptoms and pathology of the disease. Parasite burden and symptons associated with the disease can be reduced by vaccines to produces antibodies that can effectively block invasion of merozoites. This in addition can indirectly reduce malaria transmission (Miller, Baruch, Marsh, & Doumbo, 2002). In this study, PfMSP11, a novel protein found on the surface of merozoites and involved in the invasion of RBCs, was immunologically characterized using malaria clinical samples from children and healthy samples from adults. The antibody and T cell response induction potentials of the protein were evaluated using ELISA and IFN-gamma ELISpot, respectively. Overall, 27 synthetic peptides were generated by chemical synthesis from PfMSP11 full length antigen and were tested against PBMCs from 20 apparently healthy subjects to measure IFN-γ responses. Antibody responses from 283 clinical malaria samples were measured against the 20 synthetic peptides of PfMSP11.

Peptides at the C-terminus elicited relatively higher antibody responses than those at the N-terminus. Peptide 2 to Peptide 12, and Peptide 17 recorded low antibody responses across all sites. Peptides against Navrongo samples recorded the lowest responses. However, peptide responses were highest against Kintampo samples (fig 4.2). This observation of high antibody responses in Kintampo can be explained by the fact that Kintampo is a high malaria transmission zone, and individuals are expected to have constant long-term exposure to parasites. This same trend was observed in a study by Cherif (Cherif, et al., 2017) where the acquisition of the immunity against

malaria is dependent on the intensity of malaria transmission. Peptides at the C-terminus were more immunogenic than the N-terminus, with peptide 16 (CKKKNKNIKKNKKNKLRKNKKDKYEDDEYDD) being the most immunogenic B cell epitope (fig 4.2). Immunogenicity to a particular region of a protein is not new as it has been observed *P. falciparum* proteins such PfCSP. The PfCSP N-terminal region of the protein is flanked by polypeptides that elicit the production of inhibitory antibodies. This inhibitory antibody is involved in hepatocyte invasion by the sporozoites. Individuals residing in malaria-endemic regions are likely to exhibit partial protective immunity rendered by these flanked polypeptides (Bongfen, et al., 2009).

Parasite density is linked to disease, and diminished parasite counts almost certainly contribute to diminished risk of disease (Marsh & Snow, 1997). Parasite density in Kintampo was significantly higher than Accra and Navrongo (fig 4.4). This trend could be dependent on the intensity of malaria transmission as Kintampo is a high transmission area. Relationship between parasite density and antibody responses of peptides were correlated. The forest plot shows peptides that were negatively and positively correlated across all sites (fig 4.5A). Eleven of the 20 peptides (fig 4.5B-L) were negatively correlated and statistically significant (fig 4.5B-L). Pep 17 however was the most significantly correlated. Pep 2 was not statistically significant but positively correlated (fig 4.5G). Peptide 17, even though was the most significantly correlated, antibody response against the peptide was low. This trend is not new as it has been observed in *P. falciparum* proteins such RH5. Reticulocyte binding homologues (RHs) are part of the protein families identified as having crucial involvement during the invasion process. In *P. falciparum*, the RH family is made up of RH1, RH2a, RH2b, RH4 and RH5. RH5 is located in a subtelomeric region of chromosome 4, upstream from RH4 that contains species-specific genes involved in host-parasite interactions. It

lacks a C-terminal transmembrane domain and the full-length translated protein is much smaller than the other RH proteins at ~63 kDa. RH5 is not immunogenic in terms of natural exposure but they are essential for parasite survival, therefore high levels of of antibodies against RH5 in patient correlates with protection or reduced parasite density (Ord, Rodriguez, & Lobo, 2015). Immune responses against RH5 are generally low in a population because the epitope of interest is not available to the host immune system, hence it is survival mechanism (Ord, Rodriguez, & Lobo, 2015). Therefore, pep 17 might be an essential epitope for the parasite survival, and that could be a reason why the parasite is not exposing that portion to the host immune response. This is because immune response to that portion could reduce parasite survival. Hence, exposure of the peptide or antigen to host immune responses do not mean it is essential for survival. Strain specificity could account for the reason why different peptides were negatively correlated across different sites (fig 4.6-4.8) (Ogutu, et al., 2009).

The role of IFN-gamma in providing protective immunity against the malaria infection remains largely unknown even though naturally acquired T cell responses can be measure using ELISpot IFN-gamma (Offeddu, Thathy, Marsh, & Matuschewki, 2012). Peptide 7 in pool 2 against subject M10 was the only peptide that tested positive but none of the peptides in pool 2 tested positive against subject M12. This is a confirmation that, there are T cell epitopes and the Individual's HLA could only recognize peptide 7. Differences in MHC (HLA) of subjects could be the reason why subjects respond differently to the same peptides. This observation is possible because HLA genes are amongst the most polymorphic human genes and there are multiple variants of each gene within the population as a whole (Janeway, Jr, & M., 2011). As such peptide responses and the extent of response may differ from subjects to subjects. Peptides that tested negative were either not immunogenic or could not be recognized by the host HLA. One of the reasons that accounts for

the low positive response is the fact that HLAs of subjects used were unknown and peptide selection was not based on any prediction. In a study by Ganeshan *et al*., (2016), 31.4 % of study subjects' HLA were typed and 63 % of subjects responded positively to at least one peptide pool. This is because, peptides tested against these subjects were selected based on the prediction of binding to their typed HLAs, however in this study, the HLAs of the subjects used were unknown and hence peptides where not selected based on any prediction. This may be one reason for the low positive response observed in this study.

## 5.2 Conclusion

In conclusion, this study has shown that there are more antibody epitopes at the C-terminus than the N-terminus, and peptide 16 (CKKKNKNIKKNKKNKLRKNKKDKYEDDEYDD) being the B cell epitope with the highest antibody response. Finally, this study data shows Peptide 7 at the N-terminus was the most immunogenic T cell epitope. Identification of both T cell and antibody epitopes within this limited number of study participants suggest that even more epitopes are likely to be identified if the sample size is expanded. more epitopes as it depends on the HLA types of the person and the extent of response.

## 5.3 Recommendation

HLA typing of our study subjects to predict the peptides they can present for T cell binding will increase the chances of identifying additional T cell epitopes, hence increasing our positive responses as peptides selection would be based on individuals HLA types.

**REFERENCES**

Ashley, E. A., Phyo, A. P., & Woodrow, C. J. (2018). Malaria. *The Lancet, 391*(10130), 1608-1621.

Belachew, E. B. (2018). Immune Response and Evasion Mechanisms of Plasmodium falciparum Parasites.

Biryukov, S., Angov, E., Landmesser, M. E., Spring, M. D., Ockenhouse, C. F., & Stoute, J. A. (2016, July). Complement and Antibody-mediated Enhancement of Red Blood Cell Invasion and Growth of Malaria Parasites. *EBioMedicine, 9*, 207-216. doi:10.1016/j.ebiom.2016.05.015

Blackman, M. J., Heidrich, H. G., Donachie, S., Mcbride, J. S., & Holder, A. A. (1990). A single fragment of a malaria merozoite surface protein remains on the parasite during red-cell invasion and is the target of invasion-inhibiting antibodies. *J. Exp. Med., 172*, 379–382.

Bongfen, S. E., Ntsama, P. M., Offner, S., Smith, T., Felger, I., Tanner, M., & al, e. (2009). The N-terminal domain of Plasmodium falciparum circumsporozoite protein represents a target of protective immunity. *Vaccine, 27*, 28–35.

Boyle, M. J., Reiling, L., Feng, G., Langer, C., Osier, F. H., Aspeling-Jones, H., . . . Beeson, J. G. (2015). Human antibodies fix complement to inhibit Plasmodium falciparum invasion of erythrocytes and are associated with protection against malaria. *Immunity, 42*, 580–590.

Cherif, M. K., Ouédraogo, O. S., Diarra, A., Ouédraogo, A., Tiono, A., & Cavanagh, D. R. (2017). Antibody responses to P. falciparum blood stage antigens and incidence of

clinical malaria in children living in endemic area in Burkina Faso. *research notes, 10*(1), 472.

Child, M. A., Epp, C., Bujard, H., & Blackman, M. J. (2010). Regulated maturation of malaria merozoite surface protein-1 is essential for parasite growth. *Mol. Microbiol, 78*, 187–202.

Chotivanich, K., Udomsangpetch, R., Pattanapanyasat, K., Chierakul, W., Simpson, J., Looareesuwan, S., & White, N. (2002). Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria. *Blood, 100*(4), 1172–1176. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12149194

Cohen, J., Nussenzweig, V., Nussenzweig, R., Vekemans, J., & Leach, A. (2010). From the circumsporozoite protein to the RTS, S/AS candidate vaccine. *Hum Vaccine, 6*, 90-96.

Corse, E., Gottschalk, R. A., & Allison, J. P. (2011). Strength of TCR-Peptide/MHC Interactions and In Vivo T Cell Responses. *The Journal of Immunology, 186*(9), 5039–5045. doi:10.4049/jimmunol.1003650

Douglas, A., Baldeviano, G., Lucas, C., Lugo-Roman, L., Crosnier, C., Bartholdson, S., . . . Ventocilla, J. (2015). A PfRH5-based vaccine is efficacious against heterologous strain blood-stage Plasmodium falciparum infection in aotus monkeys. *Cell Host Microbe, 17*, 130–139.

Erhun, W. O., Agbani, E. O., & Adesanya, S. O. (2005). Malaria prevention: knowledge, attitude and practice in a southwestern Nigerian community. *African Journal of Biomedical Research., 8*, 25-29. Retrieved from http://www.bioline.org.br/md

Etlinger, H. M., Caspers, P., Matile, H., Schoenfeld, H. J., Stueber, D., & Takacs, B. (1991). Ability of recombinant or native proteins to protect monkeys against heterologous challenge with Plasmodium falciparum. *Infect. Immun, 59*, 3498–3503.

Felgner, P., Roestenberg, M., Liang, L., Hung, C., Jain, A., Pablo, J., . . . Sauerwein, R. (2013). Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. *Sci Rep, 3*, 3549.

Fowkes, F. J., Richards, J. S., Simpson, J. A., & Beeson, J. G. (2010). The relationship between anti merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis. *PLoS Med, 7*, e1000218.

Ganeshan, H., Kusi, K. A., Anum, D., Hollingdale, M. R., Peters, B., Kim, Y., & … Sedegah, M. (2016). Measurement of ex vivo ELISpot interferon-gamma recall responses to Plasmodium falciparum AMA1 and CSP in Ghanaian adults with natural exposure to malaria. *Malaria Journal*. doi:10.1186/s12936-016-1098-8

Gardner, M. J., Hall, N., Nelson, K. E., Peterson, J., Mungall, C., & White, O. (2002). Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature, 419*, 498–511. doi:10.1038/nature01097

Genton, B., Ai-Yaman, F., Mgone, C. S., Alexander, N., Paniu, M. M., Alpers, M. P., & Mokela, D. (1995). Ovalocytosis and cerebral malaria. *Nature, 378*(6557), 564–565. doi:10.1038/378564a0

Gowda, D. C., & Wu, W. (2018). Parasite Recognition and Signaling Mechanisms in Innate Immune Responses to Malaria.

Healer, J., Chiu, C. Y., & Hansen, D. S. (2017). Mechanisms of naturally acquired immunity to P. falciparum and approaches to identify merozoite antigen targets.

Janeway, C. A., Jr, T. P., & M., W. (2011). The Major Histocompatibility Complex and Inflammation. *Southern Medical Journal*, *93*(2), 169–172. doi:10.1097/00007611-200002000-00002

Joos, C., Varela, M. L., Mbengue, B., Mansourou, A., Marrama, L., Sokhna, C., . . . Perraut, R. (2015). Antibodies to Plasmodium falciparum merozoite surface protein- 1p19 malaria vaccine candidate induce antibody-dependent respiratory burst in human neutrophils. *Malar. J.*, *14*, 409.

Klinkenberg, E., McCall, P., Wilson, M. D., Amerasinghe, F. P., & Donnelly, M. J. (2008). Impact of urban agriculture on malaria vectors in Accra, Ghana. *Malar J*, *7*, 151.

Luckheeram, R. V., Z. R., Verma, A. D., & Xia, B. (2012). CD4 +T cells: Differentiation and functions. *Clinical and Developmental Immunology*. Retrieved from https://doi.org/10.1155/2012/925135

Marsh, K., & Snow, R. W. (1997). Host-parasite interaction and morbidity in malaria endemic areas. *Philos. Trans. R. Soc. London B*, *352*, 1385-1394.

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

Miura, K. (2016). Progress and prospects for blood-stage malaria vaccines. *Expert Rev. Vaccines*, *15*, 765–781.

Mombo, L., Krishnamoorthy, R., Ntoumi, F., Bisseye, C., Ossari, S., Lu, C. Y., & Nagel, R. L. (2003). Human genetic polymorphisms and asymptomatic Plasmodium falciparum malaria in Gabonese schoolchildren. *American Journal of Tropical Medicine and Hygiene*, *68*(2), 186–190. doi:10.4269/ajtmh.2003.68.186

Mordm¨uller, B., Surat, G., & Lagler, H. (2017). Sterile protection against human malaria by chemoattenuated PfSPZ vaccine,. *Nature*, *542*(7642), 445–449.

Nanda, N., Sobti, R. C., Sutton, P. L., Carlton, J. M., Joshi, H., Sharma, V. L., & Laishram, D. D. (2012). The complexities of malaria disease manifestations with a focus on asymptomatic malaria. *Malaria Journa*, *11*(1), 29. doi:10.1186/1475-2875-11-29

Nlinwe, O. N., Kusi, K. A., Adu, B., & Sedegah, M. (2018). T-cell responses against Malaria: Effect of parasite antigen diversity and relevance for vaccine development. *Vaccines*, *36*(17), 2237–2242. doi:10.1016/j.vaccine.2018.03.023

Offeddu, V., Thathy, V., Marsh, K., & Matuschewki, K. (2012). Naturally acquired immune responses against Plasmodium falciparum sporozoites and liver infection. *Int J Parasitol.*, *42*, 535–548.

Ogutu, B. R., Apollo, O. J., McKinney, D., Okoth, W., Siangla, J., Dubovsky, F., . . . Wittes, J. (2009). Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *PloS one*, *4*(e4708). Retrieved from http://dx.doi.org/10.1371/journal.pone.0004708

Olotu, A. .., Fegan, G., & Wambua, J. e. (2016). Seven-year efficacy of RTS,S/AS01 malaria vaccine among young African children. *N Engl J Med*, *374*, 2519–2529.

Ord, R. L., Rodriguez, M., & Lobo, C. A. (2015). Malaria invasion ligand RH5 and its prime candidacy in blood-stage malaria vaccine design. *Human vaccines & immunotherapeutics, 11*(6), 1465–1473. Retrieved from https://doi.org/10.1080/21645515.2015.1026496

Owusu-Agyei, S., Asante, K. P., & Adjuik, M. (2009). Epidemiology of malaria in the forest-savanna transitional zone of Ghana. *Malar J, 8,* 220.

Perlmann, P., & Troye-Blomberg, M. (2000). Malaria blood-stage infection and its control by the immune system. *Folia biologica*.

Pringle, J. C., Carpi, G., Almagro, G. J., & al, e. (2018). RTS,S/AS01 malaria vaccine mismatch observed among Plasmodium falciparum isolates from southern and central Africa and globally. . *Scientific Reports, 8*(1), 6622.

Rowe, J. A., Handel, I. G., Thera, M. A., Deans, A.-M., Lyke, K. E., Kone, A., . . . ... (2007). Blood group O protects against severe Plasmodium falciparum malaria through the mechanism of reduced rosetting. *Proceedings of the National Academy of Sciences., 104*(44), 17471–17476. doi:10.1073/pnas.0705390104

RTS,S Clinical Trials Partnership. (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet, 386,* 31–45.

Sinha, J. K., & Bhattacharya, S. (2014). A Text Book of Immunology. *Academic Publishers*, 314.

Sissoko, M. S., Healy, S. A., Katile, A., Omaswa, F., Zaidi, I., Gabriel, E. E., . . . Ruben, A. J. (2017, May). Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial. *Lancet Infect Dis., 17*(5), 498-509. doi:10.1016/S1473-3099(17)30104-4

Tan, Z., Zhou, T., Zheng, H., Ding, Y., & Xu, W. (2015). Malaria DNA vaccine gp96NTD-CSP elicits both CSP-specific antiantibody. *Parasitology Research, 114*(6), 2333–2339.

Tanner, M., Greenwood, B., Whitty, C. J., Ansah, E. K., Price, R. N., Dondorp, A. M., . . . Osier, F. (2015). Malaria eradication and elimination: views on how to translate a vision into reality. *BMC medicine, 13*, 167.

The original. (2017). *Types of Vaccines*. Retrieved 2017

Theisen, M., Roeffen, W., Singh, S., Andersen, G., Amoah, L., van de Vegte-Bolmer, M., . . . al., e. (2014). A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages. *Vaccines, 32*, 2623–2630.

United States Centers for Disease Control and Prevention. (2011). A CDC framework for preventing infectious diseases.

WHO. (2020). *Malaria*. Retrieved August 26, 2020, from https://www.who.int/news-room/fact-sheets/detail/malaria

Wieczorek, M., Abualrous, E. T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F., & Freund, C. (2017). Major histocompatibility complex (MHC) class I and MHC class II

proteins: Conformational plasticity in antigen presentation. *Frontiers in Immunology, 8*, 1-16. doi:10.3389/fimmu.2017.00292

Wilson, K. L., Flanagan, K. L., Prakash, M. D., & Plebanski, M. (2019). Malaria vaccines in the eradication era: current status and future perspectives. *Expert Rev. Vaccines, 18*, 133–151.

Winch, P. J., Makemba, A. M., Kamazina, S. R., Lwihula, G. K., Lubega, P., Minjas, J. N., & Shiff, C. J. (1994). Seasonal variation in the perceived risk of malarial, Implication for promotion of insecticide impregnated bednets. *Social Science and Medicine., 39*, 63-75.

Winkler, S., Willheim, M., Baier, K., Schmid, D., Aichelburg, A., & Graninger, W. &. (1999). Frequency of Cytokine-Producing T Cells in Patients of Different Age Groups with Plasmodium falciparum Malaria. *Journal of Infectious Diseases, 179*(1), 209–216.

Woehlbier, U., Epp, C., Hackett, F., Blackman, M. J., & Bujard, H. (2010). Antibodies against multiple merozoite surface antigens of the human malaria parasite Plasmodium falciparum inhibit parasite maturation and red blood cell invasion. *Malar. J., 9*, 77.

World Health Organization. (2019). *World malaria report 2019.* Geneva: World Health Organization.

Zheng, J., Pan, H., Gu, Y., Zuo, X., Ran, N., & Yuan, Y. (2019, October 3). Prospects for Malaria Vaccines: Pre-Erythrocytic Stages, Blood Stages, and Transmission-Blocking Stages. (A. Das, Ed.) *BioMed Research International, 2019*. doi:10.1155/2019/9751471

# APPENDICES

## Appendix 1.1: Preparation of Standard Solutions and buffers

### A. Preparation of 1x PBS solution

Final volume: 100 ml

PROCEDURE

One small tablet is dissolved in 100 ml of distilled water.

The tablet is dissolved totally using a magnetic stirrer.

### B. Preparation of PBST-20 solution (wash buffer)

PROCEDURE

Dissolve 500 µl of Tween-20 in PBS to achieve a 0.05% concentration.

### C. Preparation of blocking buffer (3 % skimmed milk)

PROCEDURE

A mass of 7.5 g of skimmed milk is added to 250 ml of 1 x PBS solution containing 0.05 % Tween-20 and stirred to mix thoroughly

### D. Preparation of 1 % skimmed milk

PROCEDURE

One part of the 3 % milk is diluted in two parts of 1x PBST-20 solution to a final volume of 200 ml.

This is used in diluting the samples (diluent).

### E. Standard solution preparation

PROCEDURE

For a 1:1000 dilution, 2 µl of the stock standard solution is dissolved in 2 ml of the diluent.

### F. Coating buffer for ELISPOT

Total volume: 15ml

PROCEDURE (For 1 plate)

Place 15ml of distilled water in a storage container.

Weigh out 0.04g sodium bicarbonate ($NaHCO_3$) and place into the container

Weigh out 0.02g of sodium carbonate ($Na_2CO_3$) and place into the container

Place the container on the magnetic stirrer and stir without heating till all is in solution.

Adjust pH to 9.6 by adding in drop wise concentrated NaOH or HCl

Filter sterile with 0.22µm Millipore filter

### G. Blood dilution buffer for PBMC isolation

Total volume: 500ml

PROCEDURE:

Take 500ml of RPMI-1640 medium, add 5ml of L-glutamine solution and shake well.

Take 5ml off to discard and add 5ml of Penicillin-streptomycin solution and shake well.

Store at 4ºC until ready to use

(NB: If RPMI is pre-supplemented with L-glutamine, only add 5ml of Penicillin-streptomycin).

### H. Fresh Cell Wash for PBMC Separation (5% FBS/RPMI)

Total Volume: 500ml

PROCEDURE:

Take 500ml of RPMI-1640 medium, add 5ml of L-glutamine solution and shake well.

Take 5ml off and add 5ml of Penicillin-streptomycin solution and shake well.

Take 25ml off and add 25ml of Heat Inactivated Foetal Bovine/Calf Serum and shake well.

Filter sterile with 0.22µm Millipore filter.

Store at 4ºC until ready to use

(NB: If RPMI is pre-supplemented with L-glutamine, only add 5ml of Penicillin-streptomycin)

### I. Blocking/Complete Medium for ELISPOT

PROCEDURE:

Take 500ml of RPMI-1640 medium, add 5ml of L-glutamine solution and shake well.

Take 5ml off and add 5ml of streptomycin/penicillin.

Shake well and take 50ml off.

Add 50ml of heat-inactivated Normal Human Serum and shake to mix.

Filter sterile with 0.22m Millipore filter.

(NB: If RPMI is pre-supplemented with L-glutamine, only add 5ml of Penicillin-streptomycin)

**J. Cryo-freezing mixture**

Volume: 100ml

PROCEDURE:

Add 90ml of heat inactivated Foetal Bovine/Calf Serum and 10ml of DMSO

Filter sterile with 0.22µm Millipore filter

Shake well and keep at room temperature until use.

**K. Washing Buffer for ELISPOT**

1X PBS with 0.05% Tween 20

Volume: 1L

PROCEDURE

Add 2 tablets of PBS to a beaker containing 1000 ml deionized water and place the flask on a magnetic stirrer without heating and stir until all is in solution

Add 500 µl of Tween 20 and continue stirring until all is in solution

**L. Diluent Buffer for ELISPOT**

1 X PBS with 0.5% HI Foetal Bovine/Calf Serum

Volume: 25ml

PROCEDURE

Dissolve 5g tablets of PBS in 500ml of distilled water.

Place the flask on the magnetic stirrer without heating till all is in solution

Take 50µl off and add 50µl of heat-inactivated Foetal Bovine/Calf Serum.

Keep stirring till all is in solution.

**M. Chromogenic alkaline phosphatase substrate preparation**

Volume: 10ml (1 Plates)

PROCEDURE:

Add 0.4ml buffer and 0.96 ml of distilled water.

Add 100µl of Reagent A and 100µl of Reagent B

Cover the flask with aluminium foil and place it on a magnetic stirrer without heating and stir until all is in solution.