# IMPACT OF ALLELIC POLYMORPHISMS IN *Pf*AMA1 ON THE INDUCTION OF T-CELL RESPONSES IN MALARIA ENDEMIC COMMUNITIES IN GHANA


**BY**

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**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY (MPHIL) DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES**

**JULY 2019**

# DECLARATION

I, Ebenezer Addo Ofori, hereby declare that except for references to other people's work, which have duly been 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 and Dr. Michael Fokuo Ofori, both of Immunology Department, Noguchi Memorial Institute for Medical Research, University of Ghana. Neither all nor parts of this thesis have been presented for another degree elsewhere.

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# DEDICATION

I would like to dedicate this work first of all to the Almighty God for the strength. I also dedicate this to my family for their support, Dr. Omarine Nlinwe Nfor for her mentorship and to my friends for their encouragement. I love you all. God bless you.

# ACKNOWLEDGEMENTS

I am forever indebted to Dr. Michael F. Ofori who used part of his project grant to pay for some reagents and consumable I needed to complete my work. To Dr. John Tetteh, I say a big thank you for taking special interest in my well-being and staying with me throughout the nights during conduct of cellular analysis. I am also grateful to Pastor Alex Danso-Coffie, Mr. Bernard Tornyigah, Mr. Dickson Donu, Mr. Frank Osei, Mr. Jerubbaal Gideon Agyeah, Mr. Samuel Nyanpong, Ms. Gloria Owusu-Ansah and Ms. Dorcas Bredu who assisted immensely in my sampling from the field and assistance in my laboratory schedules. I thank Mrs. Augustina Frimpong for her constant advice, encouragement and assistance in my thesis. Special thanks go to Dr. Kwadwo Asamoah Kusi for the research idea, excellent mentorship, coaching, availability and patience anytime I needed it. Doc., thanks a million. Finally, I want to acknowledge the Medical Assistant of Obom Health Centre and to the staff of the facility for their tremendous help in mobilizing the study subjects, not forgetting the study subjects who voluntarily accepted to be enrolled in the study.

Thank you all and God bless you.

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| | | |
|---|---|---|
| µl | - | microliters |
| aa | - | Amino acids |
| ACT | – | Artemisinin based combination therapy |
| AMA1 | - | Apical Membrane Antigen 1 |
| APC | - | allophycocyanin |
| BALB | - | Albino laboratory-bred strain |
| 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 |
| DNTPs | - | Dinucleotide triphosphate |
| ELISpot | - | Enzyme linked immunospot |
| EtBr | - | Ethidium bromide |
| FACS | – | Fluorescent cell separator |
| FBS | – | Fetal bovine serum |
| FITC | - | Fluorescein isothiocyanate |
| G6PD | - | Glucose 6 phosphate dehydrogenase |
| Hb | - | Haemoglobin |
| HBsAg | - | Hepatitis B virus surface antigen |
| HLA | – | Human leukocytes antigen homolog 5 |
| HVR | - | Hyper variable regions |
| IFN-γ | - | Interferon-γ |
| IgG | - | Immunoglobulin G antibodies |

| | | |
|---|---|---|
| IL | - | Interleukin |
| INF-α/γ | - | Interferon alpha/gamma |
| IRB | - | Institutional Review Board |
| iRBC | – | infected red blood cell |
| IRS | - | Indoor residual spraying |
| ITNs | - | Insecticide-treated nets |
| kDa | - | Kilo Dalton |
| LLINs | - | Long-lasting insecticidal nets |
| MACS | - | Magnetic cell separator |
| MHC | - | Major histocompatibility complex |
| min | - | minutes |
| ml | - | millilitres |
| MSP2 | – | Merozoite surface protein 2 |
| NHS | – | Normal human serum |
| NK | - | Natural killer cells |
| PBMC | - | Peripheral Blood Mononuclear Cells |

| | | |
|---|---|---|
| PBS | – | Phosphate buffered saline |
| PBST | – | Phosphate buffered saline / Tween |
| PCR | - | Polymerase Chain Reaction |
| *Pf* | - | *Plasmodium falciparum* (*P. falciparum*) |
| PfAMA1 | - | *Plasmodium falciparum* apical membrane antigen |
| *Pf*RH5 | - | *Plasmodium falciparum* reticulocyte-binding Protein |
| RON | - | Rhoptry Neck Protein |
| sfc/m | – | Spot forming cells/million |
| T regs | - | Regulatory T cells |
| TCR | – | T cell receptor |
| Th1 | - | Type 1 helper cells |
| Th2 | - | Type 2 helper cells |
| TNF | - | Tumour necrotic factor |
| WHO | - | World Health Organization |

# ABSTRACT

The eradication of malaria requires a combined effort involving all available control tools, and these efforts would be complemented by an effective vaccine. An effective malaria vaccine should be capable of inducing protective immune responses against variant forms of the parasite and in a genetically diverse population. Allelic polymorphisms in antigens that are targets of protective immune responses are a major drawback to vaccine development. This study investigated the influence of allelic polymorphisms in Apical Membrane Antigen 1 (AMA1) peptide sequences from three strains of *P. falciparum* (3D7, 7G8 and FVO) on their function as targets of immunodominant T cell responses. PBMCs were obtained from subjects from Legon (low malaria transmission zone) and Obom (high malaria transmission zone) and tested against 15 synthetic *Pf*AMA1 peptides using ELISpot assay. The peptides were also tested for induction of CD4+ and CD8+ specific T cell IFN-γ responses by ELISpot using PBMCs with depleted T cell subsets. The 15 peptides represent six sets of allelic peptides from the three parasite strains. Data was expressed as the spot forming cells (sfc) per million PBMCS and analysed at two levels; first for positivity for immunodominance based on a set of criteria, and second for statistical differences between sfc/million PBMC data between peptides with any allelic set.

None of the unfractionated PBMCs of subjects from Legon made positive response to any of the peptide tested. However, 2 subjects from Obom responded positively to 4 out of the 15 peptides used. Two of the 4 peptides were alleles and belong to the same allelic set (7G8 vs 3D7/FVO respectively) while the remaining two peptides belong to different allelic sets. In assays with CD8+ enriched PBMCs, a single subject from Legon and 3 subjects from

Obom made positive response to 4 new peptides in addition to 2 peptides that made positive responses from the unfractionated PBMCs. In similar assays with CD4+ cells, there were no positive responses to any peptide for subjects from Legon. However, subject s made a positive response to 2 peptides belonging to the same allelic set. Overall, majority of the peptides which made either positive response by the set criteria or statistically significant responses had amino acid variability at positions 1 and 4 and most of the substitutions were to a charged amino residue. Also, majority of the specific IFN-γ response were from subjects belonging to the high malaria transmission zone.

On the basis of this data, polymorphisms in *Pf*AMA1 affect the induction of T- cell responses in malaria exposed subjects, and this effect is more pronounced in areas with high parasite exposure.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Malaria is a deadly blood-borne tropical disease of great public health concern. The disease is caused by the apicomplexan parasite *Plasmodium* transmitted through the bite of an infectious female *Anopheles* mosquito during a blood meal. Traditionally, there are five species of *Plasmodium* that affect humans. This include; *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malariae* and *Plasmodium knowlesi*. In 2014, another specie, *Plasmodium cynomolgi* was reported to have naturally affected an adult patient. Among these, *Plasmodium falciparum (P. falciparum)* is the most virulent also with the highest rate of complications and mortality worldwide (Greenwood et al., 2008; Buffet et al., 2010). The World Health Organization (WHO) reported an estimated a global malaria burden of 219 million cases in 2017, an increase of about three million more cases compared to 2016 cases, with 92% and 93% incidence and death in sub-Saharan Africa respectively. In 2017, the estimated cost for malaria control and eradication enormously increased by 7% compared to 2016.

There have been a decrease in the rate of malaria related death in the sub-Saharan Africa with the increase of existing tools recommended by WHO (Chuang et al., 2013). These tools include long-lasting insecticidal nets (LLINs), indoor residual spraying with insecticides, quick diagnostic testing, preventive treatment for children and during pregnancy and effective treatment of the disease antimalarial. However, even with all these interventions in place, people are still getting sick and dying from malaria. Hence, WHO has recommended

development of a number of additional interventions including vaccines to reinforce our hope of eradicating the disease (WHO, 2015).

The malaria vaccine candidate, RTS,S is the first and only malaria vaccine till date to demonstrate protection against malaria in children in phase 3 clinical trials (Owusu-Agyei et al., 2009). RTS, S is made up of an adjuvant (AS01) and hepatitis B virus surface antigen (HBsAg) virus-like particles genetically fused to a portion of the *P. falciparum-derived* circumsporozoites protein (CSP) (Kaslow & Biernaux 2015). The efficacy of RTS, S has been reported to wane with time (Olotu et al., 2016) and this makes it unlikely for this vaccine to meet the malaria eradication goal. It is therefore required to use an alternative parasite targets or vaccination strategies in order to develop an effective vaccine.

A number of malaria vaccines have gone through field trials, however, they are mostly shown low immunogenicity and efficacy, attributable to the diversities in vaccine candidate antigens (Tanner et al., 2015). *P. falciparum* apical membrane antigen (*Pf*AMA1) is among the key candidate antigens (Healer et al., 2004) comparing the genetic sequences of *Pf*AMA1 genes from different species of malaria parasites shows that certain structural domains are highly conserved while others are extremely diverse (Cortes et al., 2003). Polymorphism in *Pf*AMA1 accounts for about 10% of its sequence and this is distributed over the three domains of the protein (Chesne-Seck et al., 2005).

Genetic diversity existing in *Plasmodium* parasites is a major drawback in the development of a successful and effective malaria vaccine (Takala & Plowe, 2009). These polymorphisms greatly enhance the parasite's ability to escape or evade recognition by the

host (Ferreira et al., 2004). This limits the adequate immune response productions to the full range of circulating variants within any population.

*Pf*AMA1 has been investigated as a recombinant protein in several clinical trials (Saul et al., 2005). Antibodies to *Pf*AMA1 have been demonstrated to inhibit red blood cell invasion by merozoites (Kusi et al., 2009), and sero-epidemiological studies have demonstrated association of anti-AMA1 antibodies with naturally acquired protection to malaria (Udhayakumar et al., 2001). *Pf*AMA1 has also been tested and found to stimulate both CD8+ and CD4+ specific T-cell, responses resulting in some level of protection against malaria (Chuang et al., 2013; Sedegah et al., 2014). CD4+ T cells are a major source of interferon-γ (IFN-γ) and tumour necrosis factor alpha (TNF-α) (Muxel et al., 2011) which are involved in both pathology and protection of this disease. Also, CD8+ T cells protect against the pre-erythrocytic stage of the *Plasmodium* parasite by production of IFN-γ which is mainly involved in intrahepatic parasites killing (Dinko & Pradel, 2016).

### 1.2 Problem statement

*Pf*AMA1 has been demonstrated play a role in erythrocyte invasion by merozoites (Kocken et al., 1998) and this function could be abrogated by antibodies (Kusi et al., 2009). However, for a polymorphic antigen like AMA1, antibodies to an allelic form have been reported to bind less with other alleles as a substantial fraction of antibodies are towards strain-specific epitopes (Kusi et al., 2009, 2010). Whether a similar effect of polymorphism exists for T cell recognition of epitopes within polymorphic antigens such as AMA1 however remains unclear. T cells are classically stimulated in the context of major

histocompatibility complex (MHC) molecules and they are known to bind to specific amino acid residues on the peptide sequence (Sant'Angelo et al., 2002). However, the MHC genes are the most diverse known genes as there are multiple variants within the population (Janeway et al., 2011). Hence, polymorphism within the peptides and/or within the MHC molecule will most likely affect MHC binding and recognition by T cells (Nlinwe et al., 2018)

### 1.3 Significance of study

The innovative and effective development of vaccine platforms and the application of potential immunotherapies that stimulate or enhance malaria protection require a deep understanding into cellular immune mechanisms against *Plasmodium* parasite. This will direct peptides selection for inclusion in a multi-epitope vaccine which will have a broader specificity. This study therefore investigated the impact allelic polymorphisms have on the induction of specific CD8+ and CD4+ T cell IFN-γ responses by stimulating PBMCs from subjects with different exposure levels to malaria parasites with allelic AMA1 peptides from 3D7, FVO and 7G8 strains of *P. falciparum*.

### 1.4 Hypothesis

Variable sequences of the *Pf*AMA1 antigen can be targets of immunodominant T cell responses.

### 1.5 Main aim

This study was aimed at assessing the effect of allelic polymorphism in *Pf*AMA1 on the induction of AMA1 specific CD4+ and CD8+ T cell responses.

### 1.6 Specific objectives

1. Assess IFN-γ induction potential of predicted polymorphic AMA1 peptides from three parasite strains (3D7, FVO and 7G8).
2. Characterise peptides for their specificity in activating CD4/CD8 T Cells.
3. Identify unique circulating AMA1 haplotypes in the study population.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Global and Regional Trend in Malaria Burden

The global estimated malaria cases in 2017 as reported by WHO was 219 million (WHO, 2018). Even though there were about 20 million reduction in global cases in 2017 compared to 2010, there has been a change in upward trend between 2015 and 2017. Transmission of malaria occurs in five WHO regions and 3.4 billion people in 92 countries are estimated to be at risk of the disease (Figure 2.1). The burden of malaria is not distributed evenly across the world as the WHO African Region (AFR) recorded about 92% of the reported cases. High global malaria burden (80%) is carried by 15 countries within sub-Saharan Africa and India and this disease claims the life of a child every 2 minutes (WHO, 2018). Despite a later increase in cases, malaria-associated death globally has declined between 2010 and 2017 from 607 000 to 435 000 cases except for the WHO Region of the Americas, largely due to a sudden rise in malaria in Venezuela (WHO, 2018). However, AFR recorded about 93% of these mortalities associated with malaria.

## 2.2 Malaria burden in Ghana

Malaria associated mortality accounted for 19% of all recorded deaths in Ghana in 2015 (WHO, 2016). However, there was a significant decline to 4.2% in 2016 (WHO, 2017). Besides this, case fatality rate among children under 5 years declined from 15% in 2010 to 11% in 2016 (WHO, 2017). However, malaria admission increased from 280,000 to 340,000 between 2000 and 2017 (WHO, 2018).

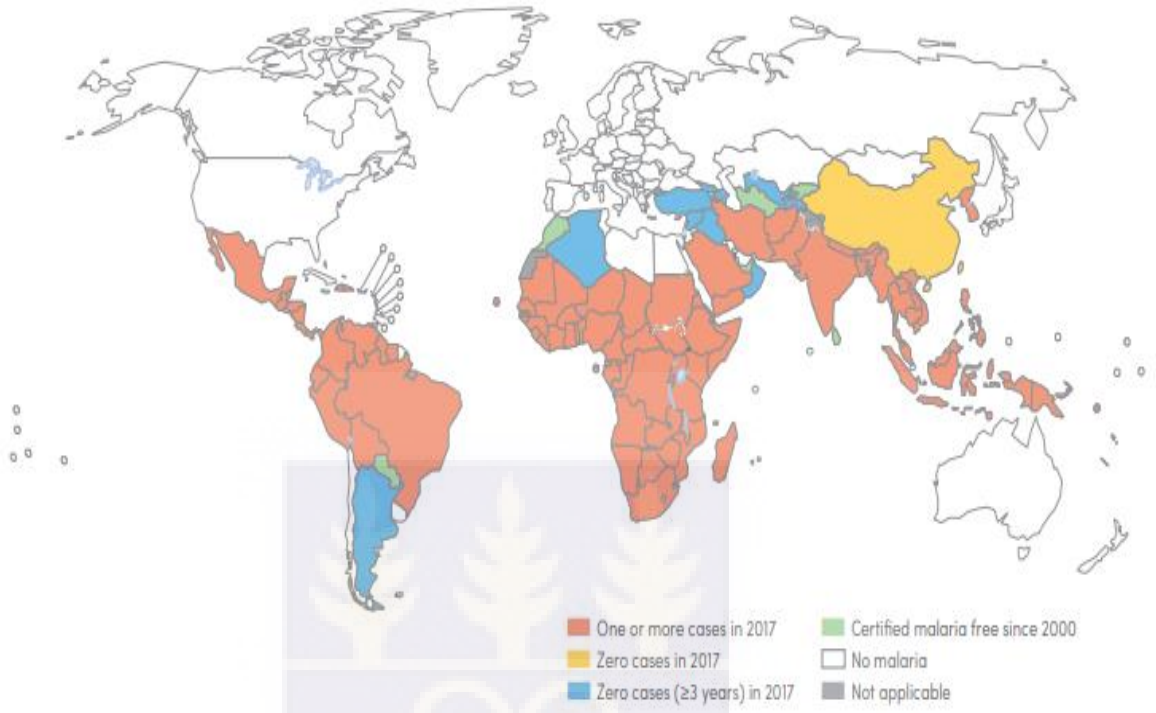


**Figure 2.1. Status of malaria cases in 2017 from countries with indigenous cases in 2000:** WHO European Region countries reported no malaria cases in 2016 and 2017. Countries with no cases over the past 3 yrs. considered to be malaria-free. Source: (WHO, 2018)**.**

## 2.3 Economic burden of Malaria

The economic devastation and human influence of malaria has been known for a long time. The estimated total cost for the control and elimination of malaria in 2017 was US$ 3.1 billion, a 7% increase from 2016. The contribution of government in countries with malaria endemicity accounted for 28% of the total investment of which US$ 0.6 billion and US$ 0.3 billion were used for malaria control activities and patient care respectively. Africa was the major WHO Region recipient of the total investment in malaria, followed by the South-East Asia, America, Western Pacific and Eastern Mediterranean (WHO, 2018).

## 2.4 Population at risk of Malaria

Report from WHO in 2017 shows that, about half of the world's population is at risk of malaria, however, 87 countries and territories are currently with ongoing transmission of malaria. Also, most of these malaria cases and death occurred in sub-Saharan Africa (WHO, 2017). Moreover, certain group of individuals are considered highly at risk of severe malaria than others. These includes; children below 5 years of age, pregnant women, HIV/AIDS patients, non-immune migrants, mobile populations and travellers (Brown, 2011). Therefore, national malaria control programs should put in place strategies for protecting populations from the infection.

## 2.5 Malaria clinical presentations

Mostly complicated or severe malaria has been the main focus because of the high level association with malaria related mortalities (Nanda et al., 2012). Severe malaria is defined by at least any of these clinical presentations: coma (caused by cerebral malaria), haemoglobinuria, hypoglycaemia, malarial anaemia, convulsions, acute pulmonary oedema, metabolic acidosis, acute renal failure, jaundice, hyper-parasitaemia, circulatory collapse, spontaneous haemorrhage, and/or high fever electrolyte disturbance (WHO, 2014). Contrastingly, clinical presentation of mild or uncomplicated malaria is usually fever and possibly one or more of the following manifestations: headache, chills and sweats, watery diarrhoea, vomiting, jaundice, anaemia and splenomegaly (Grobusch & Kremsner, 2005). The cyclical release of toxins, inflammatory mediators and merozoites from RBCs concurs with febrile illness and if this is diagnosed and treated quickly, such individual have a high recovery rate. The most lethal species of *Plasmodium* is *P.*

*falciparum* however, *P. ovale* and *P. vivax* can be difficult to treat due to relapse. *P. falciparum* however, induces high parasitaemia and also causes the iRBC sequestration in the microvasculature of vital organs. This obstructs the flow of oxygenated blood to vital organs like the brain and can lead to deep coma.

## 2.6 Malaria control and eradication strategies

### 2.6.1. Therapeutic Drugs and Insecticide

Over the years several antimalarial have been introduced to treat both symptomatic and asymptomatic individuals. These therapeutics drugs includes quinine, chloroquine, Proguanil etc. Quinine is the first antimalarial drug introduced to treat asymptomatic individuals (Achan et al, 2011). However, due to poor compliance and tolerability and resistance, a more effective artemisinin was discovered (Oaks et al, 1991). WHO therefore suggested artemisinin-based combination therapy (ACT) as first-line treatment of uncomplicated malaria caused by the *P. falciparum* (Witkowski et al, 2013). In recent years however, resistance of parasite to artemisinin has been identified to have spread across rapidly than expected (Birkett, 2016).

Another tool implemented is insecticide for vector control. Currently, the only approved insecticides for used in LLINs and ITNs are pyrethroids (Zaim & Jambulingam, 2007). Since WHO adopted LLIN as a key control strategy, the approved insecticides for LLINs and ITNs for effective malaria control have been pyrethroids (Curtis & Mnzava, 2000). However, this success has been haltered by the rapid pyrethroid resistance development in the vector mosquito (Figure 2.2). Fortunately, this resistance has not really been associated with decreased efficacy hence, ITNs still remain effective tools in almost all settings (Tun

et al, 2015). Nevertheless, with the increase in malaria control strategies, the continued selection of pyrethroid resistance may compromise malaria control programs and render this group of insecticides ineffective (N'Guessan et al., 2010).

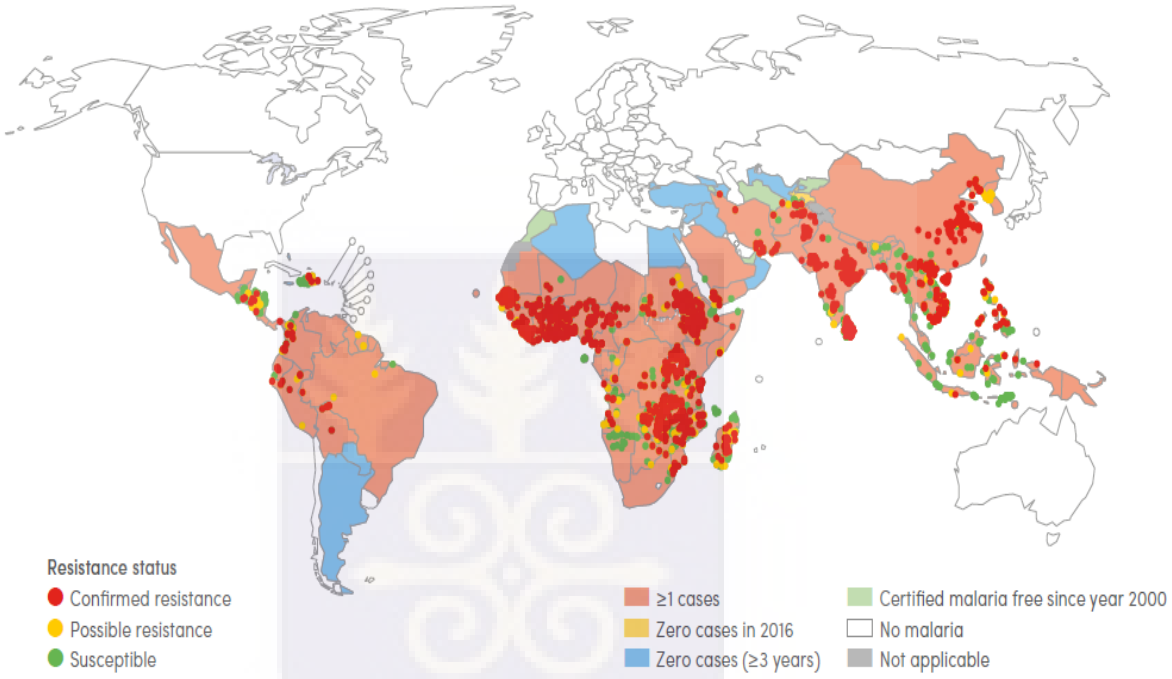


**Figure 2.2**. **Status of pyrethroid resistance of mosquitoes between 2010 and 2016**. Data obtained after multiple classes or types of insecticide as well as mosquito species and time point were tested from standard WHO insecticide susceptibility or CDC bottle bioassays. **Source**: **WHO, 2017 report**.

## 2.7 Natural Immunity to malaria

### 2.7.1 Innate immune response

Innate immunity to malaria is the inherent or natural resistance to the disease. This form of immunity is the first line of defence against any infection and can be found in most vertebrates. Innate responses to malaria have been shown to be very essential in limiting initial parasite replication in mice while in human's parasite growth can be controlled early in primary

infections (Molineaux et al., 2002). Macrophages and dendritic cells are able to bind directly to iRBC by CD36 expressed on their surfaces without any malaria-specific opsonizing antibody (Serghides et al., 2003). Also, dendritic cells respond to microbial infections by sampling site of entry for pathogen, responding to signals, uptake and processing of antigens and finally activating both naïve and memory T cells (Guermonprez et al., 2002; Sher et al.,2003). Moreover, natural killer (NK) cells is the frequent cells to respond after human PBMCs are challenged with infected red blood cells (iRBCs). Other T cell subtypes such as natural killer T (NKT) cells and γδ T cells start to make interferon- γ (IFN-γ) only after NK-cell response reaches its peak (Artavanis-Tsakonas & Riley, 2002), signifying that NK cells might initiate a cascade of innate immune responses.

### 2.7.2 Humoral immune response

In malaria, immunoglobulin G (IgG) antibodies play a key part in fighting the infection by decreasing parasitaemia and clinical symptoms (Medeiros et al., 2013; Folegatti et al., 2017). Different clinical malaria expression have demonstrated a differential regulation in antibody patterns to *Plasmodium* parasite and this is reported that even in unstable malaria transmission areas, immunity to malaria can be produced only if the right antibodies are made (Leoratti et al., 2008). IgG1 and IgG3 (cytophilic antibodies) are the most essential IgGs in mediating leukocyte activation by binding to FcγRI and FcγRIII (Kwenti et al., 2019). These antibodies are associated with lower risks of malaria complication in malaria endemic areas (Weaver et al., 2016; Cheng et al., 2018). However, non-cytophilic antibodies (especially IgG4) are pathogenic and their presence are associated with the severity of malaria (Roussilhon et al., 2007; Stanisie et al., 2009). The mechanisms of humoral protection include: inhibition of hepatocytes invasion by sporozoites (Dent et al.,

2008), avoid RBC invasion (Haynes et al., 2002), depress parasite growth (Wilson et al., 2010) and enhance phagocytosis of parasite by macrophages (Druilhe & Pérignon, 1997). Serum from immune individuals have been demonstrated to have the ability to disrupt formation of rosettes (Migot-Nabias et al., 2010) and also bind to iRBCs and prevent them from binding to endothelial cell ligands (Ricke et al., 2000).

At the pre-erythrocytic stage, antibody-mediated immune response targets the free sporozoites and infected hepatocytes. Sporozoites are targeted through surface antigens like CSP to inhibit hepatocytes invasion by the neutralization of proteins needed for traversing and invading the cell (Belachew, 2018). At the erythrocytic-stage, antibodies directly inhibit merozoite invasion of erythrocytes or by opsonizing merozoites for phagocytosis (Bouharoun-Tayoun, 1990). They also mediate cellular killing or prevents iRBCs adhesion to endothelium and prevent unregulated inflammation by neutralizing parasite (Gomes et al., 2016). Also, antibodies to gametocytes functions through complement-mediated lysis and inhibit their sequestration and maturation in the host (Belachew, 2018). Antibodies from host during blood meal by the vector mosquito are also involved in for gametocytes killing and by inhibition of gamete fusion in the mosquito through complement-mediated lysis (Dennison et al., 2015).

### 2.7.3 Cellular Immune Response to Malaria

Several lines of evidence from experimental rodent models suggest an important role for T cells in protection against malaria. CD8+ T cells, also known as Cytotoxic T-cells (CTL) are mainly involved in pre-erythrocytic stages protection against the parasite (Wang et al, 1998); however, this is thought to be CD4+ T cells dependent (Carvalho et al., 2002;

Shedlock, 2009). CD8+ T have been demonstrated to produce IFN-$\gamma$ which is mainly involved in killing of intrahepatic parasites (Dinko & Pradel, 2016). This protection was reversed and parasitaemia increased when CD8+ T cells were depleted in RAS immunized BALB/c mice prior to sporozoite inoculation (Perlaza et al., 2011). Similarly, genetically activated sporozoites (GAS) immunized mice elicited a CD8+ T cell dependent protection (Tarun et al., 2007) which was able to kill *P. yoelli* infected hepatocytes (Trimnell et al., 2009). Immunization with recombinant adenovirus expressing CSP of *P. yoelli* and depletion of CD8+ T cells showed CD8+ T cell involvement in inducing sterile immunity (Rodrigues et al., 1998). Moreover, CD4+ T cells have also been observed to be involved in pre-erythrocytic malaria immunity (Tsuji et al., 1990) It is able to kill infected hepatocyte and induce protection in naïve mice upon adoptive transfer.

At the erythrocytic stage, the CD8+ T cells role of is negligible (Dennison et al., 2015) and hence, the main T cell subset involved in immune response is the CD4+ T helper cells. They have been demonstrated to produce pro-inflammatory cytokines for activating macrophages and also mediate specific B cell clones activation (Bertolino & Bowen, 2015). Also, CD4+ T cells are essential for most *Plasmodium*-specific antibody responses (Good et al., 2004). Though cell-mediated immune (CMI) response has a vital role in immune protection, it also has the potential of causing tissue damage and contributes to severe malaria development (Clark et al., 2008). Similarly, regulatory T cells (Tregs), a subset of CD4+ T cells, play a role by regulating the host immune response and this could either result in immune-tolerance or enhance the severity of the disease. Tregs usefulness relies on the timely induction because their presence in early infection is dampens immune response and allow the parasite to thrive whilst latter induction limits immunopathology

(Hansen & Schofield, 2010). Mostly, prove of cell-mediated immunity are from animal models and these are usually poor models for studies of human malaria. In addition, reports from different animal species or strains often differ greatly and this makes it complex to conclude definitely with regards to immune effector mechanisms in malaria.

#### 2.7.3.1 T cell interaction with Peptide-bound HLA

T-cells are classically stimulated by peptides in the context of major histocompatibility complex (MHC) molecules, also known as Human Leukocyte Antigen (HLA) molecules in humans. Antibodies can bind to any part of the antigen's surface, however, T cells receptors (TCR) are restricted to sensing the presence of antigenic peptides presented by HLA I/II molecules (Figure 2.3). TCRs recognize specific amino acid residues on HLA bound peptides during presentation and this eventually determines the type of response elicited. TCRs make contact with residues 5 to 7 of a span of 8 residues of HLA I bound peptides (8-mers and 9-mers) and from 6 to 7 of a span of 9 residues of HLA II bound peptides (13-mers and 16-mers). HLAs on the other hand, also binds to amino residues at specific positions within the peptides they present (Corse et al., 2011). HLA I which presents peptides to CD8+ T cells has been reported to bind to amino acids at positions 2, 5/6 and 9 within the peptide side chain (Wieczorek et al., 2017). Other studies have also reported MHC I core anchor positions as positions 2 or 5 and the final amino acid (PΩ) in the peptide side chain (Batalia & Collins, 1997). HLA II, which presents to CD4+ T cells, binds to positions 1, 4, 6 and 9 (Wieczorek et al., 2017) or positions 1 and 9 as reported by another study (Lafuente & Reche, 2009). Hence, polymorphisms may have a significant effect on the generation of specific T cell responses to antigens and the potential vaccines efficacy based on these antigens (Nlinwe et al., 2018). Moreover, polymorphisms in T cell

epitopes and HLA molecules could cause CD8+ T cells activation during primary infection to be less effective during subsequent infections leading to liver stage infection (Nlinwe et al., 2018; Gilbert et al., 1997).

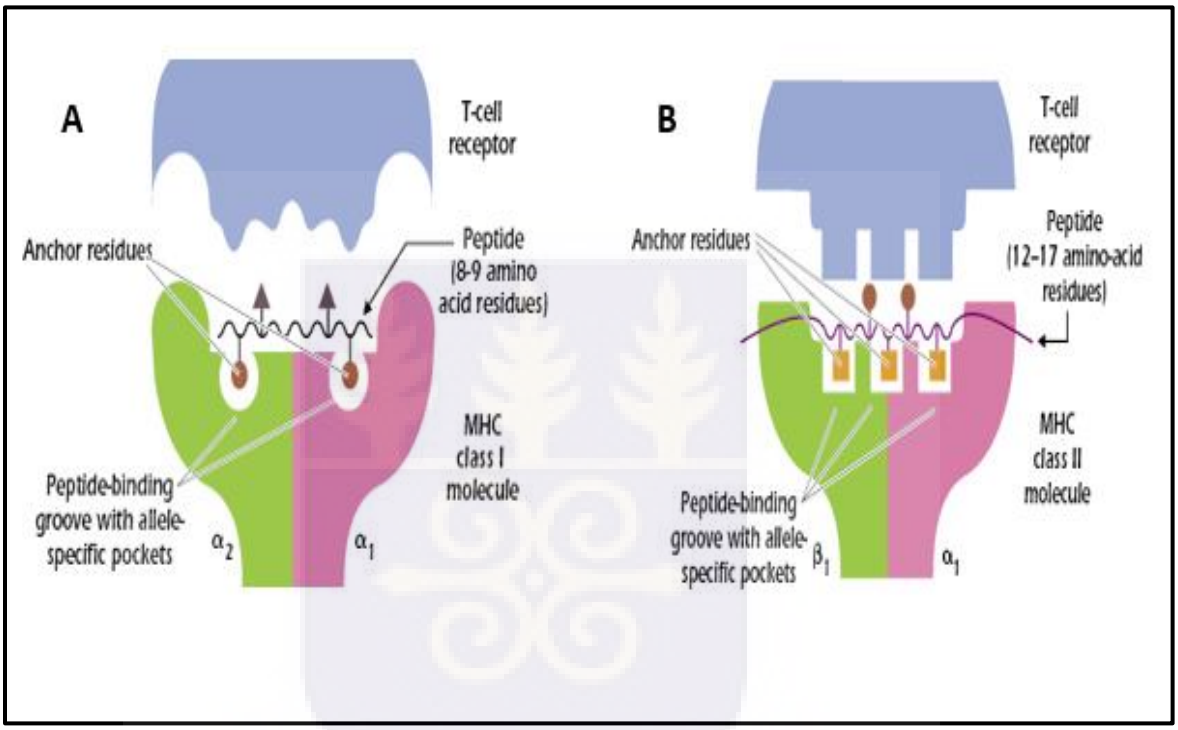


**Figure 2. 3. How T Cells Recognize Antigen:** A) MHC I interacts with peptide binding groove at positions 2 and 9. B) MHC II interacts with peptide binding groove at positions 1, 4 and 9. Anchor residues in the peptide bind to the allele-specific pockets of the MHC molecule before T cell receptor binding.

### 2.7.4 Cytokine response to malaria

Malaria is characterized by wide range production of cytokines from both the innate and adaptive arm of the immune system. Following to the innate response that regulate early malaria infection the adaptive immunity later predominates and CD4+ T-cells becomes the major producers of cytokines (Luckheeram et al., 2012). Two groups of mature CD4+ T cells are associated with distinct cytokine profiles. Type 1 helper cells (Th1) produces IFN-

α/γ, lymphotoxin-α (TNF-β), interleukin-12 (IL-12) while type 2 helper cells (Th2) produces IL-4, IL5, 6, 9, 10 and 13. Th1 and Th2 are associated with a strong cell-mediated immunity and antibody production respectfully. An early response of IFN-γ to malaria is essential protecting against severe forms of the disease (Cabantous et al., 2005). Increased IFN-γ levels is associated with the reduction of parasite in acute malaria (Winkler et al, 1998) and a interrupts re-infection (Luty et al,1999) while low levels are associated with high parasite density in children (Winkler et al, 1999).

### 2.7.5 Human genetics and immune response

Numerous RBC disorders, such as ovalocytosis (Genton et al., 1995), haemoglobin variants (Chotivanich et al., 2002), ABO blood group antigen (Hill, 1992), complement receptor 1 polymorphisms (Cockburn et al., 2004) and G6PD deficiency (Mombo et al., 2003), have been associated with some level of protection against severe malaria. G6PD A- heterozygosity protects against all malaria forms in female subjects (Mombo et al., 2003). ABO blood group distribution globally coincides with natural selection by various pathogens. Reduced rosetting and sequestration of O blood group is a mechanism of protection against malaria (Rowe et al., 2007). Other studies have also reported higher incidence of severe malaria association with blood group A (Lell et al., 2007).

## 2.8 Vaccine against malaria

Despite advances in research in malaria control and eradication, the available tools coupled with naturally acquired immunity have not been effective and this has been confirmed by an increase in the malaria cases as reported by WHO 2018. With emergence of current anti-malarial drugs and insecticides resistance and with no absolute protection with current

vaccine, there is, therefore, the need the addition of new and better tools to help eradicate malaria. An effective vaccine would be the model additional tool for control, prevention and elimination of many infectious diseases. Several approaches have been accepted in the development of malaria vaccines and these have broadly targeted the blood and liver stages of the parasite development**.**

However, the complexity of the *Plasmodium* genome has made it a challenge in developing an effective malaria vaccine so far due to (Hoffman et al., 2015). The *Plasmodium* surface proteins are more exposed to immune system and this has resulted in high antigenic diversity (Cowman et al., 2002). Due to the immune responses against AMA1 antigen, it has been included as a potential vaccine candidate antigen (Farooq et al., 2009; Kocken et al., 1998; Malkin et al., 2005).

### 2.8.1 Pre-erythrocytic vaccines

Pre-erythrocytic stage vaccines *Plasmodium* target the sporozoites to eliminate parasites during early infection and induce sterile immunity to interrupt transmission. Whole sporozoite vaccines have been demonstrated to induce protection in humans (Clyde et al., 1973). *Pf*SPZ vaccine, a weakened sporozoites vaccine induced immune responses from CD4+, CD8+ and γδ T cells, as well as antibody responses and trials of *Pf*SPZ vaccine in Mali confirm efficacy against naturally transmitted parasites (Ishizuka et al., 2016; Epstein et al., 2017). Also, it has been shown that using irradiation almost completely protected humans however, it was expensive and could not be applied on a larger scale (Hoffman et al, 2002).

The most advanced and approved malaria vaccine. Studies generally have shown modest efficacy, immunogenicity and safety with RTS, S/AS01 in infants and children in selected endemic areas in sub-Saharan Africa (Asante et al., 2011; Olotu et al., 2016). The vaccine is currently being introduced in Ghana Malawi and Kenya in an ongoing piloting implementation (Adepoju, 2019). This vaccine is a pre-erythrocytic stage vaccine based of the last 18 NANP repeats and C-terminus of the parasite CSP antigen fused to the Hepatitis B virus surface antigen, and is formulated with the proprietary AS01 adjuvant (Leroux-Roels et al., 2014). The low efficacy associated with RTS, S/AS01 vaccine makes it unlikely for this vaccine to meet the malaria eradication goal. An effective malaria vaccine would be an important tool for preventive measures hence, further studies on using of alternative parasite targets and vaccination strategies are highly recommended.

### 2.8.2 Erythrocytic stage vaccine

The challenges in developing a blood stage vaccine over the years are usually diversities in merozoites and iRBC surface proteins, difficulties expressing proteins of correct conformation and redundancy in the merozoite invasion pathways (Satchwell, 2016). However, focusing on novel antigens combinations could give synergistic activity and enhance immunogenicity of the vaccine. Moreover, vaccines from chemically weakened blood stage parasite produced immunity in a CD4 + T cell-dependent fashion (Raja et al., 2016, 2017). Contrastingly, these vaccines did not offer cross protection even after CD8+ T cells activation and protection at the blood-stage remained intact after CD8+ T cell depletion (Raja et al., 2016).

*P. falciparum* reticulocyte-binding protein homolog 5 (*Pf*RH5) is a highly conserved antigen targeted for blood stage vaccine (Douglas et al., 2011). It interacts with a receptor called basigin and is localized to the moving junction during invasion (Wright & Rayner, 2014). Protection from *Pf*RH5-based vaccines is associated with anti-*Pf*RH5 antibody concentration and neutralization of parasite activity *in vitro* (Douglas et al., 2015). Purified anti-*Pf*RH5 IgG inhibited parasite growth by about 60% *in vitro* (Douglas et al., 2015). Naturally acquired immunity to malaria is classically non-sterile, yet anti-*Pf*RH5 antibodies can potently provide sterile protection. However, there is low titres of RH5 antibodies in clinically immune adult (Patel et al., 2013) but despite that, purified antibodies from human immune serum had strong invasion inhibitory effects (Richards et al., 2013), and in a time-to-reinfection study in Mali, the presence of anti-RH5 antibodies was strongly associated with protection from malaria episodes (Tran et al., 2014). Importantly for the use of RH5 as a vaccine, the parasite's ability to evade the host antibody responses appears to be context-specific since high-titre antisera can be raised to a recombinant RH5 protein/adjuvant mix in rabbits (Bustamante et al., 2013) and mice (Douglas et al., 2011). However, the apparently low immunogenicity of native RH5 may prevent significant levels of natural boosting following vaccination, which could affect the induction or longevity of any RH5 vaccine-induced response. Nonetheless, the pan-strain dependency on the interaction of RH5 with basigin for invasion and its susceptibility to elicited antibodies make RH5 a highly promising target for a blood-stage vaccine, either alone or in combination with other synergistic targets (Williams et al., 2012), and further trials are clearly justified.

Another blood stage vaccine that inhibits merozoites invasion targets AMA1-RON2L. Antibodies against this complex inhibits the growth of virulent *P. falciparum* (Srinivasan et al., 2017). However, AMA1-only vaccines could not offer any protection against human malaria infection (Payne et al., 2016) despite reports from field studies signifying protection from AMA1 vaccines conferred activity against homologous parasites (Thera et al., 2011).

### 2.8.3 Transmission-Blocking Vaccine (TBVs)

Results from clinical trials have showed that, *Pf*s25 (post-fertilization antigen) and *Pf*s230 (pre-fertilization antigen) based vaccine induces protection against sexual stage parasites in mosquitoes (Gwadz, 1976). Immunogenicity to these antigens were further improved by the fusion of each to the immunogenic carrier protein; Exoprotein (EPA) and administered with an adjuvant (Radtke et al., 2017). Pfs25-EPA formulation with Alhydrogel® was safe and immunogenic in humans in clinical trials. However, anti-Pfs25 antibody levels rapidly waned and functional activity was short-lived (Coelho et al., 2017).

## 2.9 Vaccine candidate antigens

There is approximately 5400 genes in *P. falciparum* genome distributed over 14 chromosomes (Doolittle, 2002). About 4% of these genes encode proteins involved in immune evasion and host-parasite interactions (Gardner et al, 2002). Several of the surface antigens including Merozoite surface Protein (MSP9 2), AMA1, and CSP and have also been tested in vaccine trials (Gardner et al, 2002). CSP is one protein heavily focused on for vaccine development in the pre-erythrocytic stage of the *Plasmodium*. Its encoding gene

is one of the first cloned malaria genes from both human and animal malaria parasites (Chulay, 1989). MSP2 and AMA1 among several others are blood stage target antigens for vaccine development. Sexual stage vaccine candidate antigens also includes *P. falciparum* sexual antigens: *Pf*s230, *Pf*s48/45, *Pf*s40/10, which are mostly expressed by gametes and *Pf*s25 expressed by the zygotes (Kaslow et al, 1988).

### 2.9.1 Apical Membrane Antigen 1 (AMA1)

#### 2.9.1.1 Structure and sequence of AMA1

AMA1 is a structurally conserved type I integral membrane protein of 622 amino acid sequence (Coley et al, 2007). The *Pf*AMA1 is encoded by a single copy gene and is characterized in a as either an 83-kDa *de novo* polypeptide product (*P. falciparum*) or a 66-kDa polypeptide product (all other species) (Healer et al., 2004). The cytoplasmic and extracellular region are about 55 and 550-amino acid long respectively and extracellular region is divided into three domains (DI, DII and DIII) based on disulphide bonds by 16 conserved cysteines (Nair et al., 2002). Based on analysis from restriction fragment length polymorphism (RFLP), DI can be further divided into four subgroups termed I, II, III and IV (Marshall et al.,1996).

Despites conserved cysteine residues, there are significant polymorphisms among AMA1 alleles of *P. falciparum* representing 10% of the entire sequence (Marshall et al., 1996). DI is the most diverse region of AMA1 (Kang et al., 2018) and this region is a major target of anti-AMA1 protective antibodies (Polley et al., 2004). Multiple lines of evidence indicate that polymorphisms in the *Pf*AMA1 DI result from selective pressures from host immune

responses (Cortes et al., 2003). *Pv*AMA1 is also polymorphic, although the regions under selective pressure might differ from those in *Pf*AMA1 (Gunasekera et al., 2007).

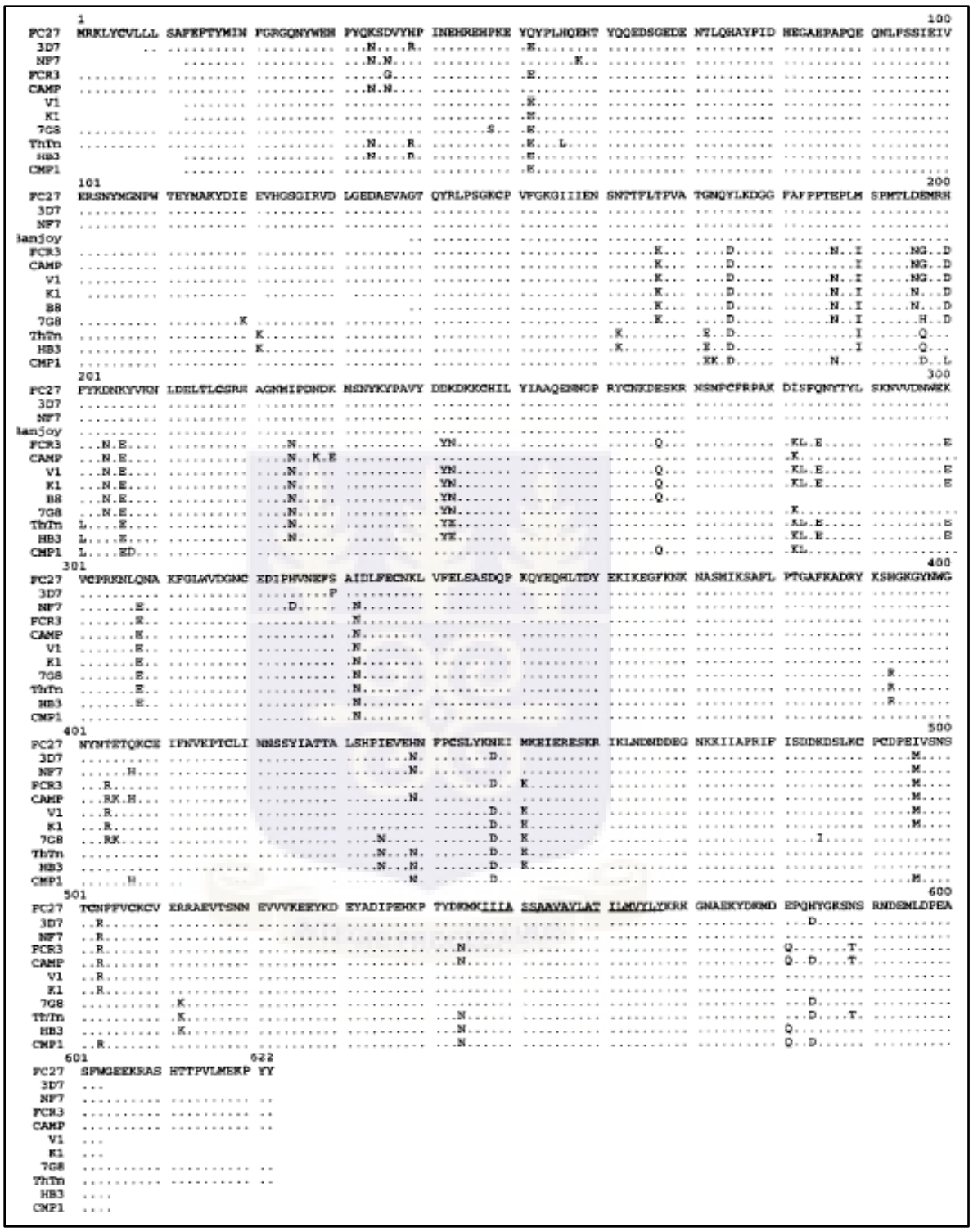

**Figure 2.4**. **Multi-sequence alignment of AMA1 proteins from various isolates of *P. falciparum***. Identical residues are indicated by dots while polymorphic residues are indicated by block letters representing the amino acid change. Putative transmembrane sequence location is underlined. Source: (Marshall et al., 1996).

#### 2.9.1.2 Role of AMA1

AMA1 has been associated with merozoites re-orientation on RBC surface (Mitchell et al., 2004). It has also been intricate in the regulation of the rhoptry secretion which mediate the junction where the parasite and host cell membrane contact during invasion (Alexander et al., 2006). AMA1 is expressed in the micronemes of the apical complex (Healer et al., 2004) and is processed proteolytically before translocation to the surface of merozoite for invasion of RBCs (Thomas et al., 1994). The protein is lost from the merozoite surface by cleavage with serine protease as the parasite undergoes invasion (Howell et al., 2003).

#### 2.9.1.3 Antibody immune response to AMA1

AMA1 has been studied in all stages of *P. falciparum*, but extensive work has focused on the blood stage of the parasite growth cycle. Studies have demonstrated AMA1 to be immunogenic in malaria-exposed individuals (Lal et al, 1996; Thomas et al, 1994) and naturally acquired antibodies to AMA1 can hinder parasite invasion *in vitro* (Hodder et al., 2001) and protect against naturally acquired malaria (Udhayakumar et al, 2001). In naturally exposed populations, antibodies to AMA1 antibody has been reported to be IgG1 predominantly with some IgG3 and a rarely high IgG2 or IgG4 levels (Corte et al, 2005). These antibodies recognize mainly DI and DII, with low responses to DIII generally, however, these levels has been seen to increase in adults (Polley et al, 2004). IgG to AMA1 constructs containing at least DI and DII is known to reduce risk of clinical malaria in parasitic individual (Polley et al, 2004).

The impact of AMA1 polymorphism on inhibitory antibodies have previously been tested using different *P. falciparum* strains against antibodies produced against recombinant AMA1 from 3D7. These antibodies were strong inhibitors of the homologous and closely related strains, however, their potency reduced when tested against more polymorphic residues (Kusi et al., 2012). Also, FMP2.1/AS01, a subunit blood stage vaccine based on the *Pf*AMA1, has shown limited success in protecting clinical malaria or parasitaemia (Thera et al., 2011) nevertheless, there was a significant allele-specific efficacy against clinical malaria caused by closely related strain to that vaccine. This observation was linked to specific positions of amino acids identified earlier as vital causes of allele-specific naturally acquired immunity *in vitro* (Dutta et al., 2007), in field trials (Takala & Plowe, 2009) and in response to vaccination with a monovalent vaccine (Ouattara et al., 2013).

#### 2.9.1.4 Cell-Mediated Immune Response to AMA1

Cellular immune response is a major mediator of protective immunity to malaria. The presence of AMA1 on sporozoite surface also makes CMI against this protein essential in inhibiting sporozoites invasion and parasite development at the pre-erythrocytic stage. AMA1-specific T cell has been demonstrated to regulate antibody production in immunized mice and this immunity is lost when the immunised mice is depleted of CD4 T cells (Xu et al., 2000). However, there was a partial protection when CD4+ T cells specific to conserved epitope of AMA1 was transferred into athymic mice (Xu et al., 2000). Also, robust T cell response to AMA1 have been reported in irradiated sporozoites immunized subjects (Krzych et al., 1995). Thus, while antibodies offer immune protection against post AMA1 immunization, antibody-independent CMI to AMA1 contributes separately to

overall protection. Despite accessibility to T cells, the high levels of polymorphisms present in this antigen are a major drawback in effective vaccine development.

# CHAPTER THREE

## MATERIALS AND METHODS

### 3.1 Materials

Penicillin streptomycin Solution, Tween 20, Trypan blue, Dimethyl sulfoxide (DMSO), Concanavalin A (Con A) were obtained from Sigma- Aldrich chemical company. St. Louis, USA. RPMI-1640, Phosphate buffered Saline (PBS) tablets and Foetal Bovine Serum (FBS) were purchased from Gibco, USA. Normal Human Serum (NHS) was supplied by Promocell, Ficoll-Paque$^{TM}$ by GE-Healthcare, Sodium carbonate ($Na_2CO_3$) from J.T Baker, USA, Sodium bio-carbonate ($NaHCO_3$) was purchased from Mallinckrodt Chemicals, USA and ELISpot plates (96-well polyvinylidene difluoride backed plates (Cat #: MA1PS4510) were from Millipore Cooperation, USA. Anti-human IFN- γ mAb 1D1k, Purified (3420-3-1000), biotinylated anti-human IFN- γ mAb 7-B6-1 mAb (3420-6-1000), streptavidin Alkaline Phosphate Conjugate (3310-8) were all purchased from Mabtech AB, USA. AP conjugate substrate kit was obtained from Bio Rad Laboratories (USA), Countess cell counting chamber slides (C10228) and Dynabeads™ Untouched™ Human CD8/CD4 T Cells Kit were purchased from Invitrogen, USA. All synthetic *Pf*AMA1 peptides were produced by Cellular technology Ltd., USA. BD heparin vacutainers were also purchased from BD logistics (UK). Multi-channel pipette were products of Thermo Labsystems (USA). HM-10P TOA pH meter was manufactured by TOA, Japan and HB502 magnetic stirrer was from Bibby Scientific (UK). Pipette aid and Vortex mixer (A GMBHutovortex SA6) were from Drummond Scientific Co. (USA) and Stuart Scientific (UK) respectively. ELISpot reader and ELISpot Reader System (ELISpot program 7.0) were from AID Autoimmune Diagnostika GMBH, Germany.

## 3.2 Methods

### 3.2.1 Ethics, Consent and Permission

The study received ethical approval from the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. Preceding enrolment, the study protocol was clarified to all subjects and informed consent was sought from the study subjects.

### 3.2.2 Study Area and Population

This study was done in the Legon and Obom both in the Greater Accra region of Ghana. In Legon (Figure 3.1). In Legon, malaria transmission follows the rainfall pattern and the average annual rainfall is less than 1000 mm and most of it is occurs between April and June (Dodoo et al., 2011). Malaria slide positivity is usually below 1% for most of the year (Dodoo et al., 2011). Obom, on the other hand, is in the Ga-South Municipality of Greater Accra Region of Ghana. Malaria transmission in Obom is perennial however, the highest malaria season coincides with the main rainy season between June and August (Ghana Statistical Service, 2011). The average yearly rainfall differs between 790 mm to 1270 mm (Ayanful-Torgby et al.,2018). The prevalence of *P. falciparum* is high in Obom compared to Legon (Amoah et al., 2016). In 2018, the estimate of malaria incidence by microscopy was 35.6 % of all out-patient visits at the Obom Health Centre (Amoah et al., 2018).

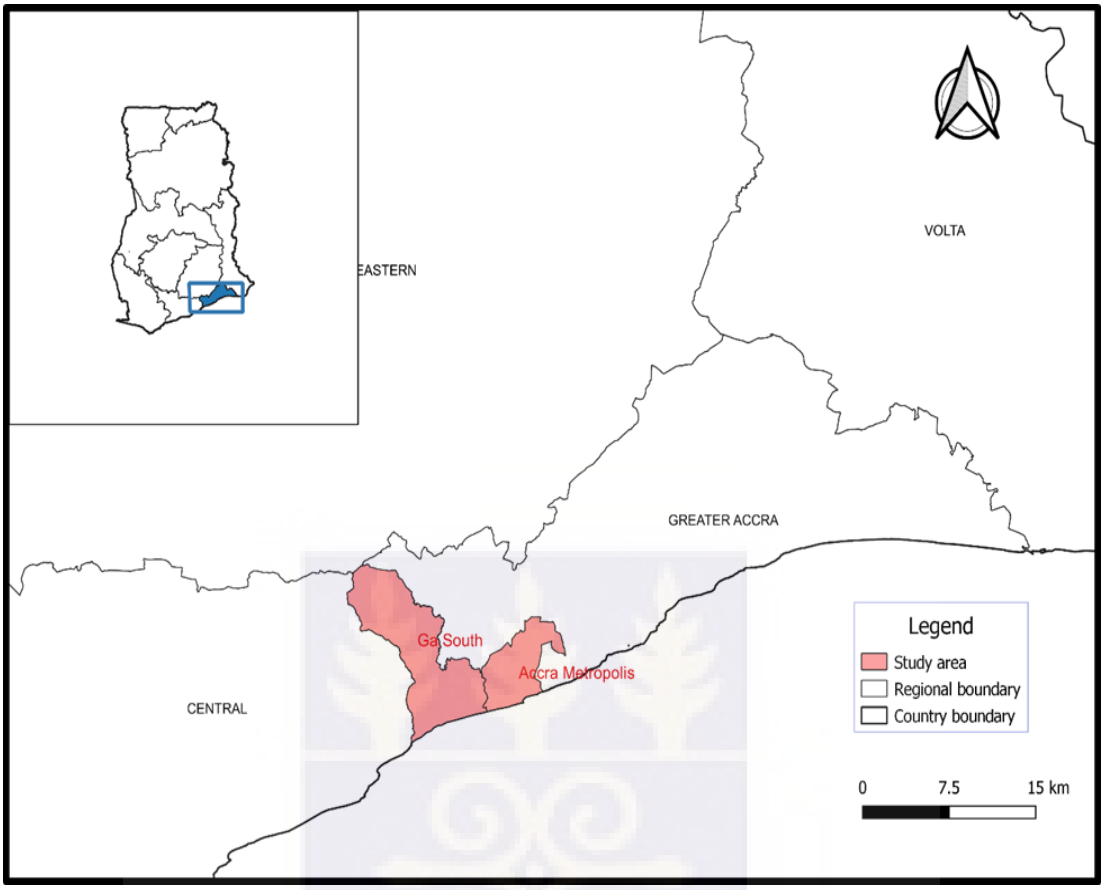


**Figure 3.1. Map of Ghana highlighting study sites.** The map highlights the Ga-south and Accra metropolitan district within which the two sites are located. Figure generated using QGIS.

### 3.2.3 Study subject's selection criteria and sample processing

The study was a cross-sectional and was conducted within March and April, 2019. Study subjects are resident in the study site involving male and female adults between 21 and 35 years. The haemoglobin (Hb) level in study subjects was determined using HemoCue (HemoCue® Hb 201 System, Sweden) and subsequently screened for malaria parasite using microscopy. Female participants were screened for pregnancy using a human chorionic gonadotropin (hCG) test strip. Subjects with haemoglobin <10 g/dl and females who were pregnant were excluded from the study. A total of 10 volunteers from each site

were included in the study after they met the inclusion criteria during the screening process. Sixty milliliters (60 ml) venous blood was collected from subjects from each study site into heparinized tubes and transported quickly on ice within 2 h to NMIMR for immunological assays. Dried Blood Spot (DBS) were made from all the 20 participants and 37 additional filter paper samples were taken and stored at -20 $^{o}$C until use for molecular analysis.

### 3.2.4 Parasite estimation by light microscopy

Thick, thin blood smears and DBS were each prepared from a drop (~50 µl) of finger-prick blood and allowed to air dry. The smears were stained with 10% Giemsa for 15 min and air dried before viewing under the light microscope using 100x oil immersion objective lens. Two different microscopists read the slides to confirm present of parasite.

## 3.3 Synthetic peptides selection

Synthetized peptides from 3D7 AMA1 strains that were predicted by bioinformatics predicted to elicit HLA-restricted T cell responses were selected and their corresponding allelic sequences from the FVO and 7G8 parasite strain AMA1s were tested alongside for comparison (Table 3.0). All peptides were synthesized commercially by Chiron Technologies (Clayton, Victoria, Australia). AMA1 amino acid sequences of the three parasite strains are presented in Figure 3.1 below.

**Table 3. 1. Sequences and locations of peptides in AMA1 antigen of *P. falciparum*.**

| Peptides | Sequence | Strain | Position of amino acids in AMA 1 protein |
|---|---|---|---|
| e1 | AKD**KL**F**E**NY | FVO | 278-286 |
| e2 | AKD**K**SFQNY | 7G8 | 278-286 |
| e3 | AKISFQNY | 3D7 | 278-286 |
| e22 | DVY**H**PINEHR | 7G8, FVO | 36-45 |
| e23 | DVYRPINEHR | 3D7 | 36-45 |
| e24 | EHREH**S**KEY | 7G8 | 43 -51 |
| e25 | EHREHPKEY | 3D7, FVO | 43 -51 |
| e46 | **D**FYK**N**N**E**YVK | 7G8, FVO | 200-209 |
| e48 | HFYKDNKYVK | 3D7 | 200-209 |
| e60 | **KL**F**E**NYTYL | FVO | 282-290 |
| e61 | **K**SFQNYTYL | 7G8 | 282-290 |
| e62 | ISFQNYTYL | 3D7 | 282-290 |
| e87 | MTL**NG**MR**D**FY | FVO | 193-202 |
| e88 | MTLD**H**MR**D**FY | 7G8 | 193-202 |
| e89 | MTLDEMRHFY | 3D7 | 93-202 |

Peptides are from the 3D7 (consensus), FVO and 7G8 strains of the *Pf*AMA1. Variable position(s) in corresponding variants of the peptide sequences are emboldened and underlined

**3D7 sequence (locus PFU65407 accession U65407.1)**

MRKLYCVLLL SAFEFTYMIN FGRGQNYWEH PYQNSDVYRP INEHREHPKE YEYPLHQEHT
YQQEDSGEDE NTLQHAYPID HEGAEPAPQE QNLFSSIEIV ERSNYMGNPW TEYMAKYDIE
EVHGSGIRVD LGEDAEVAGT QYRLPSGKCP VFGKGIIIEN SNTTFLTPVA TGNQYLKDGG
FAFPPTEPLM SPMTLDEMRH FYKDNKYVKN LDELTLCSRH AGNMIPDNDK NSNYKYPAVY
DDKDKKCHIL YIAAQENNGP RYCNKDESKR NSMFCFRPAK DISFQNYTYL SKNVVDNWEK
VCPRKNLQNA KFGLWVDGNC EDIPHVNEFP AIDLFECNKL VFELSASDQP KQYEQHLTDY
EKIKEGFKNK NASMIKSAFL PTGAFKADRY KSHGKGYNWG NYNTETQKCE IFNVKPTCLI
NNSSYIATTA LSHPIEVENN FPCSLYKDEI MKEIERESKR IKLNDNDDEG NKKIIAPRIF
ISDDKDSLKC PCDPEMVSNS TCRFFVCKCV ERRAEVTSNN EVVVKEEYKD EYADIPEHKP
TYDKMKIIIA SSAAVAVLAT ILMVYLYKRK GNAEKYDKMD EPQDYGKSNS RNDEMLDPEA
SFWGEEKRAS HTTPVLMEKP YY

**7G8 sequence (accession EU586371.1)**

MRKLYCVLLL SAFEFTYMIN FGRGQNYWEH PYQKSDVYHP INEHREHSKE YEYPLHQEHT
YQQEDSGEDE NTLQHAYPID HEGAEPAPQE QNLFSSIEIV ERSNYMGNPW TEYMAKYDIE
EVHGSGIRVD LGEDAEVAGT QYRLPSGKCP VFGKGIIIEN SNTTFLKPVA TGNQDLKDGG
FAFPPTNPLI SPMTLDHMRD FYKNNEYVKN LDELTLCSRH AGNMNPDNDK NSNYKYPAVY
DYNDKKCHIL YIAAQENNGP RYCNKDESKR NSMFCFRPAK DKSFQNYTYL SKNVVDNWEK
VCPRKNLENA KFGLWVDGNC EDIPHVNEFS ANDLFECNKL VFELSASDQP KQYEQHLTDY
EKIKEGFKNK NASMIKSAFL PTGAFKADRY KSRGKGYNWG NYNRKTQKCE IFNVKPTCLI
NNSSYIATTA LSHPNEVEHN FPCSLYKDEI KKEIERESKR IKLNDNDDEG NKKIIAPRIF
ISDDIDSLKC PCDPEIVSNS TCNFFVCKCV EKRAEVTSNN EVVVKEEYKD EYADIPEHKP
TYDKMKIIIA SSAAVAVLAT ILMVYLYKRK GNAEKYDKMD EPQDYGKSNS RNDEMLDPEA
SFWGEEKRAS HTTPVLMEKP YY

**FVO sequence (PFU84348 accession U84348.1)**

MRKLYCVLLL SAFEFTYMIN FGRGQNYWEH PYQKSDVYHP INEHREHPKE YEYPLHQEHT
YQQEDSGEDE NTLQHAYPID HEGAEPAPQE QNLFPSIEIV ERSNYMGNPW TEYMAKYDIE
EVHGSGIRVD LGEDAEVAGT QYRLPSGKCP VFGKGIIIEN SNTTFLKPVA TGNQDLKDGG
FAFPPTNPLI SPMTLNGMRD FYKNNEYVKN LDELTLCSRH AGNMNPDNDK NSNYKYPAVY
DYNDKKCHIL YIAAQENNGP RYCNKDQSKR NSMFCFRPAK DKLFENYTYL SKNVVDNWEE
VCPRKNLENA KFGLWVDGNC EDIPHVNEFS ANDLFECNKL VFELSASDQP KQYEQHLTDY
EKIKEGFKNK NASMIKSAFL PTGAFKADRY KSHGKGYNWG NYNRETQKCE IFNVKPTCLI
NNSSYIATTA LSHPIEVEHN FPCSLYKDEI KKEIERESKR IKLNDNDDEG NKEIIAPRIF
ISDKDSLKCP CDPEMVSNST CRFFVCKCVE RRAEVTSNNE VVVKEEYKDE YADIPEHKPT
YDNMKIIIAS SAAVAVLATI LMVYLYKRKG NAEKYDKMDQ PQHYGKSTSR NDEMLDPEAS
FWGEEKRASH TTPVLMEKPY Y

**Figure 3.2. *Pf*AMA1 sequences of the three varaint AMA1 antigens (3D7, 7G8 and FVO).** Coloured sequences are the selected peptides used in PBMC stimulation in this study. Variants of each peptide are highlighted with same Colour. All selected amino acids are within the N-terminus (aa 36-290) of the protein sequences which is the part exposed to the immune system.

### 3.4 Peripheral Blood Mononuclear Cells (PBMC)

#### 3.4.1 Isolation of PBMC from Whole Blood

PBMCs were isolated from whole blood by gradient centrifugation using Ficoll-Paque™ (ρ= 1.077 g/ml, Sigma-Aldrich, St. Louis, MO, USA) density gradient media as described by Anum et al., (2015). Briefly, the blood was diluted with an equivalent volume of the dilution buffer, RPMI 1640 medium (Sigma, Poole, UK), containing 1% of penicillin streptomycin. The diluted blood (25 ml) was gently overlaid on 15ml of Ficoll-Paque™ without allowing it to mix. This was spun at 2000 rpm for 15 min at 24 ºC without breaks. The uppermost layer was aspirated leaving the mononuclear cell layer. The cells were carefully transferred into a 50 ml centrifuge tube and washed thrice with three parts of RPMI 1640 supplemented with 5% FBS (5% FBS/RPMI-1640) at 2000 rpm, 24 ºC for 10 min without breaks. The cells were then finally suspended in a 10% NHS/RPMI-1640 culture medium. A 10 µl aliquot of the cell suspension was added to 10 µl of 0.4% Trypan blue (1:1 dilution) and the viability and concentration were determined using an automated cell counter (Countess II FL Automated Cell Counter, Invitrogen, USA).

#### 3.4.2 Cryo-preservation of PBMC

The isolated PBMCs were pelleted at 1500rpm for 7 min and the supernatant was discarded. The cells were loosened by gently tapping the tube and appropriate volume of freezing mix (consisting of 90% FBS and 10% DMSO). The freezing mix was added to the cells gently while swirling he tube and the cells were stored at 20 million per vial using a NALGENE™Cryo1$^0$C freezing container (ThermoFisher Scientific, USA) at -80 $^0$C overnight before transfer into liquid nitrogen until use for ELISpot assay.

### 3.4.3 Recovery of frozen PBMC from liquid nitrogen

Frozen PBMC's were moved from the liquid nitrogen on ice and thawed quickly at 37 °C in a water bath until most of the ice had melted. The thawed PBMC's were transferred into 20 ml of pre-warmed cell wash buffer (5% FBS/RPMI-1640) in a 50 ml centrifuge tube and spun at 2000 rpm for 10 min at 25 °C. The supernatant was aspirated and the pelleted cells were loosened by tapping of the tube with the fore finger (index finger). The cells were washed again after re-suspending in 10 ml of the wash buffer and the cells finally resuspended in 10 ml of complete medium (10% NHS/FBS-1640). A 10 µl aliquot of the cell suspension was mixed with 10 µl of 0.4% Trypan blue (1:1 dilution) and the cell viability and concentration were determined using an automated cell counter as previously described. Cells were subsequently rested at 37°C in 5 % $CO_2$ for 20 h. The next day, the cells were washed with 15 ml of the wash buffer and the viability and concentration were re-estimated as described above and adjusted to a final concentration of 20 x $10^6$ cells/ml. The cell volume was divided into three equal parts and one part was incubated at 37 °C in 5% $CO_2$ prior to ELISpot assay. The second and third parts were enriched for CD8+ and CD4+ T cells respectively before use in ELISpot assays alongside the unfractionated cells.

### 3.4.4 In Vitro depletion of human CD8+ and CD4+ specific T-Cells

Dynabeads (Depletion MyOne™ SA Dynabeads® Invitrogen, USA) were used to enrich human CD8+ or CD4+ T cells from PBMCs following the manufacturer's instructions. Briefly, Dynabeads was washed twice by first gently vortexing the vial for a minute and 500 µl each was transferred into 15 ml centrifuge tube labeled for each sample. The beads were re-suspended in 1 ml of isolation buffer: ($Ca^{2+}$ and $Mg^{2+}$ PBS free) supplemented

with 2% FBS and 0.6% sodium citrate) and tube was placed in a magnetic cell separator (MACS) (Mario MACS, Miltenyi Biotic, Germany) for 3 min to wash the beads by separating the beads from the buffer. The buffer was discarded and the beads again were re-suspended in 1ml of the isolation buffer for the second wash. PBMC suspension was prepared by resuspending the cells at 20 x $10^{6}$ cells/ml in 500 µl isolation buffer. A 100 µl each of heat inactivated FBS and antibody mix (mouse IgG antibodies against the non-CD8+ or non-CD4+ specific T- Cells) was added to the cells and gently vortexed to mix before incubating at 4 ºC for 40 min. The cells were washed with 4 ml of the isolation buffer by centrifuging at 4 ºC, 2000 rpm for 10 min. The buffer was discarded and the cells were then resuspended in 500 µl of isolation buffer and 500 µl of prewashed Dynabeads was added and incubated for 50 min at room temperature with gentle tilting and rotation at 300 rpm. The bead-bound cells mixture was resuspended in 4 ml isolation buffer and mixed by gentle multiple pipetting with narrow-tipped pipette to avoid foaming. The tube was placed in the MACS system for 3 min, the supernatant containing the untouched $CD8^{+}$ or $CD4^{+}$ T cells was transferred into a new tube. The Dynabeads were again resuspended in 4 ml of the isolation buffer and placed in the MACS to remove remaining untouched cells. Residual beads were removed by placing the tube containing the untouched cells in the MACS for 2 min and the cells transferred into a 15 ml centrifuge tube. The untouched cells were pelleted and finally resuspended in 2 ml of complete medium (10% NHS/FBS-1640).

### 3.4.5 Surface staining of untouched CD4+ or CD8+ specific T cells

Flow cytometry was used to confirm the isolation efficiency of untouched CD4+ and CD8+ T cells after staining CD4+ and CD8+ T cells' surfaces with specific monoclonal

antibodies. After depletion, a 300 µl aliquot of the untouched CD4+ and CD8+ specific T cells were each transferred into different FACS tubes and 300 µl of unfractionated cells were also aliquoted into another FACS tube as a control. The cells were stained with 5 µl each of monoclonal antibodies (FITC-labelled antibodies against CD3 and APC labelled antibodies against CD8) and incubated at room temperature (RT) for 15 min. The cells were washed with 2 ml of COLD FACS buffer for 5 min and spun at 2000 rpm at $4^0$C. The supernatant was discarded and the cells were loosened and re-suspended in 300 µl of the COLD FACS buffer and acquired using BD FACS Calibur (Becton Dickinson, CA, USA).

### 3.4.6 Ex-Vivo ELISpot IFN-γ Assay

IFN-γ ELISpot assay was done as described earlier by Ganeshan et al., (2016) using the unfractionated PBMCs, enriched CD8+ and CD4+ T cells. A 100 µl of 15 µg/ml anti-human IFN-γ purified antibody (Mabtech AB, USA) was used to coat each well of multiscreen plates (Millipore Corporation, USA) and incubated overnight. The plates were washed six times with 250 µl/well of RPMI-1640 and blocked for 2 h with 200 µl/well of 10% NHS/RPMI-1640. A 100 µl of cells (400,000 cells/well) from each subject were tested with 100 µl of the test AMA1 peptides (Table 1) each in duplicate at a concentration of 10 µg/ml. A 100 µl of concanavalin A (Con A, Sigma Aldrich, USA) at 1.25 µg/ml was used as positive control stimulant. Cells in negative control wells were incubated with 100 µl of 10% NHS/RPMI-1640 culture medium only to assess background responses. The cells were incubated for 36 h at 37 °C, 5% $CO_2$ and washed six times afterwards with 250 µl/well PBS/0.05% Tween 20 (PBST). The wells were incubated with 100µl/well of 1µg/ml biotinylated anti-IFN-γ polyclonal antibody (Mabtech, USA) for 3 h and subsequently with

100 µl/well of alkaline-phosphatase-conjugated streptavidin (Mabtech, USA) for 1 h after repeating the washing process in between. The IFN-γ-producing spots were developed by adding 100 µl/well of AP chromogenic substrate (Bio-Rad, USA) and the number of spots per well was estimated using an automated ELISpot plate reader (AID GmbH, Germany). The acquired data was exported and saved in Microsoft Excel for analysis.

### 3.5 *Pf*AMA1 circulating allele detection

#### 3.5.1 DNA Extraction by Saponin-Chelex extraction Procedure

DNA was extracted from stored filter paper (Whatman Int. Ltd, England) using the Chelex extraction protocol. A 5-6 mm square piece of a blood blot on filter paper was cut out with a sterile punch into labelled 1.5 ml microfuge tubes. About 1 ml of 1x PBS was added to the blot followed by 50 µl of 10% saponin. The tube was vortexed for about 10 sec and incubated at 4 $^{o}$C overnight. The tube was centrifuged at 10,000 rpm for 1 min and the reddish PBS/saponin supernatant was discarded. A millilitre of 1xPBS was added to the tubes containing the filter paper and incubated at 4℃ for 30 min before spinning at 10,000 rpm for 1 min and the supernatant discarded. A 100 µl nuclease-free water was added and spun at 10,000 rpm for 1 min. The supernatant was discarded and 30ul of 20% Chelex and 70 µl of nuclease-free water were added. The tubes containing filter paper was incubated at 95 ℃ for 10 min and vortex at 2 min interval. The tubes were finally vortexed and spun for 6 min at 13,000 rpm. The supernatant containing the DNA was transferred into sterile microfuge tubes and stored at -20°C until use for Polymerase Chain Reaction (PCR).

### 3.5.2 DNA Amplification by PCR

The oligonucleotide primers used in this study were picked from already published sequences by Farooq et al., 2009, to amplify the hyper variable regions (HVR) of *Pf*AMA1 gene. The forward primer was 5'- CCT TTG AGT TTA CAT ATA TG - 3' and reverse primer was 5' - TAT CCT CAC AAT TTC CAT CG - 3'. The following cycling conditions were used for the reaction: denaturation at 94 °C for 5 min preceded 35 amplification cycles: denaturation for 30 sec at 94 °C, annealing for 1 min 30 sec at 46.1°C, and extension for 1 min at 72 °C. The last extension was carried out for 5 min. The reactions were done in a 40 µl final volume, containing 4 µl of 1X reaction buffer, 3.2 µl of 2µM $MgCl_2$, 0.8µl of 200nM mixture of the four dNTPs, 1 µl of 250 nM concentration of each of the two appropriate primers, and 1.2 µl of 0.15 U/µl *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), 23.8 µl of nuclease free water and 5 µl of DNA template. All reactions had a DNA from 3D7 strain as positive control and molecular grade water was used as negative controls. No PCR product was obtained in the negative control. The PCR products were analysed by electrophoresis on 1.5% agarose with 2 µl ethidium bromide (EtBr) as the stain with a standard 100 bp ladder. The DNA bands were visualized using a UV trans-illuminator (VWR International, USA) and fragments obtained were compared by size with a DNA ladder loaded onto the gel.

### 3.6 Data and statistical analysis

IFN-γ activities of all peptides were calculated as the number of spot forming cells per million PBMCs (sfc/m). The assay was defined as positive if there was (a) at least a doubling of sfc/m in test wells compared to control wells, and (b) a difference of at least 10 spots between test and control wells as used by Ganeshan et al., (2016). Student t-test was used to compare differences of IFN-γ responds between two peptides within an allelic

set and ANOVA were used to compare differences between three peptides within an allelic set. Statistical analysis and graphics were done using Graph Pad prism (version 6.0, San Diego, CA, USA) and excel. A p value less than 0.05 was considered statistically significant. Flow cytometry analysis was done by Flowjo V10 software (Tree Star, San Carlos, CA, USA).

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Demographics characteristics of study subjects

A total of 20 study subjects (10 from each study site) with ages ranging from 18 -35 years were used in this study (Table 4.1). In Legon, 7 subjects were males and 3 were females with a mean age of 25.80 ± 1.24 and mean Hb level of 16.33 ± 0.40. Obom on the other hand had 9 of its subjects being males and only 1 was female. The mean age and Hb level were 29.40 ± 1.22 and 14.03 ± 0.93 respectively. In all, 4 (20%) subjects were females and 16 (80%) were males. There was no statistical difference in the gender ($p$ = 0913) and the mean age was also not significantly different between the two sites ($p$ = 0.053) however, there was significance difference in the Hb levels between the sites ($p$ = **0.036**).

**Table 4. 1 Demographics of study subjects in Legon and Obom**

| | **Study sites** | | **P value** |
|---|---|---|---|
| **Gender** | Legon (n=10) | Obom (n=10) | |
| Male | 7 | 9 | |
| Female | 3 | 1 | 0.913 |
| **Age (years)** | 25.80 ± 1.24 | 29.40 ± 1.22 | 0.053 |
| **Hb (g/dl)** | 16.33 ± 0.40 | 14.03 ± 0.93 | **0.036** |

P value for gender comparison was obtained after fisher's exact test whiles that of Age and Hb were obtained after student t test.

### 4.2 Parasite carriage among the study subjects

Parasite data was compared between the study sites (Table 4.2). A total of 21 samples were collected from Legon for molecular analysis including the 10 samples for the immunological assay. Three out of the 21 (14.3%) study subjects tested positive for *P. falciparum* by microscopy and 5 subjects (23.8%) including those positive by microscopy tested positive by PCR. In Obom however, a total of 36 samples were collected including the 10 samples from immunological assay. *P. falciparum* was detected in 11 (30.5%) subjects by microscopy and PCR also detected 18 (50%) positive samples including the 11 samples by microscopy. Overall, 57 samples were used for the molecular analysis and 14 (24.6%) subjects in total tested positive for *P. falciparum* while 23 (40.4%) tested positive for the parasite in all by PCR. Thus there 9 additional samples were detected by PCR after microscopy. Although PCR detected more infections than microscopy, these differences did not reach statistical significance.

**Table 4. 2. Proportions of individuals carrying parasite by microscopy and PCR**

| | Study site | | | |
|---|---|---|---|---|
| **Detection method** | **Legon (n=21)** | **Obom (n=36)** | **Total** | **P-value** |
| **Microscopy** | 3 | 11 | 14 | 0.167 |
| **PCR** | 5 | 18 | 23 | 0.051 |
| **P-value** | 0.432 | 0.161 | | |

P values were obtained after chi square test

### 4.3 IFN-γ responses from unfractionated PBMCs of subjects to predicted polymorphic *Pf*AMA1 peptides

The magnitude of IFN-γ responses (sfc/m) from unfractionated PBMCs of subjects against 15 synthetic *Pf*AMA1 peptides is shown in the tables below. The 15 synthetic peptides represent six groups of allelic peptides from the three *P. falciparum* parasite strains (3D7, 7G8 and FVO). In Legon (Low transmission area), no subject made positive responses to the different allelic peptides tested based on the positivity criteria (Table 4.3). However, on the basis of statistical differences between responses to allele sets, 3 subjects (s2, s3, and s5) made significant responses to 2 peptides; DVY**H**PINEHR present in the 7G8 and FVO strains and **K**SFQNYTYL from 7G8 strain compared to their corresponding allelic variants. Subjects, s3 and s5 made significant responses to the same peptide **K**SFQNYTYL ($p$ = 0.036 and 0.037 respectively) relative to the corresponding 3D7 and FVO variants, while s2 made a significant response to DVY**H**PINEHR relative to the corresponding 3D7 variant ($p$ = 0.047).

Contrastingly, 2 subjects (s12 and s16) from Obom (high malaria transmission area) made positive responses to 4 (27%) peptides tested (AKDISFQNY, DVY**H**PINEHR, EHREH**S**KEY and EHREHPKEY) based on the set positivity criteria (Figure 4.1). Two of the 4 peptides belong to the same allelic set (EHREH**S**KEY from 7G8, EHREHPKEY 3D7/FVO) while the remaining two peptides belong to different allelic sets (AKDISFQNY from 3D7 and DVY**H**PINEHR from 7G8/FVO). Also 2 peptides: **KL**F**E**NYTY and MTL**NG**MR**D**FY, (both belonging to FVO strain) that were not positive based on the set positivity criteria made statistically significant responses to 2 subjects (s12, $p$ = 0.004 and

s20, $p = 0.005$ respectively Table 4.3). All subjects made positive IFN-γ responses to Con A.

**Table 4. 3. IFN-γ responses of PfAMA1 synthetic peptides tested against PBMCs from Legon subjects**

| Responses to Study Subjects from LEGON | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | IFN-g Resp. | Synthetic AMA 1 Peptides | | | | | | | | | | | | | | |
| | | AKDKLFEVY | AKDKSFQNY | AKDVSFQNY | DVYHPINEHR | DVYRPINEHR | EHREHSKEY | EHREHPKEY | DFYKNVEYVK | HFYKDNKYVK | KLFSYYTYL | KSFQNYTYL | SFQNYTYL | MTLNQNRDFY | MTLDHNRDFY | MTLDENRHFY |
| S1 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 233 | 240 | 221 | 219 | 291 | 250 | 211 | 239 | 165 | 220 | 258 | 254 | 218 | 218 | 265 |
| s2 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg* | neg | neg | neg | neg |
| | Sfc/m | 76 | 95 | 90 | 105 | 80 | 81 | 75 | 95 | 85 | 76 | 120 | 75 | 96 | 105 | 99 |
| s3 | Response | neg | neg | neg | neg* | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 51 | 39 | 54 | 71 | 51 | 65 | 54 | 63 | 51 | 34 | 58 | 53 | 50 | 46 | 56 |
| s4 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 68 | 68 | 68 | 49 | 84 | 61 | 59 | 60 | 60 | 64 | 58 | 53 | 51 | 66 | 54 |
| s5 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg* | neg | neg | neg | neg |
| | Sfc/m | 80 | 89 | 109 | 93 | 81 | 94 | 94 | 90 | 108 | 79 | 99 | 96 | 104 | 81 | 88 |
| s7 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 4 | 8 | 10 | 4 | 5 | 9 | 10 | 8 | 10 | 4 | 4 | 5 | 1 | 1 | 1 |
| s8 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 114 | 99 | 85 | 101 | 124 | 111 | 113 | 105 | 124 | 113 | 123 | 103 | 99 | 98 | 96 |
| s9 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 79 | 54 | 46 | 55 | 56 | 81 | 85 | 56 | 76 | 50 | 73 | 51 | 70 | 54 | 71 |
| s10 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 114 | 126 | 108 | 89 | 95 | 100 | 94 | 114 | 118 | 96 | 93 | 101 | 108 | 86 | 81 |

Mutations in peptides are underlined in the sequences. s: subject; sfc/m: spot forming cells/million, * peptides within an allelic set *(outlined by rectangular shape)* with significant response (s2: DVYHPINEHR *$p$=0.036, s3: KSFQNYTYL *$p$=0.037, s5: KSFQNYTYL *$p$=0.047

**Table 4. 4. IFN-γ responses of PfAMA1 synthetic peptides tested against PBMCs from Obom subjects**

| Responses to Study Subjects from OBOM | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | IFN-g Resp. | Synthetic AMA1 Peptides | | | | | | | | | | | | | | |
| | | AKDKLFENY | AKDKSFQNY | AKDSFQNY | DVYRPVEHR | DVYRPVEHR EHREHSKEY | EHREHPKEY | DFVKNVLYVK | HFVKDVKDVK | KLFENYTYL | KSFQNYTYL | LSFQNYTYL | MTLNGMRDFY | MTLDMRDFY | MTLDEMRHFY | |
| S12 | Response | neg | neg | neg | neg | neg | neg | POS | neg | neg | neg* | neg | neg | neg | neg | neg |
| | Sfc/m | 74 | 133 | 85 | 93 | 68 | 58 | 180 | 156 | 163 | 166 | 91 | 69 | 74 | 36 | 88 |
| s13 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 143 | 159 | 171 | 183 | 255 | 228 | 261 | 209 | 148 | 169 | 173 | 234 | 255 | 234 | 305 |
| s14 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 36 | 51 | 31 | 64 | 30 | 35 | 45 | 46 | 31 | 43 | 54 | 45 | 45 | 58 | 41 |
| s15 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 96 | 86 | 116 | 73 | 114 | 113 | 101 | 79 | 88 | 83 | 80 | 103 | 111 | 93 | 78 |
| s16 | Response | neg | neg | POS | POS | neg | POS | POS | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 36 | 35 | 89 | 101 | 36 | 73 | 48 | 28 | 25 | 9 | 6 | 13 | 11 | 21 | 16 |
| s17 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 43 | 61 | 54 | 50 | 55 | 51 | 43 | 61 | 34 | 45 | 79 | 65 | 76 | 58 | 79 |
| s18 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 19 | 3 | 4 | 3 | 11 | 6 | 5 | 6 | 1 | 3 | 5 | 6 | 5 | 8 | 1 |
| s19 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| | Sfc/m | 68 | 51 | 39 | 41 | 51 | 50 | 45 | 44 | 39 | 25 | 49 | 66 | 45 | 51 | 51 |
| s20 | Response | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg* | neg | neg |
| | Sfc/m | 65 | 73 | 51 | 44 | 64 | 54 | 56 | 68 | 54 | 65 | 54 | 71 | 80 | 56 | 49 |

Mutations in peptides are underlined in the sequences. s: subject; sfc/m: spot forming cells/million, POS: Peptides with positive responses. Those with significant responses are indicated by asterisks (*). KLFENYTY ($p = 0.004$*), MTLNGMRDFY ($p = 0.005$*)

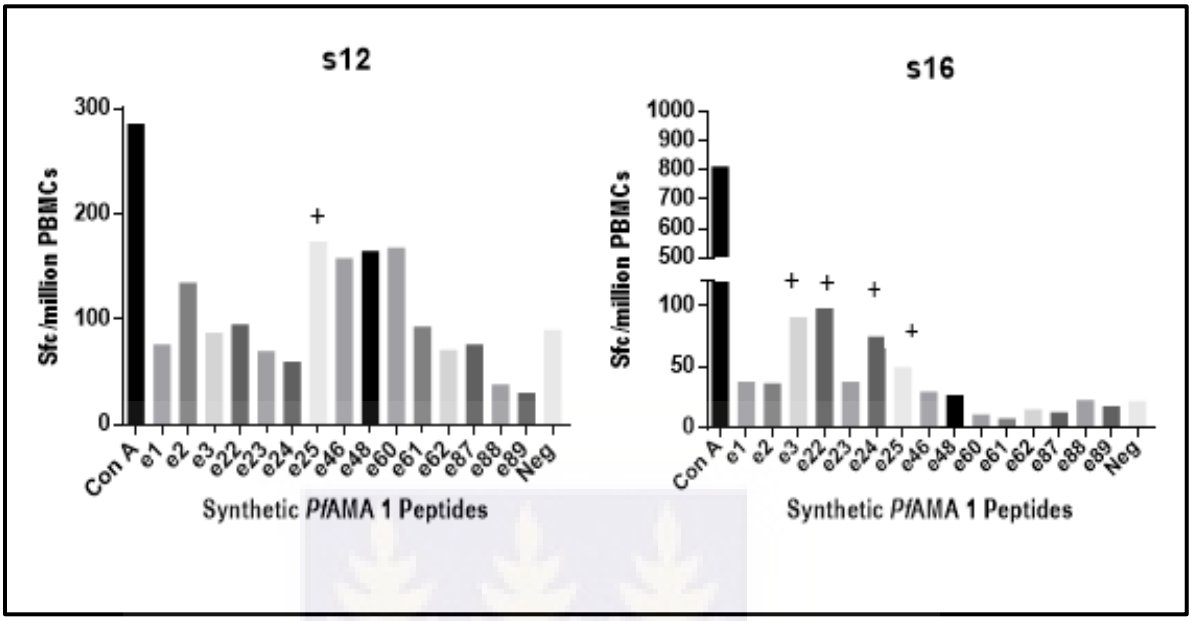


**Figure 4.1: IFN-γ responses to variant PfAMA1 synthetic peptides of s12 and s16.** +: Peptides that made positive response based on set criteria (refer to data analysis section). e3: AKDISFQNY; e22: DVY**H**PINEHR; e24: EHREH**S**KEY; e25: EHREHPKEY**.**

### 4.4 Enriched CD4+ or CD8+ T cell subsets depletion

The figure below is representative histogram displays of T lymphocytes before and after enriching for CD8+ and CD4+ T cells. Flow cytometry confirmed that CD3+CD8- (CD4+) T cell subsets were enriched in all experiments by depleting CD3+CD8+ (CD8+) T cells by about 90% (Figure 4.2 A and B). CD3+CD8+ (CD8+) T cell subsets were also enriched by depleting CD3+CD8- (CD4+) by over 28% (Figure 4.2 C and D).

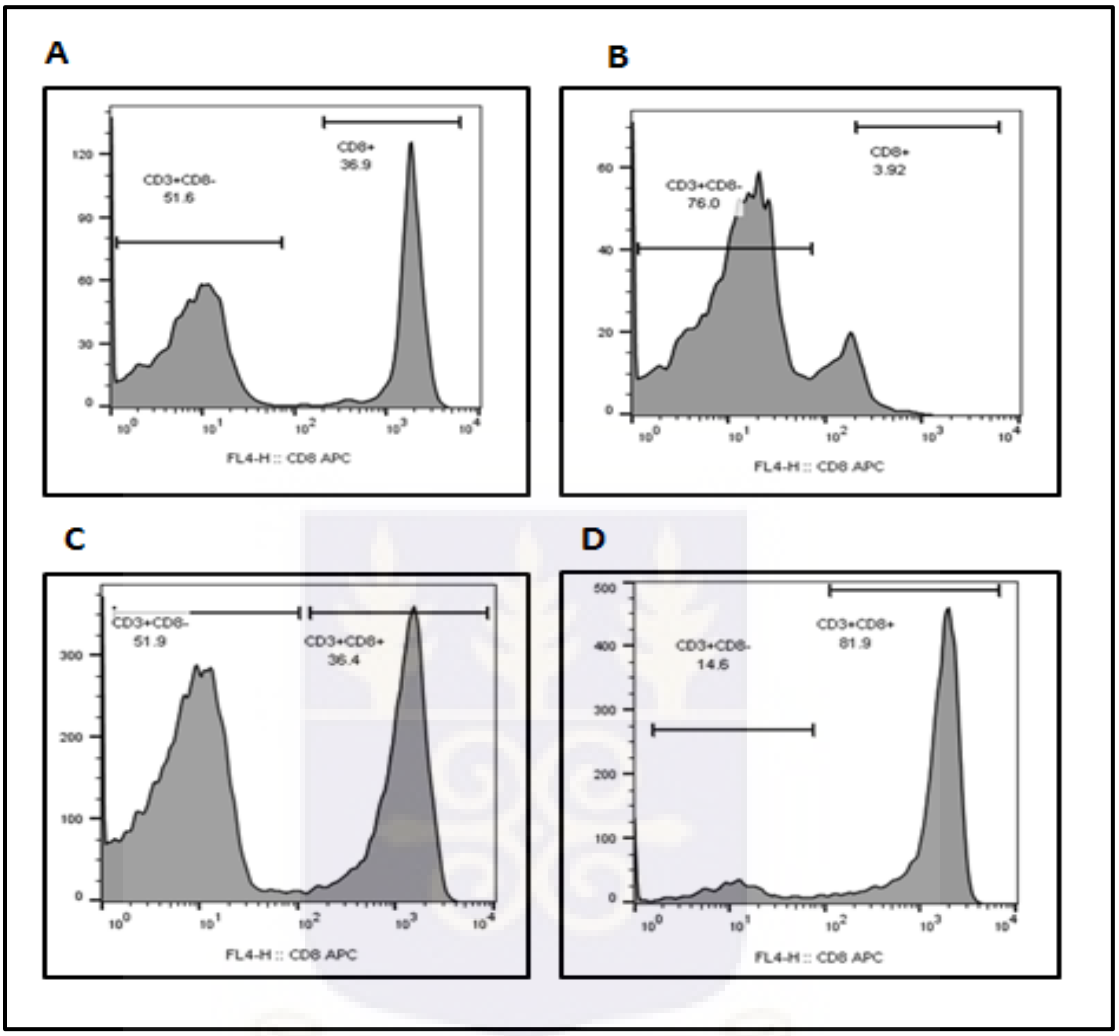


**Figure 4.2. CD4+ and CD8+ T cells subject enrichment confirmed by flow cytometry.** Gating strategy showing CD4+ and CD8+ T cell population before and after enriching CD4+ T cell subset (A and B respectively) and CD8+ T cell subset (C and D respectively).

### 4.5 Enriched CD8+ T cells IFN-γ activities of subjects to synthetic peptide

The magnitude of IFN-γ responses (sfc/m) from enriched CD8+ T cells against 15 synthetic *Pf*AMA1 peptides is shown in figures below. In Legon (Figure 4.3), subject s7 enriched CD8+ T cell made positive specific responses to a single peptide (DVYRPINEHR from the 3D7 consensus) by the set positivity criteria with no positive response from the

corresponding 7G8/FVO variant. However, a single peptide (**K**SFQNYTYL (7G8) with no positive response by the set criteria made statistically significant IFN-γ responses to the same subject's enriched CD8+ T cells relative to its corresponding variants form (3D7/FVO, $p = 0.005$).

In comparison to Legon, enriched CD8+ T cell of 3 subjects' (s13, s15, s17) from Obom made positive responses to 6 peptides in all (Figure 4.4). Subjects s15 and s17 made positive responses to 3 peptides each (s15: DVYRPINEHR (3D7), EHREHPKEY (3D7/FVO), MTLDEMRHFY (3D7); s17: AKD**K**SFQNY (7G8), DVY**H**PINEHR (7G8/FVO), DVYRPINEHR (3D7). Moreover, both subjects, s15 and s17 made positive response to a single peptide DVYRPINEHR (3D7). Subject s13 on the other hand, made positive response to only one peptide HFYKDNKYVK (3D7) by the set criteria. In addition, s13 made a statistically significant response to a peptide AKD**KL**F**E**NY (FVO) variant ($p = 0.005$*), although this was not a positive response by the set criteria.

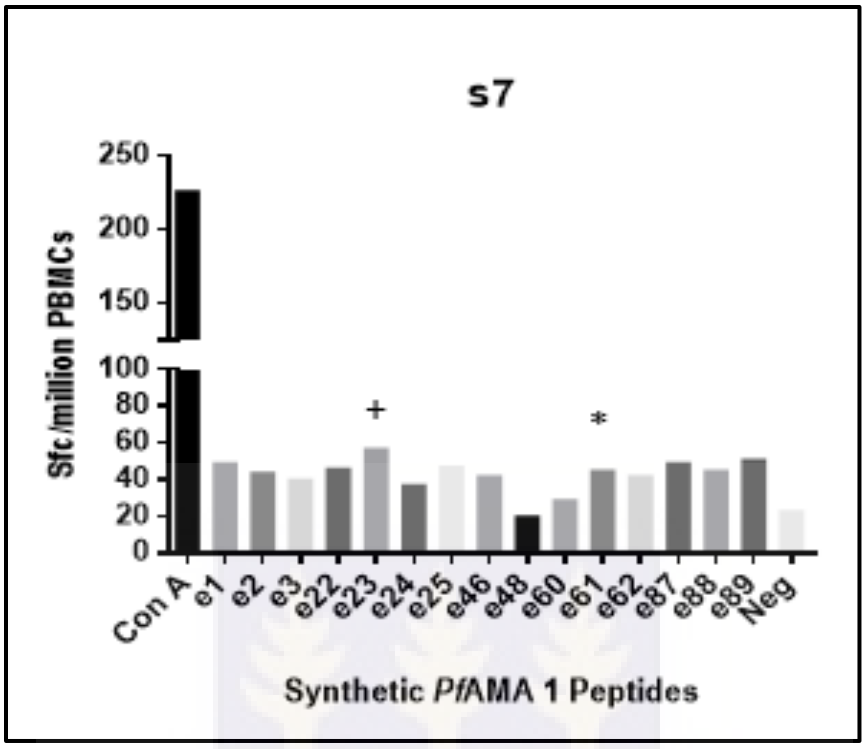


**Figure 4. 3. IFN-γ responses of CD8+ T cell of subject from Legon to variants of PfAMA1 synthetic peptides.** +: Peptide that made positive response. * Peptide with statistically significant response. e23: DVYRPINEHR (3D7); e61: **K**SFQNYTYL (7G8).

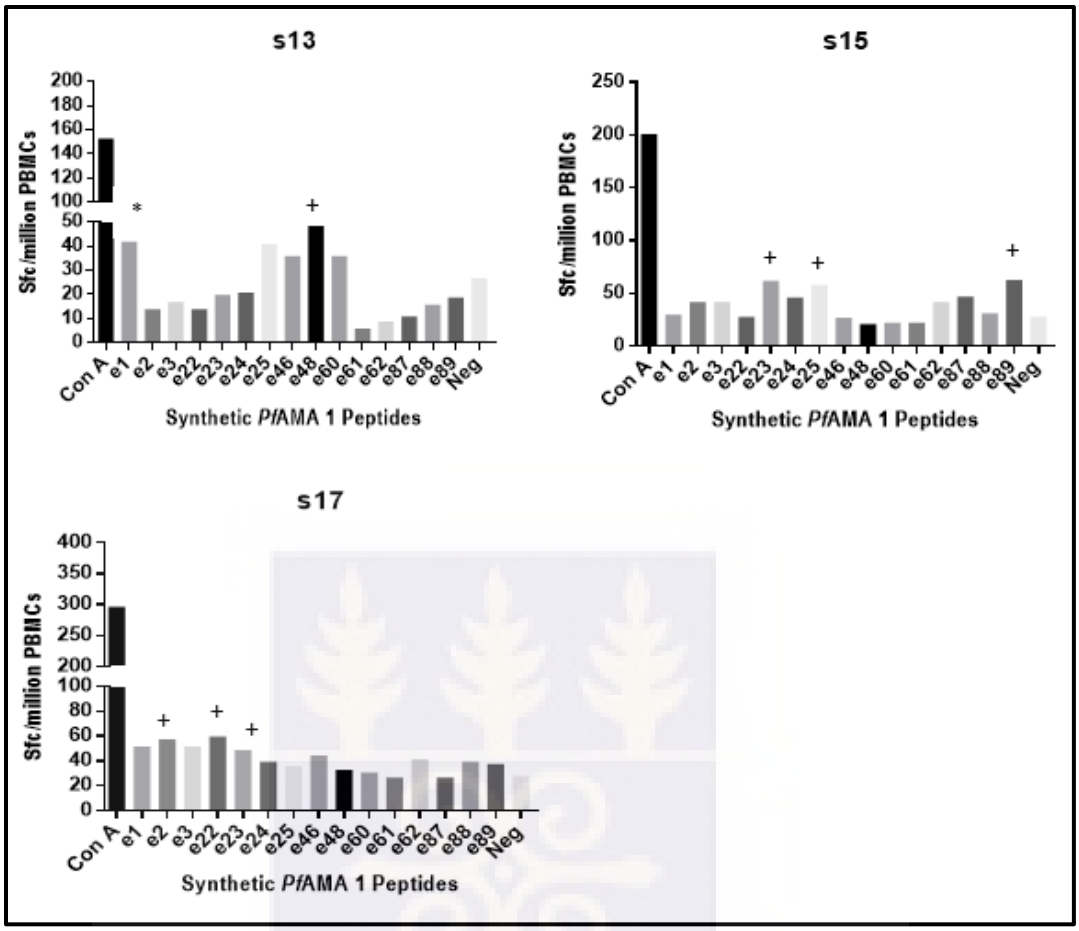


**Figure 4. 4. IFN-γ responses of CD8+ T cells from Obom to variants of *Pf*AMA1 synthetic peptides.** + = Peptides with positive IFN- γ activity, * = Peptides with significant IFN- γ activity. e1: AKD**KL**FENY (FVO), e2: AKD**K**SFQNY (7G8), e22: DVY**H**PINEHR (7G8, FVO), e23: DVYRPINEHR (3D7), e25: EHREHPKEY (3D7/FVO), e48: HFYKDNKYVK (3D7), e89: MTLDEMRHFY (3D7).

### 4.6 Enriched CD4+ T cells IFN-γ activities of subjects to synthetic peptide to synthetic peptide

The magnitude of IFN-γ responses (sfc/m) from enriched CD4+ T cells against 15 synthetic *Pf*AMA1 peptides is shown in Figures below. No CD4+ T cells of subjects from Legon made positive response to any of the peptides tested by the set positivity criteria (Figure 4.5). However, 2 peptides, ISFQNYTYL from 3D7 consensus and MTLD**H**MR**D**FY from 7G8 strain, made statistically significant IFN-γ responses ($p$ = 0.026 and 0.028

respectively) to a single subject's (s7) CD4+ T cells relative to their corresponding variants from FVO/7G8 and 3D7/FVO respectively.

Comparatively, there were 2 peptides; EHREH**S**KEY (7G8) and EHREHPKEY (3D7/FVO) that made positive responses to a single subject's (s12) enriched CD4+ T cell from Obom (Figure 4.6). However, these peptides belong to the same allelic set. Also, there were statistically significant differences in IFN-γ activities from 2 peptides (HFYKDNKYVK (3D7), $p$ = 0.008, **KL**F**E**NYTYL (FVO), $p$ = 0.03) which made no positive responses to a single subject's (s14) enriched CD4+ T cells.

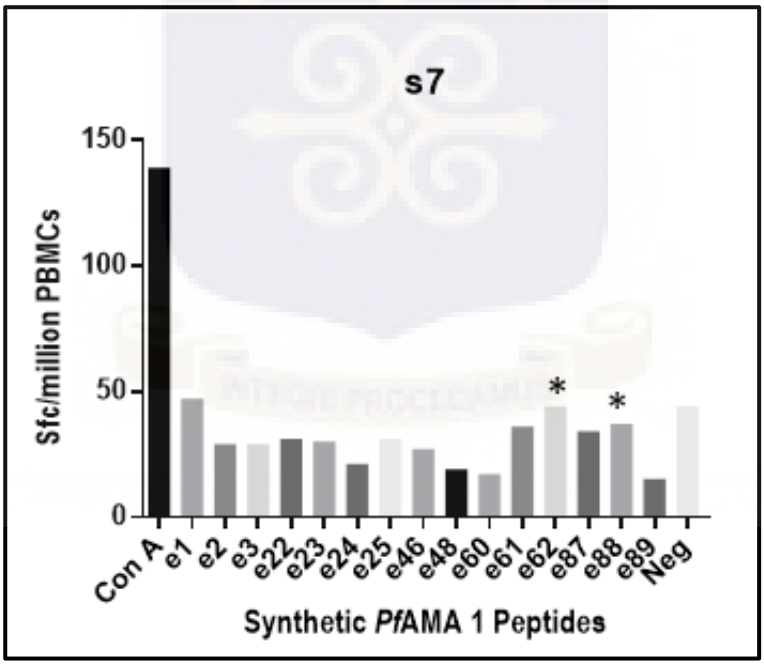


**Figure 4. 5. IFN-γ responses of CD4+ T cells to variants of *Pf*AMA1 synthetic peptides of s7.** * = Peptides with significant IFN-γ activity.e62: ISFQNYTYL (3D7); e88: MTLD**H**MR**D**FY (7G8).

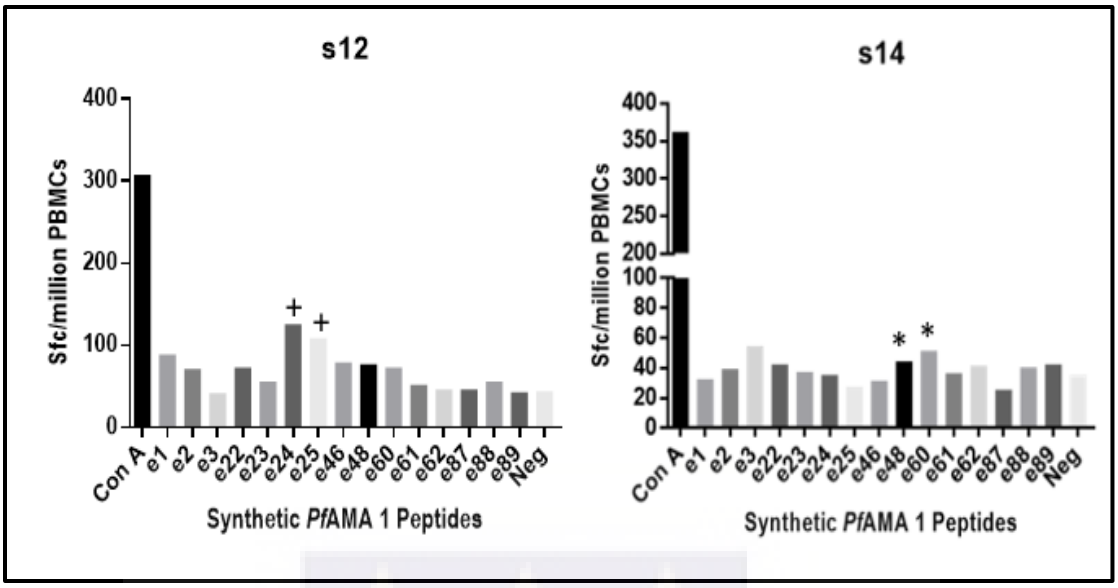


**Figure 4.6. IFN- γ responses from subject enriched CD4+ T cells against synthetic *Pf*AMA1 peptides.** + = Peptides with positive IFN- γ activity, * = Peptides with significant IFN-γ activity. e24: EHREH**S**KEY (7G8), e25: EHREHPKEY (3D7/FVO), e48: HFYKDNKYVK (3D7), e60: **KL**F**E**NYTYL (FVO).

### 4.7 Positions and frequency of amino acids substitution

Positions and frequencies of peptides with either positive responses by the set criteria or statistically significant responses to study subjects are shown in Figure 4.7. In Legon, a total of 5 substitutions occurred at 4 different positions (1, 4, 5 and 8). Three substitutions occurred at position 1 and one substitution each occurred at the positions 4, 5 and 8. However, for Obom, peptides with positive or significant responses had 12 substitutions occurring at positions 1, 2, 4, 5, 6, 7 and 8. A total of 7 substitutions occurred a position 7, two substitution occurred at positions 1, 5 and 6 each and a single substitution occurred at positions 2, 7 and 8 each. Overall, peptides with response to Obom subjects had more substitutions occurring at several different positions than that of peptides with response to Legon subjects.

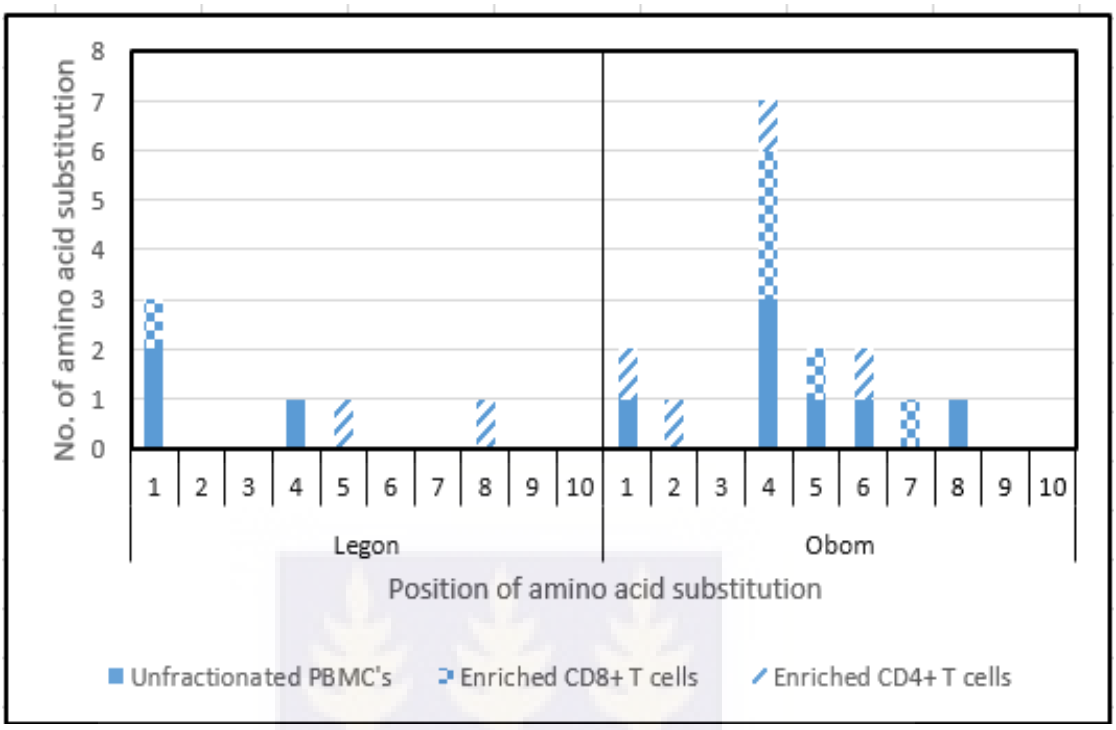


**Figure 4.7.** Positions and frequency of amino acid substitutions in peptides with responses to unfractionated PBMC's or enriched CD4+ or CD8 + T cells.

### 4.8 Amino acid substitution(s) at variable position(s) of *Pf*AMA1 polymorphic peptides

Substitutions occurring between lysine (K) and isoleucine (I) were found in 6 peptides and resulted in 12 positive responses or statistically significant responses. Four of these peptides substitutions were from I (in 3D7 strain) at the variable position in the peptide with no positive/ statistically significant response to K (in 7G8 and/or FVO strains) resulting in a positive/ statistically significant response. However, the remaining 2 peptides had substitution and response rather in the reverse. Similarly, substitution between glutamine acid (E) and glutamine (Q) occurred in 5 peptides and accounted for 11 positive responses or statistically significant responses. Three of the peptides changed from E (in FVO strain) at the variable position in the peptide with no positive/ statistically significant

response to Q (in 7G8 or 3D7) resulting in positive/ statistically significant response and the reverse in substitution and response occurred in 2 peptides. Also, substitution between serine (S) and leucine (L) occurred in 5 peptides and resulted in 10 positive response or statistically significant response. Out of this, 3 peptides with no positive/ statistically significant response and had L (in FVO strain) at the variable position responded positively or made statistically significant response when replaced with S (in 3D7 of 7G8 strains) at the variable position. However, the reverse in substitution and response occurred in 2 peptides. Again, 7 peptides had substitutions occurring between amino acid residues asparagine (N) and aspartic acid (D). Four of peptides had substitutions from N (in the FVO strain) to D (in 3D7 or FVO strains) in variable position of peptides with no positive/ statistically significant response to a positive/ statistically significant response respectively. Conversely, 3 peptides has substitution and response in the reverse. Finally, 2 peptides with substitution between histidine (H) and arginine (R) made 6 positive response or statistically significant response. Three of them had substitution from H (in 7G8/FVO strains) at the peptides' variable region to R (in 3D7 strain) resulting in positive/ statistically significant response and the reverse in substitution and response also occurred in 3 peptides. (Table 4.5).

The majority of the substitutions observed were to a basic residue (6) at the variable position within the peptide sequence, followed by substitutions to an acidic residues (5). Of the 6 basic substitutions, 4 (66.7%) were from isoleucine (I) in the 3D7 sequence to lysine (K) in the 7G8 sequence accounting for positive/ statistically significant response. However, 2 (33.3%) substitutions were from arginine (R) or glutamic acid (E) in the 3D7 sequence to histidine (H) in the 7G8/FVO strains. Also, 3 of the 5 (60%) acidic

substitutions were from glutamine (Q) in the 7G8 sequence to glutamic acid (E) in the FVO sequence resulting in positive/ statistically significant response. However, the remaining 2 (40%) acidic substitutions were the vice versa.

**Table 4. 5** Amino acid substitutions in responsive *Pf*AMA1 peptides and their variants.

| Cells | Amino acid residue(s) at variable position(s) in peptides with **NO** positive/statistically significant response | Amino group of residue(s) at variable position(s) of peptides with **NO** Positive/statistically significant response | Amino acid residue(s) at variable position(s) in peptide with Positive/statistically significant response | Amino group of residue(s) at variable position(s) of peptides with Positive/statistically significant response |
|---|---|---|---|---|
| | | **LEGON** | | |
| Unfractionated PBMCs | 3D7 (I),<br>FVO (L, E) | Aliphatic<br>Aliphatic, acidic | 7G8 (K*),<br>7G8 (S*, Q*) | Basic<br>Hydroxyl, acidic |
| | 3D7 (R) | basic | 7G8/FVO (H*) | Basic |
| | 3D7 (I),<br>FVO (L,E) | Aliphatic<br>Aliphatic, acidic | 7G8 (K*),<br>7G8 (S*,Q*) | Basic<br>Hydroxyl, acidic |
| | | **OBOM** | | |
| | 7G8 (S) | hydroxyl | 3D7/ FVO (P) | Cyclic |
| | 3D7 (I,S,Q),<br>7G8 (S,Q) | Aliphatic, hydroxyl, acidic<br>Hydroxyl, acidic | FVO (K*,L*,E*),<br>FVO (L*,E*) | Basic, aliphatic, acidic<br>aliphatic, acidic |
| | 3D7 (D,E,H),<br>7G8 (D,H) | Acidic, acidic. Acidic<br>Acidic, acidic | FVO (N,G,D),<br>FVO (N,G) | Acidic, aliphatic, acidic<br>Acidic, aliphatic |
| | 7G8 (K),<br>FVO (K,L,E) | Basic<br>Basic, aliphatic, acidic | 3D7 (I*),<br>3D7 (I*,S*,Q*) | Aliphatic<br>Aliphatic, hydroxyl, acidic |
| | 3D7 (R) | basic | 7G8/ FVO (H*) | Basic |
| | | **LEGON** | | |
| Enriched CD8+ T cells | 7G8/ FVO (H*) | basic | 3D7 (R) | Basic |
| | 3D7 (E,H),<br>FVO (N,G) | Acidic, basic<br>Acidic, aliphatic | 7G8 (H,D),<br>7G8 (D,H) | Basic, acidic<br>Acidic, acidic |
| | | **OBOM** | | |
| | 3D7 (I,Q),<br>7G8 (S,Q) | Aliphatic, acidic<br>Hydroxyl, acidic | FVO (K*,E*),<br>FVO (L*,E*) | Basic, acidic<br>Aliphatic, acidic |
| | 7G8/FVO (D,N,E) | Acidic, acidic, acidic | 3D7 (H,D,K) | Basic, acidic, basic |
| | 7G8/FVO (H) | Basic | 3D7 (R*) | Basic |
| | 7G8 (S) | Hydroxyl | 3D7/FVO (P) | cyclic |
| | 7G8 (H,D),<br>FVO (N,G,D) | Basic, acidic<br>Acidic, aliphatic, acidic | 3D7 (E,H),<br>3D7 (D,E,H) | Acidic, basic<br>Acidic, acidic, basic |
| | 3D7 (I),<br>FVO (L,E) | Aliphatic<br>Aliphatic, acidic | 7G8 (K*),<br>7G8 (S*,( | Basic<br>Hydroxyl, acidic |
| | 3D7 (R) | Basic | 7G8/FVO (H*) | Basic |
| | 7G8/FVO (H) | Basic | 3D7 (R*) | Basic |

| | | | | |
|---|---|---|---|---|
| **LEGON** | | | | |
| Enriched CD4+ T cells | 7G8 (K),<br>FVO (K,L,E) | Basic<br>Basic, Aliphatic, acidic | 3D7 (I*),<br>3D7 (I*,S*,Q*) | Aliphatic<br>Aliphatic, hydroxyl, acidic |
| | 3D7 (E,H),<br>FVO (N,G) | Acidic, basic<br>Acidic, aliphatic | 7G8 (H,D),<br>7G8 (D,H) | Basic, acidic,<br>Acidic, basic |
| | **OBOM** | | | |
| | 7G8/FVO (D,N,E) | Acidic, acidic, acidic | 3D7 (H,D,K) | Basic, acidic, basic |
| | 3D7<br>(I,S,Q),<br>7G8 (S,Q) | Aliphatic, hydroxyl, acidic<br>Hydroxyl, acidic | FVO (K*,L*,E*),<br>FVO (L*,E*) | Basic, aliphatic.<br>Acidic<br>Aliphatic, acidic |

*= Amino acid residue in variable position(s) of peptide with most positive response or statistically significant response.

### 4.9 Amplification of hypervariable region of *PfAMA1* gene

Size polymorphisms were seen in some targets of the gene. The molecular weight of PCR products of HVR of *Pf*AMA1 gene ranged from 318 to 900 bp (Figure 4.8). On the basis of molecular weight of the amplicons, the isolates were categorized into 4 allelic types (318 bp, 500 bp, 700 bp and 900 bp). The dominant allele observed in all infected subjects was at 500bp in all corresponding to the 3D7 control used. Overall, 5 (21.7%) subjects had all 4 alleles and 10 (43.5%) subjects showed 2 circulating alleles including the 3D7-like strain (500bp). Thus, 15 (65.2%) subjects had multiple alleles of the *AMA1* gene.

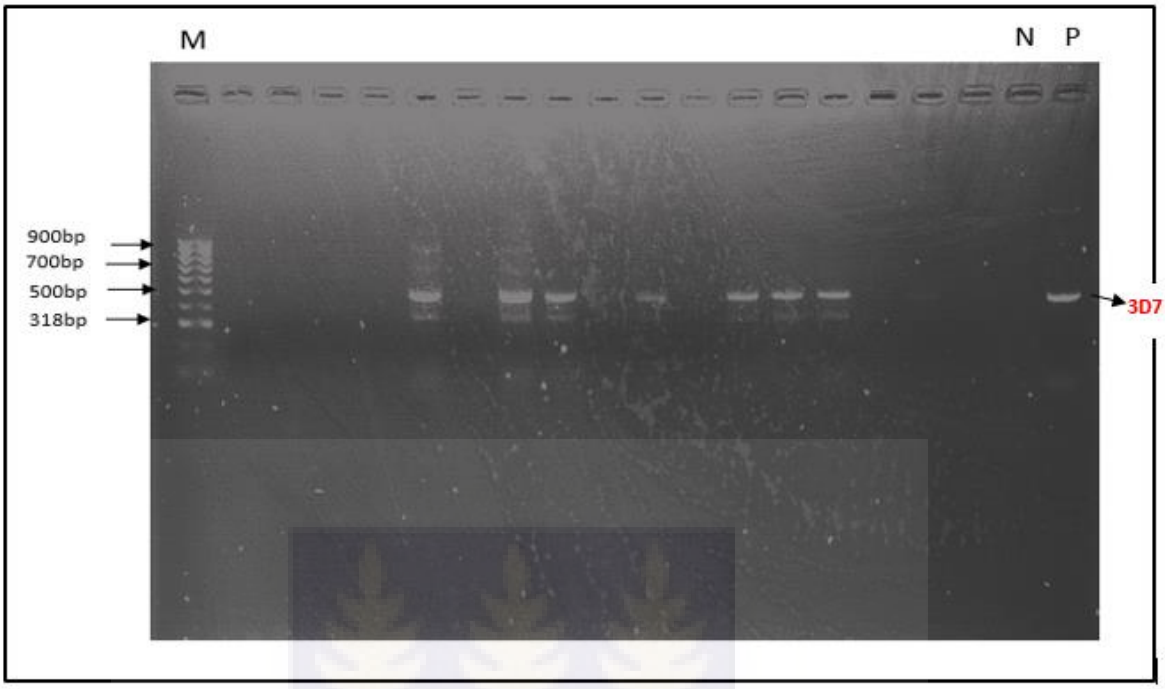


**Figure 4. 8. Gel electrophoresis image of the PCR product of *Pf*AMA1 gene.** Present of bands are samples with *P. f* infection while those with no bands are no P. f infection. M= Molecular weight marker (100 bp), N: Negative control, P: Positive control (3D7).

# CHAPTER FIVE

## 5.0 DISCUSSIONS, CONCLUSION AND RECOMMENDATION

### 5.1 Discussion

T lymphocytes recognition of foreign antigens is essential component to the adaptive immune response for limiting *Plasmodium* infection in humans. It is driven by specific T-cell receptors (TCRs) binding to antigenic peptide–major histocompatibility complex (pMHC) molecules on nucleated cells. However, this peptide-MHC complex could invariably be affected by polymorphism in any of its members. This study was aimed at investigating the impact of polymorphism in *Pf*AMA1 on the induction of potent T cell responses in individuals who have a history of natural exposure to malaria. Synthetic 8-10mer peptides from *Pf*AMA1 were tested against three different fractions of cryopreserved PBMCs from subjects living in low and high malaria transmission areas in Ghana. The three fractions were i) intact or unfractionated PBMCs, ii) CD4+ T cell enriched PBMCs and iii) CD8+ T cell enriched PBMCs. Cell enrichment was done by a negative selection protocol which removes either the CD4+ or CD8+ T cell fraction from PBMCs. The study also assessed the density and diversity of circulating parasite strains in the two study communities.

By both microscopy and PCR, the proportions of study participants who were parasite-positive were not significantly different at both sites. Although PCR detected more infections than microscopy, these differences did not reach statistical significance, mostly likely due to the low frequencies (Table 4.2).

IFN-γ induction potential of predicted polymorphic AMA1 peptides was assessed by ELISpot assay. None of the subjects from Legon (low transmission area) made positive responses to any of the peptides tested against their unfractionated PBMCs by the set positivity criteria (Table 4.3). However, in Obom, a high transmission zone, two subjects' (s12 and s16) unfractionated PBMCs responded positively to 4 peptides (Figure 4.1). One of the four peptides with positive responses belong to the 3D7 reference strain (AKDISFQNY) while another is from the 7G8 strain (EHREH**S**KEY). The two other positive peptides are conserved between at least two of the three strains assessed (DVY**H**PINEHR in the 7G8/FVO strains, and EHREHPKEY in the 3D7/FVO strains). The observation of positive responses in Obom but not in Legon can be justified by the fact that Obom is a high malaria transmission area, and subjects may have been exposed to multiple strains circulating in the community. The observation of responses to multiple allelic variants in the Obom community is supported by the findings of Koepfli et al. (2011, 2015), who showed that high transmission drives diversity due to multiple exposures to diverse clones of the malaria parasites. Hence, it was not surprising that subjects from Obom made responses to diversity strains of the peptides tested.

Moreover, after testing all the synthetic peptides against subjects' enriched CD8+ T cells, a single subject (s7) from Legon (Figure 4.3) responded to a single peptide (DVYRPINEHR from the 3D7 strain) while 3 subjects (s13, s15, s17) from Obom (Figure 4.4) made positive response to 6 peptides. Two subjects (s15 and s17) from Obom and s7, from Legon made a positive response to a single peptide (DVYRPINEHR from 3D7 strain). This could suggest the 3D7 parasite strain is a common strain circulating in the two

sites, hence these subjects may have been exposed to it and their committed T cells were able to recall for the specific response. Aside the common peptide, the 3 subjects from Obom responded positively to 5 additional peptides. Three of these peptides (EHREHPKEY, HFYKDNKYVK and MTLDEMRHFY) were from the 3D7 strain while a single peptide (AKD**K**SFQNY) belong to the 7G8 strain. However, the remaining peptide (DVY**H**PINEHR) belongs to both 7G8 and FVO strain. Responses to peptides from different strain from subjects belonging to Obom could be an indication that there are different circulating strain within the population. Therefore, these subjects may have been naturally primed to eliciting responses against diverse parasite strains. This argument supports the observation with the unfractionated PBMC where subjects from Obom made more positive responses to diverse parasite relative to those from Legon.

In a similar assay with enriched CD4+ T cells, none of the subjects from Legon responded positively to any of the tested peptides (Figure 4.5). However, a single subject (s12) from Obom made a positive response to 2 peptides (EHREH**S**KEY (7G8), EHREHPKEY (3D7/FVO) belonging to the same allelic set (Figure 4.6). This observation could mean two things: firstly, that the polymorphic residues existing in the two variant peptides may not be essential for TCR/pMHC binding. Thus, unlike the 3D7/FVO, the single substitution occurring in the 7G8 variant was not so much of importance to affect the interactions existing between the TCR/pMHC complexes. Secondly, positivity to the two peptides in the same allele set could suggest subject s12, may have been exposed to multiple parasite strains. This could be a possibility because s12 belong to the high malaria transmission area and as explained earlier that high transmission drives diversity (Koepfli et al. 2011,

2015). Hence, it is likely s12 have been exposed to multiple strain of the parasite and hence possesses T cells that are committed to recalling immune response against those strains. Moreover, EHREHPKEY from 3D7/FVO responded positively to both unfractionated PBMCs and enriched CD4+ T cells of the same subject (s12). This infers that, response from this peptide is CD4 specific.

Overall, unlike assays with either unfractionated PBMCs or enriched CD4+ T cells, that of enriched CD8+ cells made positive responses to more peptides against more subjects' cells. This could have simply resulted from the reduction in frequency of regulatory T cells (T regs). T regs are a subset of CD4+ T cells (Hobeika et al., 2011) and have been shown to increase in frequency during natural malaria infections (Frimpong et al., 2018). *In vitro* studies in both human and mice have shown that, T regs suppress proliferation and cytokine production of responder T cells (Thornton & Shevach, 1998; Seddon & Mason, 2002). Hence depletion of these subsets may have led to unregulated stimulation of CD8+ T cells resulting in higher IFN-γ activities.

On the other hand, 8 peptides made no criteria-based positive response to any of the subjects, however, there were statistically significant T cell specific IFN-γ activities from these peptides relative to their corresponding variants. This indicates that, though per our positivity criteria (explained in data analysis section), these groups of peptides may not be immunogenic enough, substitution(s) existing within the sequences may have influenced TCR/pMHC interactions or improved on their immunogenicity. The argument is supported by data from Jordan et al. (2010) who pointed out that substitutions that suitably change

the spatial structure of peptides may enhance the immunogenicity of epitopes and improve the binding of TCRs to pMHC ligands. From this study, peptides within an allele set that made statistically significant responses relative to the other variants within the set suggest an essential substitution(s) for TCR/pMHC interactions or improved immunogenicity due to substitution(s).

Substitution(s) to peptides with positive response by the set criteria or statistically significant responses occurred at positions 1, 2, 4, 5, 6, 7 and 8 (Figure 4.7). MHCs have been shown to bind to amino acids at specific positions within the peptides sequences before peptide presentation to specific T cell subsets (Corse et al., 2011). MHC I which presents peptides to CD8+ T cells has been reported to bind to amino acids at positions 2, 5/6 and 9 while MHC II presenting to CD4+ T cells binds to positions 1, 4, 6 and 9 ( Batalia & Collins, 1997; Lafuente & Reche, 2009; Wieczorek et al., 2017). Hence, substitutions in these positions are useful to predict whether a peptide will be able to bind to MHC class I or II. On the basis of this, it can be inferred from this study that immune response from CD4+ T cells are most likely to be affected by the positional substitutions observed. This was because, majority of the peptides which made either positive or significant responses had substitutions occurring at positions 1 and 4 (Figure 4.7), core anchor positions for MHC II binding. The substitutions at these positions may be so essential that it may have affected the strength of TCR/pMHC II interactions.

The majority of the positive responses by the set criteria or statistically significant responses resulted from peptides with lysine (K) at the variable position(s) within the

peptide sequence followed by glutamic acid (E), leucine (L), glutamine (Q), serine (S) and Isoleucine (I). The dominant substitutions observed were to a charged amino residue (basic or acidic) (Table 4.5). This infers that, the presence of these charged residues seems to be associated with immunogenicity. This is in contrast with the finding of Calis et al., (2013) who identified aromatic residue within presented peptides as essential in determining immunogenicity. This study however, found that, charged residues at these contact positions is also essential determining factor of immunogenicity.

In addition, few positive responses or different responses of subjects to the same peptides may be as result of different MHC (HLA) inherited by the subjects. This is possible because HLA genes are the most polymorphic genes known as there are multiple variants of each gene within the population as a whole (Janeway et al., 2011) and therefore there is a likelihood that these subjects may vary in their MHC molecules. Hence responding to the peptides may vary from person to person and may also differ in strength of peptide binding for presentation to TCR. In a study from Ganeshan et al., (2016), who typed 31.4% of the study subjects' HLA and also used AMA1 peptide pools, 63 % responded positively to at least one of the AMA1 peptide pool.. However, in this study, only 10% of the study subjects made positive responses to the single peptides. Moreover, after enriching for both CD8+ and CD4+ T cells, 20% responded positively by their CD8+ T cells while 10% made responses by their CD4+ T cells. This was because, in the earlier study, 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.

Finally, in this study, 4 allelic types of AMA1 gene in 23 isolates were identified on the basis of size polymorphism after amplifying the HVR (Fig. 4.8). Similarly, Eisen et al., 1999 and Marshall et al., 1996 observed 4 allelic types of AMA1 at different study sites. However, these were based on the absence or presence of a restriction site. Contrastingly, Farooq et al., 2009 observed and categorised alleles into 3 on the basis of molecular weight of amplicons (800 bp, 950 bp and 1000 bp) due to inability to identify diversity with restriction enzymes. In this present study, also on the basis of molecular weight, the four alleles identified were 318 bp, 500 bp, 700 bp and 900 bp which are diverse from what had been reported by Farooq et al., though the same primers were used. The observed dominant allele was at 500 bp and this was seen for all parasite-positive subjects which is at the same size to the 3D7 control used suggesting that the dominant allele in circulation could be a 3D7-like parasite. This may also explain the higher frequency of immunodominant epitopes from the 3D7 strain that were identified in the study area. However, in addition to this size, 5 (21.7%) subjects each showed an allele at 700 bp and 900 bp while 15 (65.2%) subjects also showed an allele at 318 bp. Overall, 15 (65.2%) of the subjects were positive for more than one allele and most of the subjects belongs to the high transmission area. This further confirms the argument that high transmission drives polymorphism, so people living in such communities have T cells that are most likely committed to making responses to diverse clones of the parasite.

## 5.2 Conclusion

In conclusion, this study has provided some evidence that polymorphisms existing in peptides from malaria vaccine candidate antigens, like *Pf*AMA1, influence the response of specific T cells. Again, this study has shown a possible significance of amino acid positions 1 and 4 in a presented peptide for immune recognition by MHC II. In addition, the presence of charged residues seemed to be associated with immune recognition. This will further our understanding of cellular immune mechanisms that govern anti-*Plasmodium* T cell responses and help direct peptide selection as constituents of multi-epitope vaccines that can offer cross-strain protection. Finally, the study data shows that the most predominant circulating parasite variants were the also the ones whose antigens were predominantly identified by subject immune cells.

## 5.3 Recommendation

Since there were other alleles different from the 3D7 control used, further analysis could be done employing tools like restriction fragment length polymorphism (RFLP) where enzymes with restriction site within the *Pf*AMA1 can help in identifying other variants. Also, sequencing is another tool which can be used. Sequenced data from these amplicons can be aligned with known references. Also, the current data sets are too small to investigate the preferences at each position to immunogenicity separately. Hence more peptides involving substitutions at diverse positions should be tested and immunogenicity analysed in respect to the position of substitution.

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# APPENDICES

## Appendix 1.1: Preparation of Standard Solutions and buffers

### A. 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

### B. 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 4$^{o}$C until ready to use

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

### C. 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)

### D. 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)

### E. 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.

### F. 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

### G. 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.

### H. 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.
-

### I. Isolation buffer for T cell subset enrichment

1X PBS with 2% FBS and 0.6% sodium citrate

Volume: 400ml

PROCEDURE

- Add 8ml of FBS in 392 ml of 1X PBS
- Add 2.4g of sodium citrate ($Na_3C_6H_5O_7$) to FBS/PBS solution
- Filter using 0.22µm Millipore filter

### J. 1.5g agarose gel preparation

- Weigh 1.5g of agarose in 100ml of 1X TAE buffer and mix until in solution
- Heat the solution in microwave for about 4 min until solution become clear
- Add 2µl of ethidium bromide o the solution and swirl unto in solution.
- Pour solution into the gel tank containing the comb and allow to solidify.

**Appendix 1. 2: NMIMR-IRB CONSENT FORM**

**General Information about Research**

*Dear Volunteer,*

This consent form contains information about the above titled research that seeks to determine how the occurrence of different forms of malaria parasite affects the way your body or immune system responds to these infections. Determining this will help in the development of malaria vaccine which will be effective against the different forms of malaria parasite. You will have to participate in this study just once, and to qualify as a participant, you must be at least eighteen (18) years and at most forty-nine (49) years of age. Your blood pressure will be determined. You will be expected to donate 60mL (4 table spoons full) of blood at the time of participation. Blood will be drawn from a vein in your arm and subsequently analysed in the laboratory. This blood drawn will also be used to determine your haemoglobin measurement. If you are a female, you will also donate urine for pregnancy test. To ensure that you are well informed about your role in this research, we request that you read this Consent Form (or have it read to you) and sign it (or make your mark in front of a witness) if you wilfully agree to participate in the study. You will be given a copy of the signed form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand.

**Possible Risks and Discomforts**

The amount of blood collected will not pose any harm to you, although there may be slight discomfort resulting from pain and bruising at the bleeding site. This should however

resolve in a day or two. All participants will receive appropriate treatment as necessary. Sterile techniques and disposable, single-use equipment will be used at all times to ensure safety.

**Possible Benefits**

There are no direct benefits to you from this study. Your participation may however help us make valuable inputs towards the development of a malaria vaccine. This will benefit the world at large and the inhabitants of malaria endemic countries like Ghana, in particular, because sickness and deaths caused by malaria will be highly reduced.

**Confidentiality**

All information gathered during the course of this study will be treated in strict confidentiality. We will protect information about your participation in this research to the best of our ability. Your name will not be used in any reports, although the study staff including study physician, laboratory and data analysts may sometimes look at your research records. If you have any questions, please feel free to ask the study staff.

**Compensation**

You will not be paid for participation in this study but you will receive a little compensation for the time you devoted to participate in this study. This token will be given to you after you have consented to be part of the study and made the required blood donation.

**Voluntary Participation and Right to Leave the Research**

We would like to stress that this study is strictly voluntary and should you decide not to participate there will be no consequences whatsoever for you. If you at any point during the study decide that you do not wish to participate any further, you are free to withdraw, effective immediately. Any such decision will be respected without any further discussion. Your decision will not affect the health care you would normally receive.

**Contacts for Additional Information**

If you ever have any questions about the research study or study-related problems, you may contact Dr. Kwadwo Asamoah Kusi (Tel. 026 3112715) of the Noguchi Memorial Institute or Ebenezer Addo Ofori of the Noguchi Memorial Institute (Tel. 0509207695).

**Your rights as a Participant**

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0288736513 or email addresses: nirb@noguchi.ug.edu.gh .You may also contact the Office through mobile number 0302916438 when necessary.

**VOLUNTEER AGREEMENT**

The above document describing the benefits, risks and procedures for the research title **Effect of Allelic Polymorphism on Ex-vivo Malaria Parasite Specific IFN-Gamma**

**Responses to *Pf*AMA1 in a Malaria Exposed Population** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

…… ……

Date Name and signature or mark of volunteer

**If volunteers cannot read the form themselves, a witness must sign here:**

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

……. ……..

Date Name and signature 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 Name Signature of Person who obtained consent