The expression of PF3D7_1146100; a novel, essential *Plasmodium falciparum* antigen, and its evaluation as a target of protective immunity to clinical malaria

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university for examination.

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DEDICATION

To our Blessed Mother, The Virgin Mary, for Her tender love and care thus far.

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LIST OF ABBREVIATIONS

ACT	:	Artemisinin based Combination Therapy
ADCC	:	Antibody dependent cellular cytotoxicity
AMA1	:	Apical membrane antigen 1
APS	:	Ammonium persulfate
cDNA	:	Complementary DNA
CDS	:	Coding sequence
CHMI	:	Controlled Human Malaria Infection
CMV	:	Cytomegalovirus
CSP	:	Cicumsporozoite antigen
DALYs	:	Disability-adjusted-life years
DNA	:	Deoxy ribonucleic acid
DEG	:	Database of Essential Genes
EBA 140	:	Erythrocyte binding antigen 140
EBA 175	:	Erythrocyte binding antigen 175
ELISA	:	Enzyme Linked Immunosorbent
GOI	:	Gene of interest
GPI	:	Glycophosphatidyl inositol
GST-DBL 1 α:		Glutathione-S-Transferase- Duffy Binding Like domain 1 α
HEK 293	:	Human Embryonic Kidney cells 293
HIV	:	Human Immunodeficiency Virus
IPTi	:	Intermittent Prophylaxis Treatment for infants
IPTp	:	Intermittent Prophylaxis Treatment for pregnant women
iRBCs	:	Infected Red Blood Cells
IRS	:	Indoor Residual Spraying
ITNs	:	Insecticide Treated Bed Nets
KDHSS	:	Kilifi Demographic Health Surveillance System
LB	:	Luria- Bertani

LMICs	:	Low and Middle Income Countries
LSA 3	:	Liver Stage Antigen 3
MSP 1	:	Merozoite Surface Protein 1
MSP 7	:	Merozoite Surface Protein 7
NAI	:	Naturally Acquire Immunity
NEB	:	New England Biolabs
OPA	:	Opsonic phagocytosis assay
PAGE	:	Polyacrylamide Gel Electrophoresis
PBS	:	Phosphate Buffered Saline
PVDF	:	Polyvinylidene difluoride
RBCs	:	Red Blood Cells
RH 5	:	Reticulocyte binding homolog 5
SERU	:	Scientific Ethical Review Unit
SDS	:	Sodium Dodecyl Sulphate
SOC	:	Super Optimal Broth with Catabolite Repression
TBST	:	Tris Buffered Saline with Tween 20
TEMED	:	Tetramethylethylenediamine
TLP	:	Tripartite Leader Peptide
TMHMM	:	Transmembrane Helices; Hidden Markov Model
WGCFS	:	Wheat Germ Cell Free System
WHO	:	World Health Organization

DEFINITION OF TERMS

Antigenic polymorphisms refers to allelic or genetic variations at a given gene loci that result in protein forms with distinct antigenic properties in different parasite clones or strains.

Antigenic variation refers to alterations in the phenotype of an antigen as a result of regulated expression of diverse family of genes in a clone of parasites during the natural course of an infection.

Codon bias is the high preference and frequent use of a certain codon that codes for an amino acid in an organism.

Codon optimization is the process of changing the codons of the gene of interest to conform to the codon preference of the organism used for its recombinant expression.

Essential gene is a gene described to be important for growth and survival of an organism through results of its disruption in loss-of-function studies.

Functional genomics involves the use of genomic data to study gene function and host pathogen interaction.

Heterologous expression system is a system in which a protein of interest is produced in an organism that is different from the natural host (origin of the target gene).

Reverse vaccinology is the process of discovering antigens through *in silico* screening of the genomic data encoding a complete antigenic repertoire of an organism.

ABSTRACT

The use of reverse vaccinology in vaccine candidate antigen discovery has led to identification of many *Plasmodium falciparum* novel antigens. Some of these antigens are essential for parasite survival and could be evaluated as targets of protective immunity to clinical malaria that is acquired naturally. Targeting essential antigens identified through functional genomic studies involving loss-of-function, could aid in the development of an effective next generation sub-unit vaccine. For these studies to be successful, the antigens must be expressed in their correctly folded structure in a heterologous expression system. This study aimed at expressing PF3D7_1146100, one of the novel, essential *P. falciparum* antigens, and assess the protective role of antibodies against it in clinical malaria using sera from a prospective cohort of children (N=343) from Junju, Kilifi, Kenya. PF3D7_1146100 was sub-cloned into pTT28, an expression vector, and transfected into Expi 293F cells for recombinant protein expression. The antigen was purified and characterized using SDS PAGE and Western blotting. Immunogenicity of the recombinant protein and the correlation between its immune responses and protection against clinical malaria was determined using indirect ELISA. In a univariate logistic regression analysis, anti-PF3D7_1146100 antibodies were correlated with protection against clinical malaria at an odds ratio of 0.63(0.42-0.95) at 95% C.I and *p value*=0.027. After adjusting for age and exposure, the odds ratio was 0.73(0.44-1.16) at 95% C.I with a *p-value*=0.176. The essentiality and immunogenicity of this antigen indicates that it is a potential vaccine candidate targeting the blood stage of the parasite. The successful expression of PF3D7_1146100 and its analysis as a target of the immune system, provides a basis for studying other novel essential and conserved antigens. This also provides an opportunity to study its function, a knowledge which could contribute to vaccine and drug development not only against *P. falciparum* but also other *Apicomplexa* organisms

CHAPTER ONE: INTRODUCTION

1.1 Background

Malaria is among the world's most common infectious diseases disproportionately affecting poverty-stricken regions within the tropics and sub-tropics. It claims the lives of more than 400,000 individuals with over 200 million cases being reported every year(WHO, 2020). The populations mostly at risk are children aged under five years, HIV infected individuals, pregnant women, travellers, refugees, displaced persons and migrant workers from malaria naïve areas (WHO, 2018). In 2018, the mortality of children between 0 to 5 years accounted for approximately 67% of all the reported deaths due to malaria, with sub Saharan Africa accounting for most of them(WHO, 2019).

The protozoan parasites of the genus *Plasmodium* are the agents causing malaria. Only five *Plasmodium* species are involved in infecting humans: *Plasmodium knowlesi, Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium ovale*. The major parasite causing infection in sub-Saharan Africa is *P.falciparum* and is responsible for over 90% of all reported cases globally (Figure 1.1), hence the attention it has received in the malaria vaccine development research(WHO, 2019).

In the quest to eradicate and eliminate malaria, a number of control strategies have been put in place. Artemisinin based combination therapy (ACTs), indoor residual spraying (IRS) and insecticide treated bed nets (ITNs) among others, have made a major contribution to the reduction in deaths and cases due to malaria (Bhatt et al., 2015). However, the resistance developed by parasites to antimalarial drugs and vectors to insecticides, pose a challenge towards achieving zero malaria. Lack of an effective vaccine has been a drawback to efforts put forward for malaria elimination. In fact, the current licensed vaccine being rolled out in Ghana, Malawi and Kenya demonstrated

moderate efficacy at best in Phase III trials (Neafsey et al., 2015; Draper et al., 2018). Furthermore, only a limited number of *P. falciparum* antigens in the erythrocytic stage have been assessed in their protective role against clinical malaria despite the fact that >5400 antigens are encoded in its genome (Tuju et al., 2017). The few that advanced to clinical trial stages, have failed mainly due to their antigenic polymorphisms and variations. Antigenic polymorphisms induce strain specific immunity against clinical malaria which does not protect an individual from infection by other strains. There is therefore a need for discovery of novel targets of protective anti-malarial immunity that are less polymorphic and essential for parasite survival. Assessing the role of the novel recombinant antigens in naturally acquired immunity to malaria will hasten their prioritization and validation as vaccine candidates.

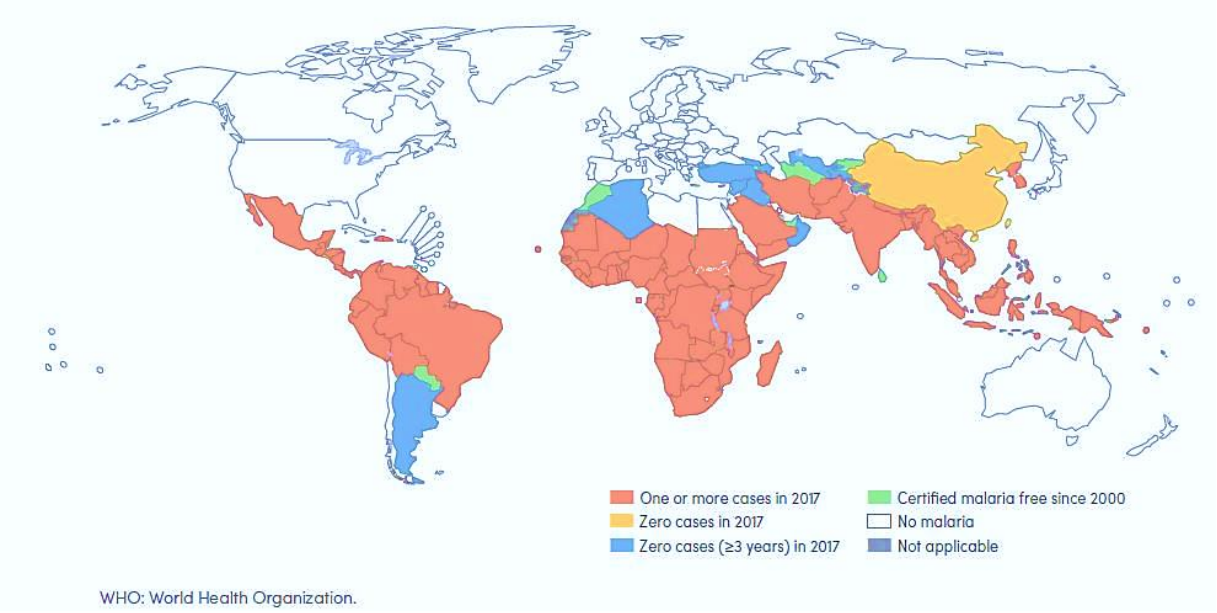


Figure 1.1 : The global prevalence of malaria in 2017 (WHO, 2018)

1.2 Problem Statement

The rising cost of malaria intervention and poverty resulting from malaria case management, calls for development of an effective malaria sub-unit vaccine (El-Houderi et al., 2019; Haakenstad et al., 2019; Winskill et al., 2019). Reverse vaccinology has enabled the identification of many novel antigens that have a potential of being vaccine candidates (Doolan et al., 2008; Crompton et al., 2010; Trieu et al., 2011; Davies et al., 2015; Kamuyu et al., 2018). Gene knock-out studies have also been done to identify proteins/ antigens essential for parasite survival especially in the invasion of host cells and tissues (Zhang et al., 2018). To date, the application of these two strategies in identifying novel essential *P. falciparum* antigens that have a potential of being vaccine candidates, and even assessing their role in the context of naturally acquired immunity, has not been explored. Targeting these antigens for vaccine development could help overcome the constraint posed by antigenic polymorphisms.

1.3 Justification of the Study

Passively transferred antibodies from individuals immune to malaria residing in the Gambia, were able to eliminate parasitemia and clinical symptoms in West and East African children as well as Thai adults suffering from malaria (Cohen et al., 1961; McGregor et al., 1963; Sabchareon et al., 1991).This indicates that these antibodies could be targeting parasite proteins that have limited polymorphisms (Bull and Marsh, 2002; Duffy et al., 2005; Doolan et al., 2009). Targeting multiple and highly conserved antigens as vaccine candidates could overcome the constraint of antigenic polymorphisms currently observed in blood stage vaccine candidates, which contribute to their failure in clinical trials (Draper et al., 2018). Reticulocyte binding homolog 5 (RH5), a recent blood stage vaccine candidate in the making, is highly conserved, essential for parasite survival and has been shown to illicit a strain transcending immunity to some *P. falciparum* strains (Douglas et al., 2011; Douglas et al., 2015).

Exposure to multiple antigens has been linked with protection against malaria and that antibodies against them, do work in synergy (Osier et al., 2008; Osier et al., 2014b; Bustamante et al., 2017). A vaccine made up of multiple conserved proteins will help alleviate the malaria burden majorly felt in sub-Saharan Africa and reduce the mortality of the most susceptible populations (Plowe et al., 2009; Tanner et al., 2015).

1.4 General Objective

To assess the role of recombinant PF3D7_1146100; a novel, essential *P. falciparum* vaccine candidate antigen in naturally acquired immunity to malaria

1.4.1 Specific Objectives

1. To optimize the recombinant expression of PF3D7_1146100 in Expi 293 F cells, a mammalian system.
2. To evaluate the association between immune responses to the recombinant antigen and protection against clinical malaria.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria vaccine development

As mentioned earlier, the heavy Falciparum malaria's burden is highly felt in sub-Saharan Africa(WHO, 2019). Every hour, malaria claims the lives of 30 children aged five years and below. Although the control measures put in place have had a positive impact towards reduction in malaria transmission, they have been faced with the challenge of resistance developed by both the vector and the parasite (Dhiman, 2019). In addition, the cost incurred in the deployment of these interventions to every individual residing in malaria endemic areas is estimated to be US$ 6.6 billion(WHO, 2019). In comparison to what was spent in 2016, the cost of malaria intervention is on the rise (Winskill et al., 2019). Taking a look at malaria case management in malaria prone areas especially in sub-Saharan Africa, malaria has impoverished many families through high out-of-pocket expenditure on treatment and loss of income resulting from absence at work (El-Houderi et al., 2019; Haakenstad et al., 2019). As a result, there is an urgency to develop a malaria vaccine that is effective.

Vaccines have been evidenced to be the most efficient and economical public health interventions able to eradicate and eliminate an infectious disease (Rappuoli, 2000; Bustreo et al., 2015; Zerhouni, 2019). Through vaccination, in 1987, the world was declared small pox free, and polio, bacterial and other viral diseases have been controlled (Berkley, 2019; Zerhouni, 2019). Apart from contributing to improved population health, mathematical models have shown the impact of vaccines on the reduction (in millions) of deaths and disability-adjusted-life years (DALYs)) and the number of medical impoverished cases in low and middle income countries (LMICs) (Chang et al., 2018; Li et al., 2021).

2.2 Current malaria vaccine status

To date, RTS,S/AS01B that consists of the central repeat and C-terminal epitopes of the circumsporozoite (CSP) antigen, is the most advanced of all malaria vaccine candidates. It is undergoing implementation trials in children in Malawi, Ghana and Kenya, despite its modest efficacy which wanes with time (Neafsey et al., 2015; Draper et al., 2018; Frimpong et al., 2018). It does not meet the threshold outlined in the WHO Malaria Vaccine Roadmap of 2030 which requires a vaccine to have a protective efficacy of > 75% against clinical malaria, and reduce incidences of malaria infection by lowering the transmission rates (Chatterjee and Cockburn, 2021). Consequently, the search for an effective malaria vaccine which could be used together with or improve the efficacy of RTS,S remains a priority. It is thought that such a vaccine would be multi-component and multi-stage. This could prevent malaria and block transmission by preventing the invasion and replication of merozoites in red blood cells (Chauhan et al., 2010; Draper et al., 2018).

The feasibility of a malaria vaccine is based on two models (Hoffman 1998). The first one is an observation in nature where adults residing in malaria endemic regions have developed protection against malaria resulting from multiple and recurrent exposure to *P. falciparum* over a long period of time (Cohen, 1979; Marsh, 1992; Doolan et al., 2009). This protection attributed to naturally acquired immunity (NAI), majorly mediated by antibodies, is effective against high parasite density, death and clinical malaria (Richards and Beeson, 2009).The other basis is the development of sterile and lasting immunity observed in animal and human challenge models in which healthy participants were infected with attenuated sporozoites and merozoites (Hoffman et al., 1998; Duffy et al., 2005; Stanisic et al., 2018).

2.3 Life cycle complexity and malaria vaccine development

Malaria vaccine development has proved to be challenging due the antigenic complexity of *P. falciparum* (Anders, 2011) whose genome encodes > 5400 antigens which are differentially expressed in the different lifecycle stages(Gardner 2002) taking place in the female anopheles mosquito (sexual stages) and the human host (asexual stages). Due to this, sub-unit vaccine approaches have been classified into the life cycle stages that they do target (Vaccines, 2011). To prevent liver stage infection, pre-erythrocytic vaccines are being developed that target sporozoites. As the fatality of the disease is manifested at the blood stage cycle of *P. falciparum*, blood stage vaccines aim to prevent red blood cell (RBC) invasion, clinical disease and development of sexual stages that infect mosquitos. A successful vaccine targeting this stage will help reduce the mortality and morbidity of children aged below five years in areas that are highly prone to malaria (Hoffman et al., 1998; Stowers et al., 2001). Transmission blocking vaccines aim to break the transmission cycle by targeting gametocytes and mosquito stages. There are suggestions on developing a multi stage vaccine which could be effective against all the *P. falciparum* life cycle stages (Draper et al., 2018). Figure 2.1 shows the vaccine candidates that target *P. falciparum* life cycle stages.

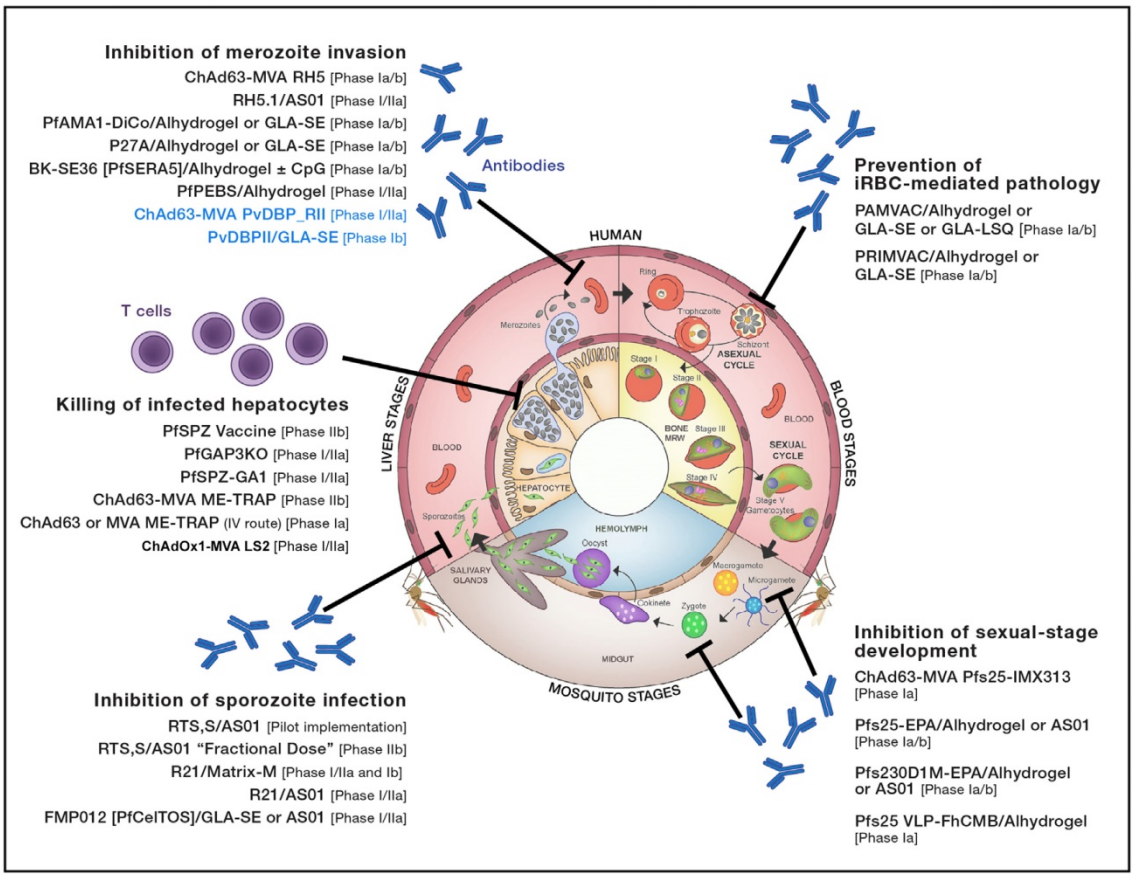


Figure 2.1: Malaria vaccine candidates in clinical development that target the life cycle stages of *P. falciparum* (Adapted from Draper et al. (2018))

2.4 The *Plasmodium falciparum* life cycle

A complete *P. falciparum's* life cycle is dependent on two different hosts: the definitive host or vector, which is the female Anopheles mosquito, and an intermediate host that is a vertebrate. It is in the female Anopheles mosquito where the sexual stages take place while the asexual stages occur in the vertebrate host, the human.

In the human host, the asexual life cycle of *P. falciparum* is commenced by an infectious mosquito bite where haploid sporozoite stages are injected in to the skin and migrate to the liver (Doolan et al., 2009; Cowman et al., 2017). They traverse the kuppfer cells and

invade the hepatocytes where they establish an infection (Cowman and Crabb, 2006; Doolan et al., 2009; Cowman et al., 2017). In the hepatocytes, sporozites are transformed into liver stages or exoerythrocytic forms. They undergo replication and after an incubation period of approximately 7 days, each infected hepatocyte releases up to forty thousand merozoites in to the bloodstream through the budding of merosomes (Doolan et al., 2009; Nilsson et al., 2015; Cowman et al., 2016).

The red blood cells (RBCs) that are in circulation are invaded by the released merozoites, initiating the repetitive blood stage cycle. This 48 hour cycle begins with the ring stage followed by the trophozoite stage and finally the schizont stage where merozoites undergo mitotic division in to 16-32 daughter merozoites (Doolan et al., 2009; Nilsson et al., 2015). These merozoites lyse the parasitized RBCs and egress initiating another blood stage cycle. This triggers the manifestation of malaria clinical symptoms due to the rupture of both infected and uninfected RBCs, and cytoadherence of infected RBCs in the vasculature and major organs evading their circulation which protects them from splenic clearance (Richards and Beeson, 2009; Boddey and Cowman, 2013). During the progression of the blood stage cycle, some trophozoites develop in to male and female gametocytes and move into the extracellular space of the bone marrow. Once in the bone marrow they undergo development through stage I-V which then move back into the peripheral blood and are ready to infect female anopheles mosquitos when they take up a blood meal(Nilsson et al., 2015).

In the mosquito midgut, the female and male gametocytes mature into female and male gametes, a process denoted as gametogenesis. The male gametes divide and undergo exflagellation to form up to 8 flagellated microgametes while only one macrogamete is formed from the female gamete (Cowman and Crabb, 2006; Nilsson et al., 2015). Fertilization takes place and a zygote is formed which undergoes meiosis and differentiates in to an invasive form, the ookinete and crosses the midgut epithelium, developing into an oocyst on the basal lamina (Doolan et al., 2009; Boddey and

Cowman, 2013). Sporogony takes place in the oocyst in which the parasite undergoes mitosis forming thousands of sporozoites which are released upon its rupture (Cowman and Crabb, 2006; Doolan et al., 2009; Nilsson et al., 2015). The haploid sporozoites migrate to the mosquito salivary glands ready to progress to the asexual stages when a parasitized mosquito bites its vertebrate host (Cowman and Crabb, 2006). Figure 2.2 shows the *P. falciparum's* life cycle.

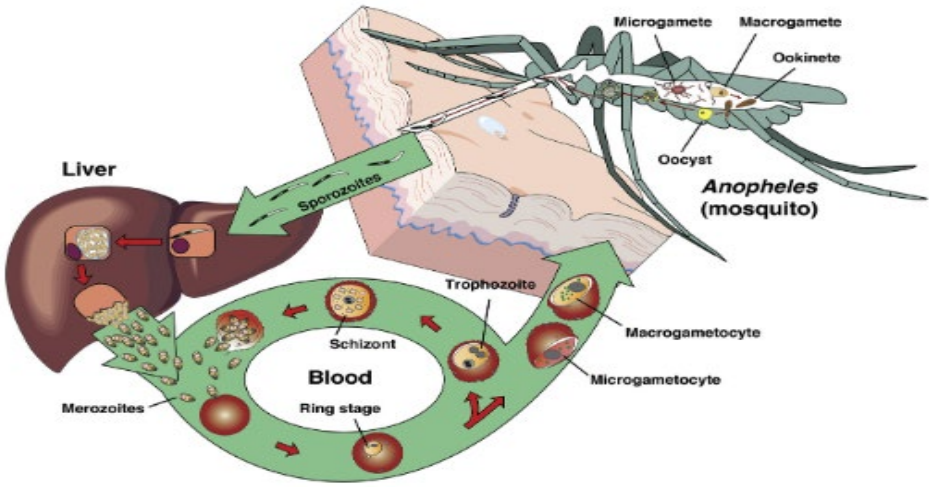


Figure 2.2: The *P. falciparum* life cycle depends on transmission cycle between humans and Anopheles mosquito (Adapted from Cowman et al. (2017))

2.5 Pathogenesis of *P. falciparum* occurs in the blood stage cycle

The pathogenesis of *P. falciparum* is mainly observed at the blood stage where schizonts burst and release merozoites that invade other RBCs resulting in their exponential increase. The release of the erythrocytic material and malaria parasites into blood triggers the pathophysiology process and onset of malaria symptoms due to the activation of the cytokine cascade (Cowman et al., 2016). RBC invasion is a very crucial step for *P. falciparum* hence its rapid and efficient entry has been the driver of its

evolution (Cowman and Crabb, 2006). Consequently, the parasite takes advantage of its highly polymorphic surface antigens and redundant RBC invasion pathways to accomplish the evasion of host's immune system.

Once the parasite gains entry into RBC, it modifies it to suit its development and survival as it acts as a source of nutrients and enables the parasite's evasion of the host immune system (Boddey and Cowman, 2013). This immune escape mechanism results from *P. falciparum* exporting proteins to the surface of infected RBCs (iRBCs) (Cowman et al., 2016) that enable cells to adhere to the vasculature and sequester in other organs to avoid clearance by the splenic macrophages, a process called cytoadherence (Boddey and Cowman, 2013). Accordingly, it results in cerebral malaria (Duffy et al., 2005), a severe syndrome that is the cause of mortality in children and which affects their cognitive development. iRBCs attract other non-infected RBCs, a process known as rossetting and are both cleared by the immune system resulting in severe malarial anaemia. Severe malarial syndromes are prevalent in children aged below five years due to the slow rate at which immunity to malaria is acquired naturally.

2.6 Naturally acquired immunity (NAI) to clinical malaria

In areas where malaria is endemic, individuals do develop immunity to the disease , a phenomenon which most blood stage vaccines being developed aim to mimic (Hoffman et al., 1998). This however, is due to the multiple and repeated exposure to *P. falciparum*. In the erythrocytic stage, naturally acquired immunity is majorly mediated by antibodies due to the brief exposure of merozoites to the immune system before gaining entry into RBCs (Cohen et al., 1961; Cohen et al., 1969). The antibodies' role in immunity against clinical malaria was shown by Cohen and colleagues by treating children suffering from malaria with purified gamma immunoglobulins obtained from malaria immune adults in the Gambia (Cohen et al., 1961). Their results were supported

by passive transfer studies in children and adults with clinical malaria from East Africa and Thailand respectively (McGregor et al., 1963; Sabchareon et al., 1991).

Acquisition of NAI is dependent on age and exposure (Cohen, 1979; Marsh et al., 1989; Baird, 1995). In malaria risk areas, children aged below five years are susceptible to malaria due to lack of immunity resulting from little or no exposure to *P. falciparum,* coupled to an immature immune system (Baird, 1995). Adults acquire partial immunity from clinical malaria due to development of long lived plasma cells that produce antibodies able to recognise the strains which they have been exposed to in their entire life time (Baird, 1995; Akpogheneta et al., 2008). The long-term exposure to *P. falciparum* of individuals in areas of malaria endemicity, protects them from the diverse strains of *P.falciparum* through acquisition of cross-reactive and strain specific antibodies (Osier et al., 2010; Rono et al., 2013). The stability and intensity of exposure to *P. falciparum,* however, affects the acquisition rate of immunity to severe and mild malaria (Gupta et al., 1999; Marsh and Kinyanjui, 2006). In areas of high malaria transmission, the acquisition of anti-disease immunity is rapid.

In conferring protection against clinical malaria, antibodies block merozoites from invading the RBCs preventing the blood stage cycle of *P. falciparum* from taking place. They also opsonize merozoites for phagocytosis, induce the antibody dependent cellular inhibition mechanisms and complement activation (Cohen et al., 1969; Bouharoun-Tayoun et al., 1990; McCallum et al., 2008; Osier et al., 2014a). To perform their function, they target antigens that the parasite secretes or expresses on its surface, some of which facilitate the invasion of RBCs (Osier et al., 2014b). This makes NAI a suitable model for prioritizing potential vaccine candidates that are known or predicted for development of vaccines (Richards and Beeson, 2009; Fowkes et al., 2010). A major challenge to prioritization and validation of vaccine candidates using this model has been the conflicting findings of epidemiological studies on the protective effect of naturally acquired antibodies (Fowkes et al., 2010), despite being proposed as correlates

of protection . It is still not clear which of the > 5400 *P. falciparum* antigens could be targets of protective antibodies (Tuju et al., 2017) as the definition of protection from clinical malaria in cohort studies, has been a challenge (Marsh and Kinyanjui, 2006).

2.7 Reverse vaccinology and antibody targets discovery

Very few *P. falciparum* antigens, (~ 22), have been under evaluation in clinical trial studies. Of these vaccine candidates, 82% were discovered during the pre-genomic error (Tuju et al., 2017). The advances in high throughput technologies in the omics era have enabled generation of data (Carucci, 2002; Doolan et al., 2014), used in the discovery of novel *P. falciparum* antigens that have a potential of being vaccine candidates. The genome is viewed as an 'entry' of antigens expressed by a parasite hence containing the necessary information for vaccine development (Rappuoli, 2000). Reverse vaccinology takes advantage of the available computer programs that scrutinize genomic data for presence of features that characterize potential vaccine candidates. These features constitute signal peptides, transmembrane domains, glycophosphatidyl anchors, export signals and adhesive properties (Sette and Rappuoli, 2010).

Genomic data has been utilized in various ways. For instance, Mu and colleagues employed genome-wide population genetics on the *P. falciparum* genomic data and identified potential novel vaccine candidates under balancing selection (Mu et al., 2007). P48/45 antigen, a potential candidate for transmission-blocking vaccine in *P. falciparum*, was identified through functional comparative genomics as the disruption of its homologue in *P. berghei* showed that it plays a role in the fertility of male gametes (van Dijk et al., 2001). Through structural bioinformatics, P27A, was discovered and was able to induce protective antibodies facilitating its formulation as a peptide vaccine for clinical trials (Villard et al., 2007).

Novel vaccine antigens that are stage specific have also been identified through *in silico* analysis of transcriptomic data (Aguiar et al., 2004; Trieu et al., 2011; Davies et al., 2015). In their study, Zenonos et al. (2014) established the transcription patterns of 4 well-studied merozoite vaccine candidates which include: RH5, EBA 140, EBA 175 and AMA 1. Using this expression pattern, function prediction and domain mapping on *P. falciparum* transcriptomic data, 14 putative antigens were identified.

2.8 Functional genomics and discovery of essential vaccine candidate antigens

The functional genomics data obtained from genome-wide saturation mutagenesis of the *P. falciparum* blood stage, can be utilized in identifying not only drug targets, but also vaccine candidates. Zhang and colleagues identified antigens that were essential for *P.falciparum* viability (Zhang et al., 2018). Most of the vaccine candidates in clinical trials were shown to be dispensable for parasite's survival (Zhang et al., 2018), a possible explanation of their high polymorphisms and failure to proceed in clinical trial stages. Using reverse vaccinology, potential vaccine candidate antigens that are essential and conserved in the parasite's blood stage have been identified and await pre-clinical validation. Other studies have identified essential and conserved vaccine candidates and drug targets through *in silico* prediction of antigen essentiality to the parasite and using the database of essential genes (DEG) (Vilela Rodrigues et al., 2019; Rout et al., 2015).

2.9 Recombinant expression of *P. falciparum* antigens

Pre-clinical evaluation of both known and novel potential vaccine candidate antigens necessitates their expression in heterologous systems (Tuju et al., 2017; Crosnier et al., 2013), since sufficient amounts cannot be isolated from the parasite despite their superiority in structure and immunogenicity (Fernandez-Robledo and Vasta, 2010). Recombinant proteins, therefore, must be expressed in their native conformation with post-translational modifications mimicking the organism's native protein, as this is a key determinant of immunogenicity (Stowers et al., 2001; Fernandez-Robledo and Vasta,

2010; Cowan et al., 2014). Attempts to express *P. falciparum* antigens in heterologous systems has met technical difficulty that has posed a challenge to understanding not only the function of novel antigens, but also their assessment as vaccine candidates.

The genome of *P. falciparum* is A-T rich (~ 80%) (Gardner et al., 2002), a feature that has been implicated in the difficulty encountered in recombinant expression of its antigens due to the codon usage incompatibility. In addition, high molecular weight of its proteins (> 56 kDa) and low complexity regions made up of repetitive amino acids also contribute to low or lack of heterologous expression (Birkholtz et al., 2008; Vedadi et al., 2007; Mehlin et al., 2006). Other features that have been shown to impact negatively on expression include: signal peptides, transit peptides, transmembrane domains and Plasmodium export signal (PEXEL) motifs (Crosnier et al., 2013; Vedadi et al., 2007; Mehlin et al., 2006).

To date, a number of heterologous systems including bacteria (Salinas et al., 2019; Singh et al., 2018; Aguiar et al., 2015; Reddy et al., 2014; Vedadi et al., 2007; Mehlin et al., 2006), yeast (Milek et al., 2000; Stowers et al., 2001), protozoa (Reymond et al., 1995; van Bemmelen et al., 2000; Cowan et al., 2014), plants (Ghosh et al., 2002), cell free systems (Oda-Yokouchi et al., 2019; Ito et al., 2019; Tsuboi et al., 2008; Doolan et al., 2008; Mu et al., 2007), and mammalian cells (Kamuyu et al., 2018; Zenonos et al., 2014; Crosnier et al., 2013) have been explored in the expression of *P. falciparum* antigens.

Due to the low cost of production, scalability and the ease of genetic manipulation, *E. coli* has been the most preferred heterologous system. However, most proteins expressed by *E. coli* are not soluble requiring refolding procedures. Many studies have reported difficulty in expressing full-length ecto-domains (Aguiar et al., 2015; Vedadi et al., 2007; Mehlin et al., 2006) in *E.coli*, contrary to Reddy and colleagues who expressed a full-length *P. falciparum* RH5 protein (Reddy et al., 2014). Cell free systems, especially

the wheat germ cell free system (WGCFS), are being highly explored despite the expression of recombinant proteins taking place in a reducing environment which negatively impacts protein folding. On the other hand, mammalian cells enable the expression of full-length ecto-domains in their soluble and native conformation (Kamuyu et al., 2018; Zenonos et al., 2014; Crosnier et al., 2013). Since a correctly folding protein is able to interact with other proteins, Crosnier et al. (2013) showed the MSP1-MSP7, EBA175–Glycophorin C and EBA140-Glycophorin A protein interactions after the recombinant expression of the antigens in HEK 293 cells. These protein-protein interactions are usually exploited by the parasite when invading RBCs. The low protein expression levels and high cost of production of this system has resulted in their slow adoption (Fernandez-Robledo and Vasta, 2010; Birkholtz et al., 2008).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

This was a study conducted in Junju, a village in Kilifi sub-county, Kilifi County in Kenya which is within the Kilifi Demographic Health and Surveillance System (KDHSS). In this area, peak malaria transmission is experienced in two seasons (May to July and November to December). This study is part of a long-term longitudinal immunological cohort set up to identify immune correlates of malaria. Briefly, in May 2014 (before the season of malaria transmission began), a cross-sectional bleed was done and children (N=343) were followed up for one year where cases of clinical episodes of malaria were actively and passively detected. To monitor the episodes of malaria, the participants were visited every week in their homes (as illustrated in Figure 3.1), where temperature was recorded. In children with fever, parasitemia was checked by examining thick and thin blood smears using light microscopy and malaria rapid diagnostic test (MRDT). Individuals with fever resulting from parasitemia were treated and had a free access to the local District Hospital. The definition of malaria was a fever (an auxiliary temperature >37.5 ^{0}C) with a parasite density of 2500/µl of blood.

Antibody levels to a specific antigen measured using ELISA at the beginning of the malaria season, were used to infer a correlation with protection against clinical malaria in the first six months of follow-up.

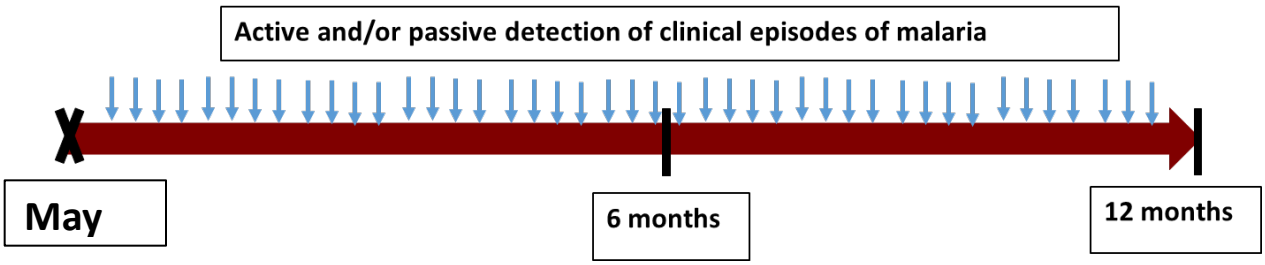


Figure 3.1: An illustration of the longitudinal Junju cohort study set up.

Cross sectional bleed done in May (2014), a month before the onset of a malaria season in Junju village, Kilifi sub-county. The participants were followed up for one year to detect malaria cases. Antigen specific antibodies present prior to the malaria transmission season were used to determine correlation with protection against malaria in the first six months of follow up.

3.2 Ethical Approval

The ethical approval of this study was obtained from the Kenya Medical Research Institute (KEMRI) Scientific Ethical Review Unit (SERU) with protocol number SCC3139.

3.3 Methods

3.3.1 Essential Gene Selection

PF3D7_1146100 was selected through combining dataset from PhenoPlasm (Functional genomics) database and PlasmoDB (for Reverse vaccinology). PhenoPlasm database contains information on *P. falciparum* gene knockout or genetic manipulation experiments while PlasmoDB contains integrated data on genomics, transcriptomics and proteomics of *Plasmodium* species. PF3D7_1146100 antigen was prioritized for evaluation in the laboratory since it is small in size, ~20.3 KDa (easy to clone and express), had never been expressed and studied as a target of protective immunity to malaria before. Briefly, a query was submitted in PlasmoDB database (https://plasmodb.org/) using PF3D7_1146100, its gene identifier. Transmembrane

domain, signal peptide or a glycophosphatidly inositol (GPI) anchor presence was used to determine whether it was an exported or a surface protein.

3.3.2 Sequence retrieval and construct design

The ectodomain region spanning amino acid 20 to 175 was selected for expression. SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP-4.1/) was used to check for signal peptide presence. For transmembrane domain, the TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to determine whether it was present. Sites for N-glycosylation were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).Codon optimization of the ectodomain for expression in human cells was done. Unique Nhe I and Bam HI recognition sites were flanked to the 5' and 3' ends of the ectodomain respectively to enable cloning in pTT28 expression vector (Figure 3.2). Following its design, the construct was synthesized by a commercial gene synthesis provider, Invitrogen™ GeneArt™ and contained an ampicillin resistance gene, AmpR, which was used for selection (Figure 3.3).

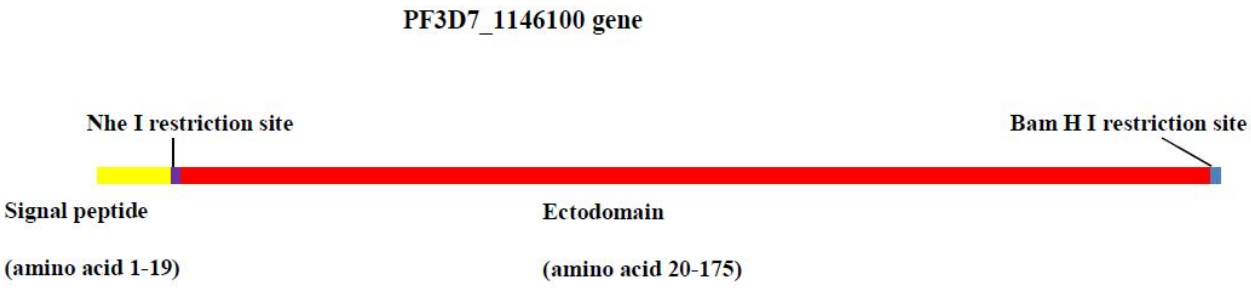


Figure 3.2: The PF3D7_1146100 gene cassette.

The predicted signal peptide constitutes the first 19 amino acids (yellow) and an ectodomain spanning the 20th to the 175th amino acid (red). Nhe I (purple) and Bam HI (blue) sites are flanked on the ectodomain to facilitate cloning in to the expression vector.

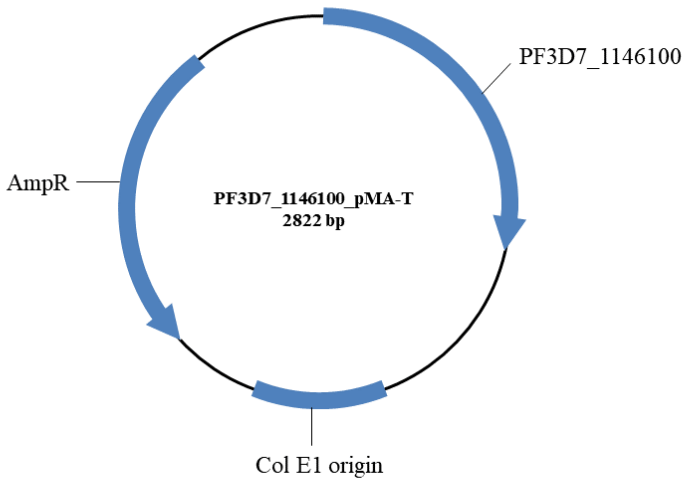


Figure 3.3: A plasmid map of the PF3D7_1146100 construct

The plasmid contains an AmpR gene for selection using ampicillin and Col E1 origin which is enables the replication of the plasmid by *E. coli* bacteria (pMA-T vector adapted from https://www.thermofisher.com)

3.3.3 Transformation and Plasmid Midiprep

One hundred microliters of TE buffer was used to resuspend 5 µg of the lyophilized PF3D7_1146100 gene construct and vortexed for 1 minute. Addition of plasmid DNA, at a volume of 2 ul, to 25 µl of competent Top 10 *E.coli* cells (Invitrogen) was done. This mixture was incubated on ice for 30 minutes then rapidly transferred to a waterbath for 30 seconds at 42ºC, after which it was incubated again on ice for 2 minutes. 250 µl of Super Optimal Broth with Catabolite Repression (SOC) media (at a temperature of 37 ºC) was added to the heat-shocked cells followed by a 1 hour incubation while shaking at 200 rpm after which they were incubated overnight at 37 ºC/180rpm in 300ml of LB broth with ampicillin in New Brunswick Scientific™ Innova™ 42 incubator shaker

(Eppendorf, USA). After harvesting *E.coli* cells, Qiagen midiprep kit was used in the extraction of plasmid DNA as per the instruction of the manufacturer.

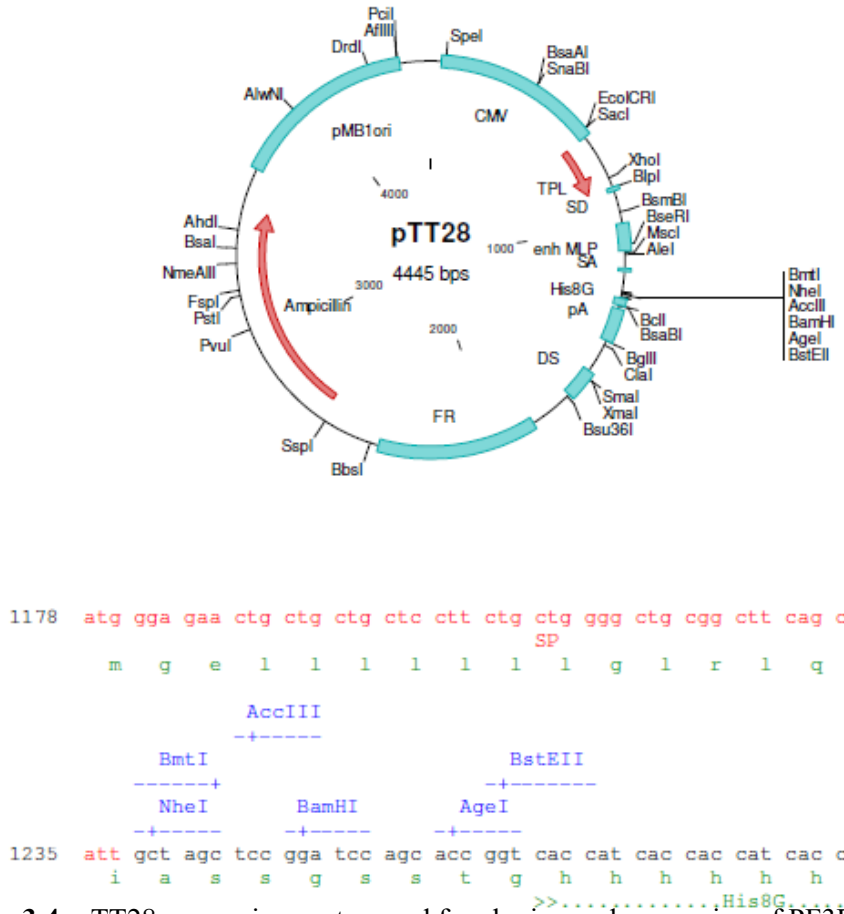
3.3.4 Restriction digest

Half a microliter each of NheI HF and BamHI HF (New England Biolabs (NEB)) restriction enzymes (whose recognition sequences are 5'-GCTAGC-3' and 5'-GGATCC-3'respectively) were added to 10 µl of PF3D7_1146100 plasmid construct, 2 µl of 10X cutsmart buffer (NEB). To make the mixture to a volume of 20 µl, 7 µl of DNAse/RNAse free water was added. Incubation was done overnight at 37 ºC. The reaction mixture was resolved on a 1.5% agarose gel containing redsafe DNA stain at 100V. Smart ladder (Eurogentec), a DNA marker that separates into bands ranging from 200-10000 base pairs, was used. ChemiDoc MP system (Bio-Rad) was used to visualize the agarose gel. The band with the expected molecular size was cut out from the gel and gel extraction performed using the QIAquick gel extraction kit (Qiagen) as per the instruction of the manufacturer. NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific) was used to quantify the DNA extracted from the gel. The same restriction enzymes were used for expression vector digestion to facilitate sub-cloning of the Gene of Interest (GOI) downstream of the mouse IgG kappa signal peptide and upstream of its hexa histidine tag.

3.3.5 PF3D7_1146100 ligation to the expression vector

The amount of insert DNA required for the ligation reaction was calculated using the NEBiocalculator (http://nebiocalculator.neb.com). Using an expression vector (Figure 3.4) that is 4445 base pairs (bp) in size at a mass of 50 ng and a 468 bp length of GOI at a 3:1 insert/vector molar ratio, the amount of the required insert DNA was 15.79 ng. Briefly, 50 ng of expression vector was combined with 15.79 ng of the insert DNA and the reaction volume topped up using distilled water to 10 µl. To the vector-DNA mix, 2X quick ligation buffer (NEB) at a volume of 10 µl was added followed by 1 µl of

quick ligase. The tube containing the reaction mixture was gently tapped after which the mix was incubated at room temperature for 30 minutes. Using this reaction mix, competent Top 10 *E.coli* cells were transformed as in section 3.3.3 and plated on LB-agar containing 100 µg/ml of ampicillin, and incubated overnight at 37 ºC. After incubation, eight colonies per plate were selected randomly and inoculated into 3 ml of LB broth supplemented with ampicillin and incubated at 37 ºC overnight. Plasmid extraction was done from 1.5 ml culture from each colony using the plasmid miniprep extraction kit (Promega) as per the instructions of the manufacturer. Nhe1 HF and Bam H1 HF restriction enzymes were used to digest the extracted plasmids as described in section 3.3.4. If ligation was successful, two bands of 4445 bp and 468 bp were to be observed. If not, only a single band would be observed at 4445 bp. Colonies observed to contain the GOI were grown in 400 ml of LB-Broth. Following overnight incubation, plasmid extraction from *E.coli* was done using the Qiagen maxiprep kit (Qiagen) as per the instructions of the manufacturer. TE buffer was used to resuspend the extracted plasmids prior to quantification using NanoDrop.




```
1178  atg gga gaa ctg ctg ctg ctc ctt ctg ctg ggg ctg cgg ctt cag ctg agt ctt gga
                                        SP
        m   g   e   l   l   l   l   l   l   l   g   l   r   l   q   l   s   l   g

                  AccIII
                  -+-----
          BmtI                          BstEII
          ------+                       -+-------
            NheI        BamHI         AgeI
            -+-----     -+-----       -+-----
1235  att gct agc tcc gga tcc agc acc ggt cac cat cac cac cat cac cac cac ggg tga
        i   a   s   s   g   s   s   t   g   h   h   h   h   h   h   h   h   g   -
                                        >>.............His8G.............>>
```

Figure 3.4: pTT28 expression vector used for cloning and expression of PF3D7_1146100 pTT28 contains a human cytomegalovirus (CMV) promoter, tripartite leader peptide (TLP), multiple cloning sites, a histidine tag, polyadenylation signal and an ampicillin resistance gene among others. PF3D7_1146100 was cloned downstream of the signal peptide and upstream of the histidine tag sequence (pTT vector derived from Durocher et al. (2002)).

3.3.6 Transfection of Expi 293 cells

The plasmid DNA isolated in section 3.3.5, were transfected using the Expi293™ *Expression System* Kit (Thermo Fisher Scientific, USA) as per the manufacturers' instructions. Briefly, Expi 293™ cells were diluted to 3.0 x 10^6cells/ml with 100ml of Expi293™ media following the determination of cell count and viability using an automated cell counter. Into 5 ml of Opti-MEM™ media, 264 µl of Expifectamine™ 293 Reagent (Thermo Fisher Scientific, USA) was diluted and incubated for 5 mins. To

5 ml of Opti-MEM™ Medium, 100 µg of DNA (0.5-5µg/µl) was added and incubated for 5 min too. DNA and ExpiFectamine™ 293 Reagent were mixed gently and incubated at room temperature for 20 min. The DNA reagent complex was added gently to the cells while swirling and incubated for 16-18 hours at 37 ^{0}C, 8% CO_2 and 125 rpm in a New Brunswick™ shaker incubator (Eppendorf, USA). A volume of 500 µl and 5 ml of ExpiFectamine™ 293 transfection enhancer 1 and ExpiFectamine™ 293 transfection enhancer 2 was added respectively to the cells. The cells were incubated for 4 days at 37 ^{0}C, 8% CO_2 and 125 rpm in a New Brunswick™ shaker incubator (Eppendorf, USA). Cells were pelleted and the culture supernatant stored at 4^0 C.

3.3.5 Protein purification

The ProBond™ Purification System (Invitrogen™, USA) was used to purify recombinant PF3D7_1146100 antigen from the culture supernatant. Briefly, 300 µl of ProBond™ Nickel-Chelating resin (Invitrogen™, USA) was resuspended in distilled water and centrifuged at 514 x g (in a fixed rotor) for 5 minutes using Centrifuge 5810 R (Eppendorf, USA) and the supernatant discarded. The pelleted resin was resuspended in 100 µl of native binding buffer (10 mM Imidazole, 50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0) and added to 50 ml of culture supernatant containing 1 mM of Nickel II chloride (1 M). The mixture was incubated with agitation at 4 ^{0}C for 1 hour and transferred to purification columns where the resin settled by gravity and the supernatant aspirated and stored at 4 ^{0}C. The resin was washed 6 times with 5 ml of native wash buffer (20 mM Imidazole, 50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0). Protein elution was done four times using 300 µl of native elution buffer (250 mM Imidazole, 50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0).

3.3.6 SDS PAGE

Following protein purification, proteins were separated based on molecular weight using SDS Poly Acrylamide Gel Electrophoresis. In order to linearize the protein, gels were

run under reducing and denaturing conditions. Briefly, 14 % Separating gel was prepared by adding 2.5 ml of stock solution A (48 ml 1M HCl, 36.3 g Tris, 0.23 ml TEMED and distilled water to 100 ml), 4.7 ml stock solution C (Protogel ^{TM}) and distilled water to 10 ml and poured into a gel cassette. Isopropanol at 50 % (v/v) was overlaid on the gel until it polymerised. Isopropanol was removed and stacking gel poured above the separating gel. Stacking gel was prepared by adding 0.95 ml of stock solution B (48 ml 1 M HCl, 6.0 g Tris, 0.48 ml TEMED and distilled water to 100 ml), 1.25 ml of stock solution C and 5.0 ml distilled water. A volume of 10 µl loading dye was added to 10 µl of sample and incubated for 10 min at 100 ^{0}C on a thermoblock (Eppendorf, USA). In the first well, 6 µl of Rainbow^{TM} marker- Full range (Amersham^{TM}) was loaded, followed by 6 µl of Benchmark His-tagged protein standard (Thermo Fisher, USA) and 10 µl of sample in the second and third wells respectively. Electrophoresis was done at 100 V for 120 minutes and the gel incubated in instant blue dye (Expedeon Ltd, UK) overnight with agitation.

3.3.7 Western Blot

The his-tagged recombinant protein transfer to polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, USA) was done by Western blotting for detection with anti-his antibodies. Briefly, PVDF membrane was activated in methanol for 1 minute, washed with water and placed in transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol). The transfer stack was placed in a cassette (BioRad Mini Trans-Blot^{TM} System) and proteins transferred at 100 V for 1 hour with tank placed on ice. The membrane was blocked at room temperature for one hour using 5 % (w/v) blotto (Thermo Fisher Scientific, USA) in Tris Buffered Saline with Tween 20 (TBST) followed by washing with TBST buffer 5 times for 6 min each with agitation on a shaker. Conjugated antibody was added to the membrane in the ratio of 1: 5000 blotto for 1 hour. A volume of 3ml substrate was added in the dark and an image obtained using the gel imager (Bio-Rad laboratories Inc, USA).

3.3.8 Indirect ELISA

The recombinant antigen's immunogenicity, coating concentration and evaluation of immune responses to it were determined using indirect ELISA.

3.3.8.1 Determination of PF3D7_1146100 coating concentration and schizont ELISA

To determine the coating concentration of PF3D7_1146100, a Dynex 4HBX immunolon ELISA plate was coated with 100 µl of a two-fold serially diluted recombinant protein in coating buffer (15mM Na2CO3, 35mM NaHCO3, pH 9.4). The coating concentration ranged from 48 µg/ml to 0.01µg/ml respectively. For schizont ELISA, 100 µl of schizont extract at a dilution of 1: 8000 in coating buffer, was coated per well of the Dynex 4HBX immunolon ELISA plates. The plates were incubated at 4 ^{0}C overnight. Following incubation, each well of the plates was washed with wash buffer (0.05 % Tween 20 in 1X PBS) at a volume of 200 µl five times, and blocked at room temperature for 5 hours using blocking buffer (1 % skimmed milk in washing buffer). Again, washing of the plates was done as above. For coating concentration determination, 100 µl of positive and negative controls diluted in coating buffer at 1:200 were added. The positive and negative controls are pooled hyperimmune sera (PHIS) acquired from adults immune to malaria residing in Kilifi and sera from malaria naïve adults in Europe respectively. To the plates coated with schizont extract, 100 µl of serum samples diluted in coating buffer at 1:1000 was added to the respective well. Plates were incubated at 4 ^{0}C overnight. After washing five times as above, addition of 100µl of rabbit polyclonal anti-human IgG with a horseradish peroxidase conjugate (Dako) diluted in blocking buffer at 1:5000, to each well was done. Plates were then incubated at room temperature for 3 hours. After washing, 100 µl of o- phenylenediamine dichloride (OPD) (Sigma) was added per well and the plates developed by 15 minutes of incubation in the dark and at room temperature. To stop the reaction, 25 µl of 2 M sulphuric acid was added in each well. Using an ELISA plate reader, optical density readings were taken at 492 nm.

3.3.8.2 Determination of PF3D7_1146100 epitopes and anti- PF3D7_1146100 antibody levels

To find out the epitopes present in recombinant PF3D7_1146100, 400 µl of the protein diluted in coating buffer to a final concentration of 1.5 ug/ml was heated at 98 ^{0}C for 15 minutes. A Dynex 4HBX immunolon ELISA plate was coated with 50 µl of heat-treated and native PF3D7_1146100 in each of the 12 wells. To determine anti-PF3D7_1146100 antibody levels in sera, 50 µl of the antigen diluted in coating buffer as above, was used to coat each well of the Dynex 4HBX immunolon ELISA plates. After incubating the plates for five hours at 4 ^{0}C, they were washed as described in section 3.3.8.1 above and blocked using 5% skimmed milk in wash buffer for 3 hours at 37 ^{0}C. To the ELISA plate coated with heat-treated and native PF3D7_1146100, malaria immunoglobulins (MIG) and naïve sera were diluted at 1:200 in blocking buffer and 100 µl of MIG, naïve sera and blocking buffer added to the wells respectively. For the PF3D7_1146100 ELISA, 100 µl of serum samples at a 1:200 dilution in blocking buffer was added to every well. The plates were incubated overnight at 4 ^{0}C after which they were washed and rabbit polyclonal anti-human IgG at a volume of 100 µl added per well. The secondary antibody was diluted 1:5000 in blocking buffer. The plates were incubated again at 37 ^{0}C for 2 hours. Following incubation and washing, 100 µl of OPD substrate was added to each well and incubated for 17 minutes in the dark, at room temperature. To stop this reaction, 25 µl of 2 M sulphuric acid was used and readings taken using the ELISA plate reader at 492 nm.

3.3.9 Statistical analysis

Statistical analysis was done in R, version 3.5.2 (2018-12-20) using R statistical packages: ggplot2, ggbeeswarm, ggpubr and ggiraphExtra (Kassambara, 2019; Team, 2018; Moon, 2018; Clarke and Sherrill-Mix, 2017). Non-parametric statistical tests were adopted since antibody responses for the cohort of children did not follow a normal distribution. Wilcoxon rank-sum test was explored in assessing the statistical

significance of the differences in median anti-PF3D7_1146100 and anti-schizont antibody responses between malaria positive and malaria negative individuals. The difference in median anti- PF3D7_1146100 antibody responses in the respective age categories and in the tertile responses (low, medium and high antibody responses), were subjected to Kruskal-Wallis test. Tertile variables were categorized in to three equal groups based on OD values. The difference between individual sample ODs and the mean plus 3 SD of malaria naïve sera OD, was used to determine the seropositivity for each sample.

Because malaria outcome is binary in nature, logistic regression model was used in determining the correlation between anti- PF3D7_1146100 antibodies and protection from clinical malaria after 6 months of follow up. The logistic regression models described here were built using the forward method selection, with malaria positivity as the response variable. Tertile level, age of participant at recruitment and schizont OD were the predictor variables and *p-value* was used in determining the statistical significance of the association with protection.

Model 1, independent variable: Tertile (Low v/s Medium)

In this univariate model, low v/s medium tertile was the explanatory variable which described the influence of being in the low or medium tertile on malaria positivity.

Model 2, independent variable: Tertile (Medium v/s High)

This univariate model assessed the impact of medium v/s high tertile on malaria positivity.

Model 3, independent variable: Tertile (Low v/s High)

In this model, low v/s high tertile was the predictor variable. Whether belonging to the low or high tertile had any impact on malaria positivity, was determined using this model.

Model 4, independent variables: Tertile (Low v/s High), Age of a participant

The effect of belonging to the low or high tertile on malaria positivity after adjusting for age was described in this model. Antibody acquisition is affected by age. Age is therefore a confounder.

Model 5, independent variables: Tertile (Low v/s High), Age of a participant, Exposure (Schizont OD)

In this model, the influence of being in the low or medium tertile on malaria positivity after adjusting for age and exposure of the participant to *P. falciparum* was explored. Antibody responses to the schizont stage of the parasite represented exposure. Exposure to the parasite impacts antibody acquisition against a *P. falciparum* antigen.

CHAPTER FOUR: RESULTS

4.1 Construct design

The first 19 amino acids (as highlighted in yellow on the sequence) made up the predicted signal peptide, and the cleavage site was between the 19th and the 20th amino acid. The sequence did not have any transmembrane domains as predicted by the TMHMM server. Two sites where N-glycosylation could take place were predicted as shown by the amino acids highlighted in red in the output below. As this post-translation modification is not common in *P. falciparum*, the threonine and serine residues were substituted with alanine to inhibit protein glycosylation.

Name: Sequence Length: 175

MKCLIFCFSIFLLLTLSLCSTHKEQKYCNSTHRGILKYHKKKDNNEYVTVSA
ILKNNALELFKGSKFFEKFSLYDIITPIVILSTDVECLTLNFVNDDSVILCCDT
EESINNWWLYLTKQILCLHKGELRNENDNKTLEEEQNNIINNNNLNEVSINI
TEDDLDNIPNVLIKTES

A sequence output showing Asn-Xaa-Ser/Thr (N-X-S/T) sequons which are highlighted in blue and the Asparagines (N) highlighted in red were predicted to be N-glycosylated. Part of the sequence highlighted in yellow is the predicted signal peptide with the cleavage site between the 19th and 20th amino acid.

4.2 Successful sub-cloning, expression and characterization of PF3D7_1146100 antigen

The GOI was successfully excised from the GeneArt vector after plasmid isolation and restriction digestion using NheI and BamHI enzymes. After gel purification, ligation and colony screening, PF3D7_1146100 was cloned successfully in to the pTT28 expression vector as indicated by the confirmatory digest (Figure 4.1). The GOI, PF3D7_1146100,

and expression vector pTT28 were of the expected fragment sizes (468 bp and 4445 bp respectively) as shown in Figure 4.1.

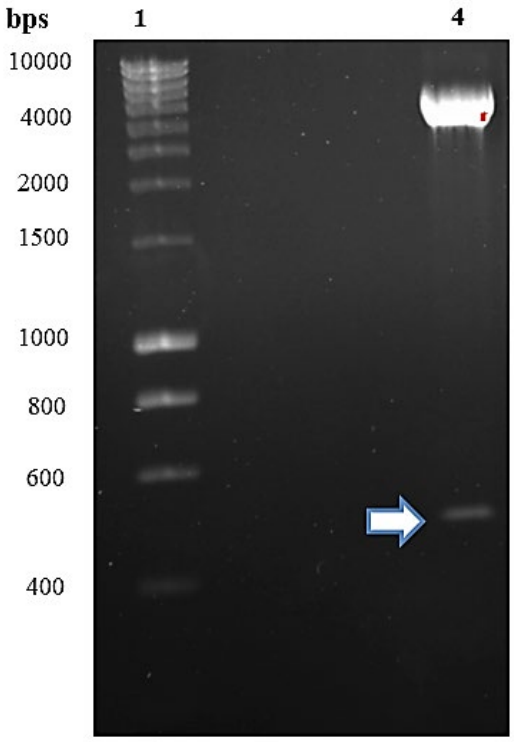


Figure 4.1: A confirmatory digest of the successfully cloned PF3D7_1146100 gene (indicated by the arrow) in a pTT28 expression vector.

Plasmids isolated from TOP 10 *E. coli* transformed cells were transfected into Expi 293F cells. The purified protein was characterized by SDS PAGE, where a band ranging within the predicted molecular weight (20.3 kDa) was observed as shown in Figure 4.2 A. To confirm that the band on the SDS PAGE was PF3D7_1146100, the protein was transferred to PVDF membrane by Western blot. Anti-his antibodies recognized the his-tag on PF3D7_1146100 as depicted in Figure 4.2 B.

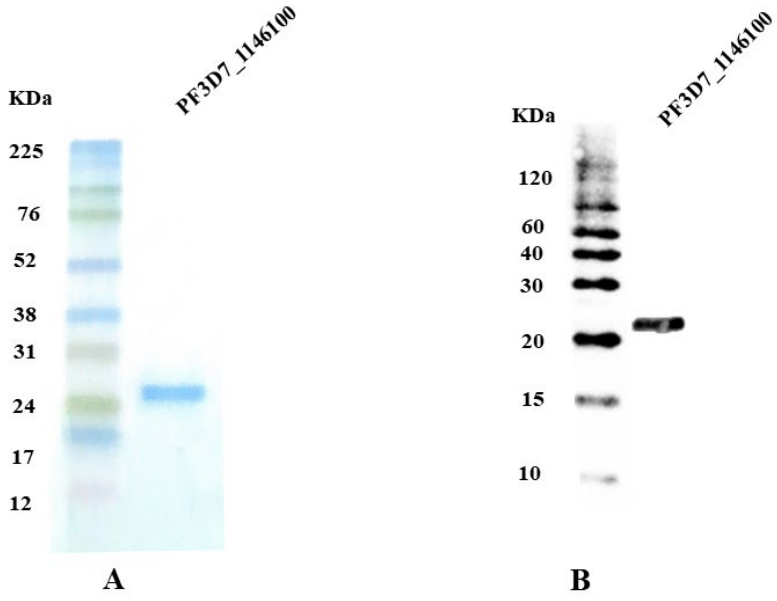


Figure 4.2: Characterization of the purified recombinant PF3D7_1146100($\approx \boldsymbol{20.3kDa}$) protein as indicated on SDS PAGE (A) and western blot (B) images.

4.3 Concentration of recombinant PF37_1146100 required for an optimal reaction with immune sera

PHIS optical density (OD) readings were higher compared to those of the naïve sera in the serially diluted PF3D7_1146100 protein (from a concentration of 48 µg/ml to 0.01 µg/ml). PHIS OD readings increased with the increase in protein concentration up to1.5 µg/ml, after which increasing protein concentration resulted in a negligible increase in OD. This concentration (1.5 µg/ml at an OD value of 1.277) was then taken as the coating concentration as shown in the titration curve (Figure 4.3). The curve was generated by plotting concentration in µg/ml against ODs of the naïve and PHIS sera. In the naïve sera, the optical density readings did not increase with increased protein concentration. PHIS contain antibodies against *P. falciparum* antigens, which have been

acquired naturally. The increase in OD readings of PHIS showed that the antibodies present recognised PF3D7_1146100, indicating the immunogenicity of the protein.

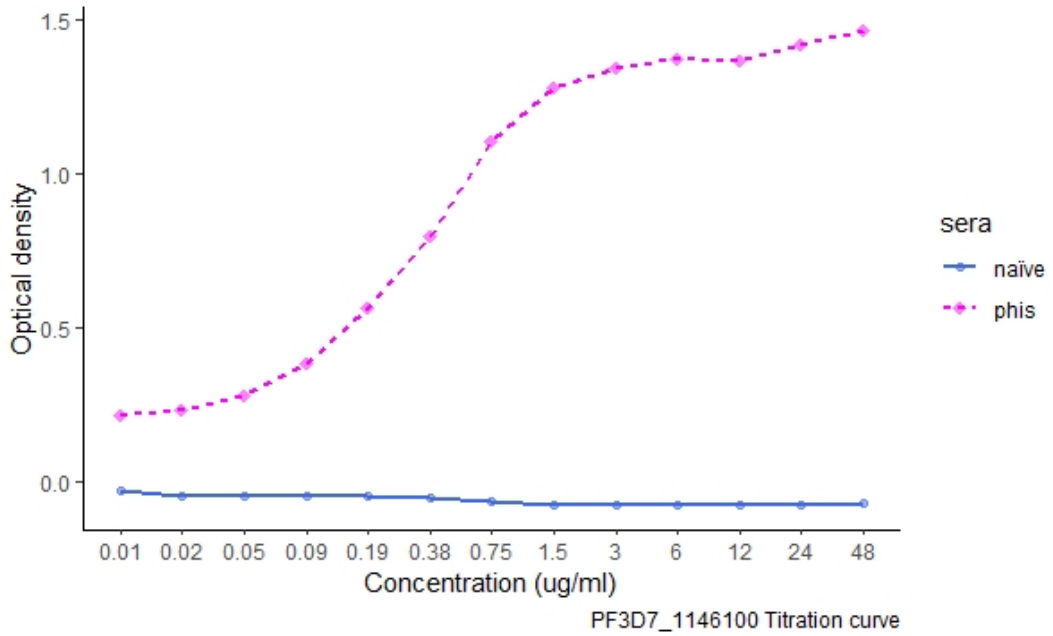


Figure 4.3: A titration curve of PF3D7_1146100 protein**.**
A curve of optical density against concentration of PF3D7_1146100 protein to determine the concentration of the protein optimally recognized by antibodies from malaria immune adults (PHIS).The magenta and royal blue curves represent the OD readings of PHIS and naïve sera respectively at the serial dilutions of PF3D7_1146100.

4.4 The epitopes present in recombinant PF3D7_1146100 antigen

Properly folded proteins are able to illicit an immune response (Birkholtz et al., 2008) as antibodies do bind to their epitopes on the protein which could either be conformational or linear. The epitopes present were determined in both the native and heat-treated protein. Malaria immunoglobulins (MIG) and naïve sera were used as a source of antibodies that could bind to the epitopes present in both the heat-treated and the native proteins. The optical densities for the blank (blocking buffer) and naïve sera were

similar in both the heat-treated and the native samples (Figure 4.4). A difference in optical densities was observed with MIG. The OD readings in the native protein were slightly higher than those of the heat-treated sample. This showed that the predominant epitopes in PF3D7_1146100 could be linear.

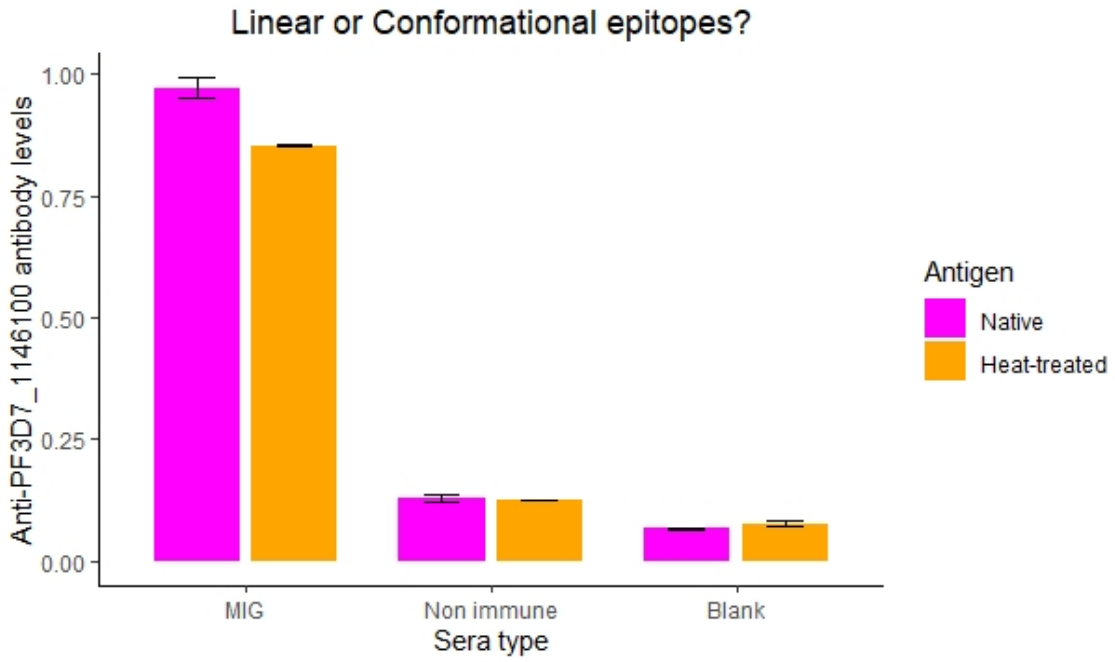


Figure 4.4: Antibody levels against native (magenta bars) and heat-treated (orange bars) PF3D7_1146100 antigen**.**

The slight decrease in the response of MIG to the heat-treated compared to the native antigen indicates the possible presence of linear epitopes. No response was observed with non-immune sera and blocking buffer (blank).

4.5 Sero-epidemiological information on the Junju 2014 cohort of children

In this cohort, 343 individuals were recruited but only 329 were included in statistical analysis because the rest lacked complete epidemiological data. Of the 329, 48.9% (161/329) were female. The mean age in years of the participants was 8.04 at an IQR of (4-11). Number of episodes summed up to 595 while the mean parasitemia at the bleed was 3607 parasites/µl of blood. The prevalence of anti-PF3D7_1146100 as defined by

seropositivity was 34.04%. The positivity cut-off was determined using sera from 26 malaria naïve individuals from the UK where Mean +3SD was an OD of 0.542. Samples with OD value greater than 0.542 were seropositive. With this cut-off, only 112 out of 329 individuals were anti-PF3D7-1146100 positive. Table 1 shows the characteristics of the cohort.

Table1: Characteristics of the Junju 2014 cohort as obtained from their epidemiological data.

Characteristics	0-3 years (n=64)	4-6 years (n=64)	7-9 years (n=55)	10-12 years (n=88)	>12 years (n=58)
Mean age in years at enrollment	2.36	5.06	7.96	10.93	13.28
Gender(female %)	35(54.7)	26(40.6)	28(50.9)	47(53.4)	25(43.1)
Mean parasitemia/µl of blood	52.5	12,383	433.5	4,018	289.1
Individuals with at least one malaria episode	48	53	37	64	21
Cumulative cases	126	151	112	159	47
Anti-PF3D7_1146100 antibody prevalence(%)	9(14.1)	21(32.8)	22(40)	37(42.1)	23(39.7)
Exposure(schizont)(%)	25(39.1)	44(68.8)	38(69.1)	74(84.1)	50(86.2)

4.6 Presence of naturally acquired antibodies against recombinant PF3D7_1146100 antigen

Naturally acquired anti- PF3D7_1146100 antibodies were observed in this cohort. Compared to the pooled hyper immune sera (PHIS) and the purified immunoglobulin from adults immune to malaria, anti- PF3D7_1146100 antibody levels were lower in the experimental cohort (Figure 4.5). However, a difference between anti- PF3D7_1146100 antibody levels in the children cohort compared to the controls (naïve sera and blanks) was observed confirming the presence of these antibodies within the population.

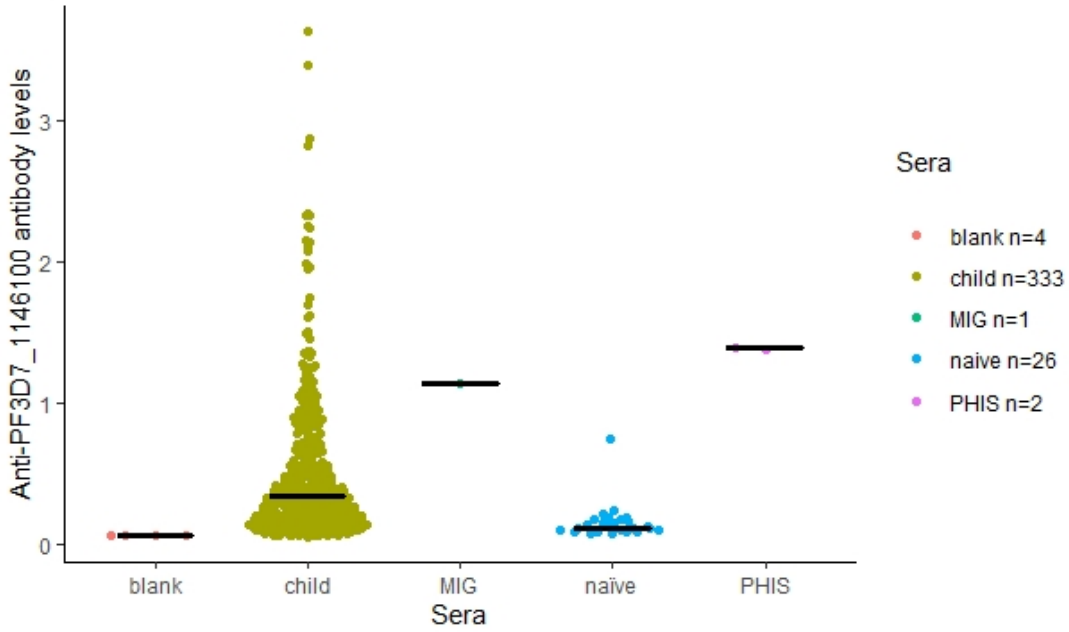


Figure 4.5: Interaction of antibodies from the children cohort sera with the recombinant PF3D7_1146100 antigen.
Malaria immunoglobulin (MIG) and PHIS were positive controls, while sera from individuals naïve to malaria and blank wells (wells in which neither sera nor malaria immunoglobulin were added to sample buffer) were the negative controls.

4.7 Naturally acquired anti-PF3D7_1146100 antibodies' variation with age

Anti- PF3D7_1146100 antibodies were observed to increase with age (Figure 4.6). Anti-PF3D7_1146100 antibody levels were lower in the 0-3 years category and higher in the 10-12 years category. The anti- PF3D7_1146100 antibody levels in the five age categories was compared using Kruskal-Wallis test. The differences in the age category were found to be statistically significant (p=0.00063).

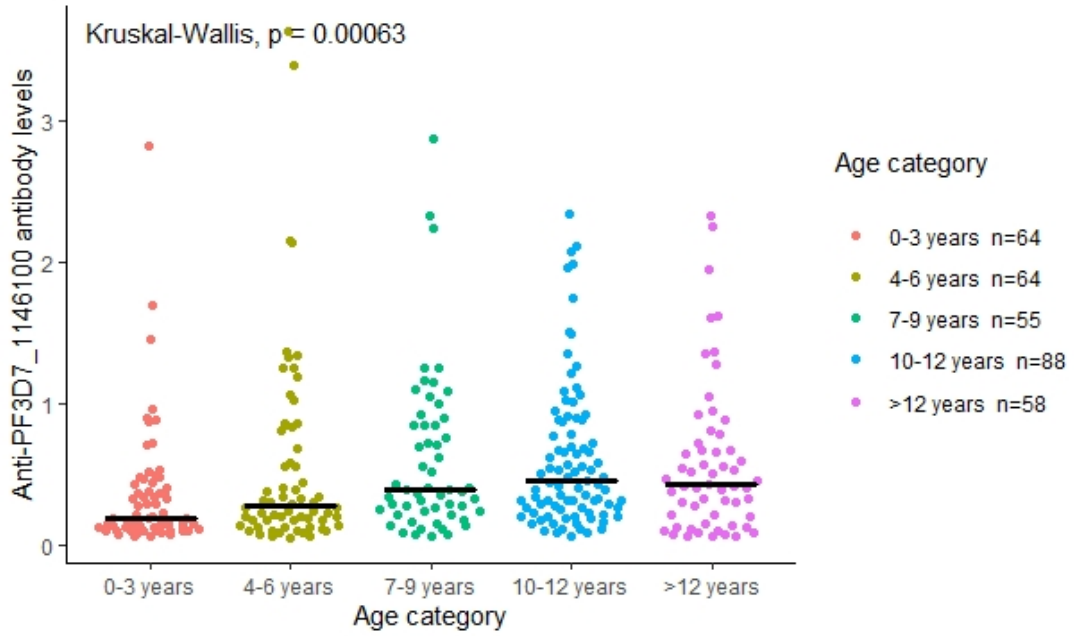


Figure 4.6: Antibodies against the recombinant PF3D7_1146100 antigen increased with age. The ages of children ranged from 1 - 14 years and were divided into 5 categories. The antibody levels varied in the age categories.

4.8 Anti-PF3D7_1146100 antibody responses and malaria outcome

Individuals who did not have malaria throughout the follow up period were observed to have high anti-PF3D7_1146100 antibody levels. Low levels were however observed in malaria positive individuals (Figure 4.7). The Wilcoxon rank sum test showed that this observed outcome was statistically significant (p=0.0097). This prompted the evaluation of whether these antibodies were correlated with protection from malaria.

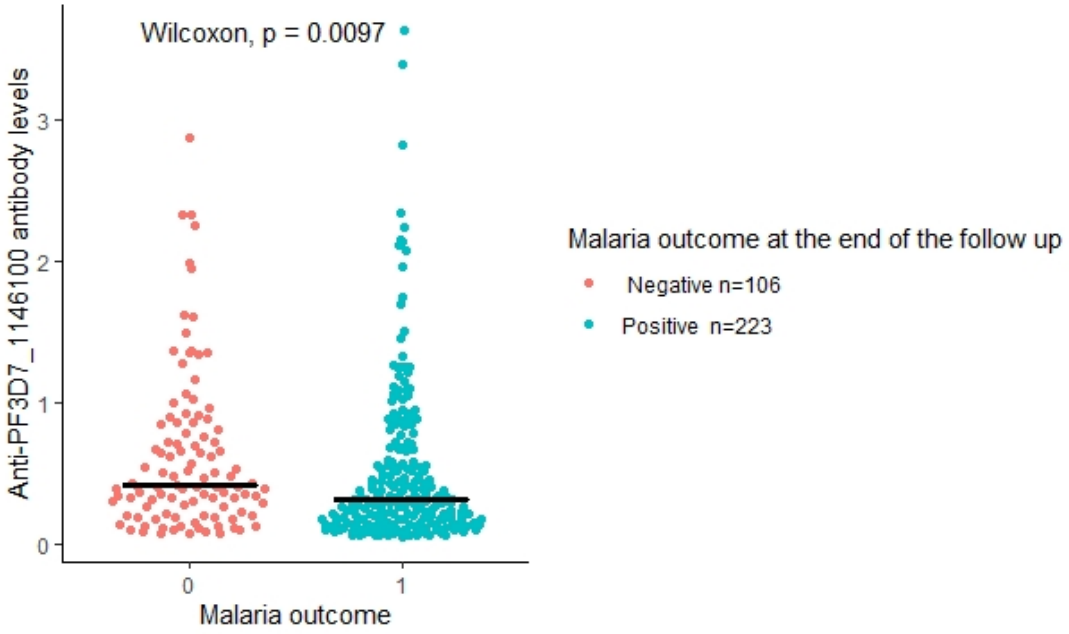


Figure 4.7: Variation in anti-PF3D7_1146100 antibody levels between malaria negative individuals (0) and malaria positive individuals (1).
Anti-PF3D7_1146100 antibody levels were higher in children who were malaria free during the follow up.

4.9 Malaria outcome and levels of anti-schizont antibodies

Individuals who were malaria free at the end of the follow-up period were found to have higher level of antibodies against the schizont extract in comparison to individuals who had at least a single malaria episode (Figure 4.8). The median difference was however slightly statistically significant as shown by the p-value (p=0.064) obtained using the Wilcoxon rank sum test.

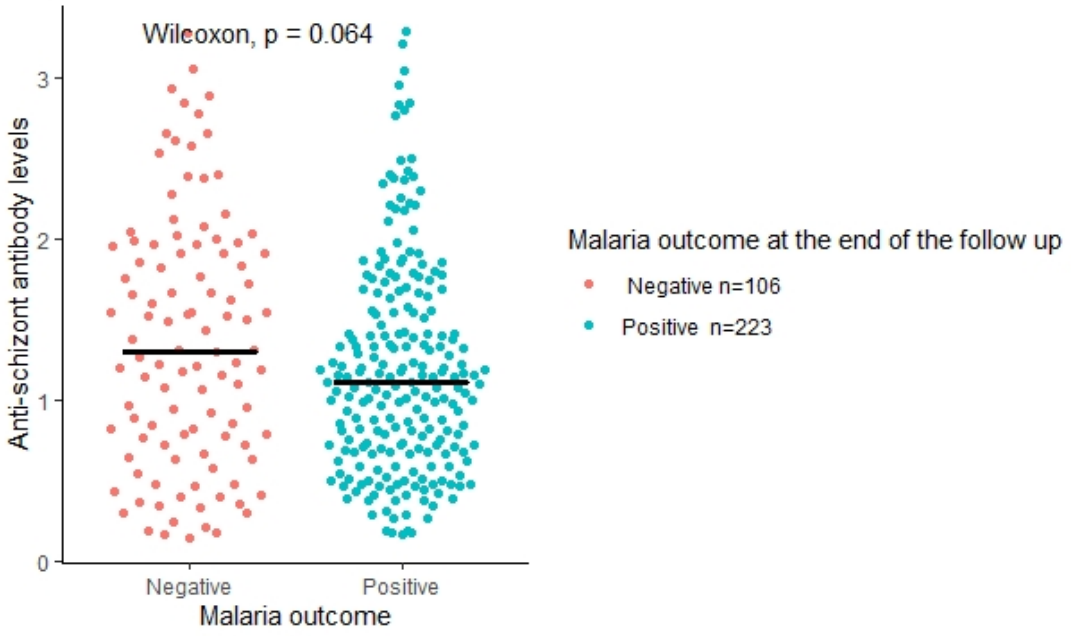


Figure 4.8: Anti-schizont antibody among malaria positive and disease free individuals. Variation in anti-schizont antibodies between malaria positive and malaria negative individuals.

4.10 Anti-PF3D7_1146100 antibodies' association with protection against clinical malaria

Evaluation of the correlation between anti-disease immunity and presence of anti-PF3D7_1146100 antibodies, necessitated the classification of the participants into low, medium and high responders, in accordance with their antibody levels. Tertile was the name of the variable with low, medium and high antibody levels (Figure 4.9).

When the effect of being low, medium and high responders to the count of malaria clinical episodes an individual had in the course of the follow up was assessed, low responders had the highest number of episodes compared to both medium and high responders. However, in the medium and high responders the difference in episode numbers was insignificant (Figure 4.10).

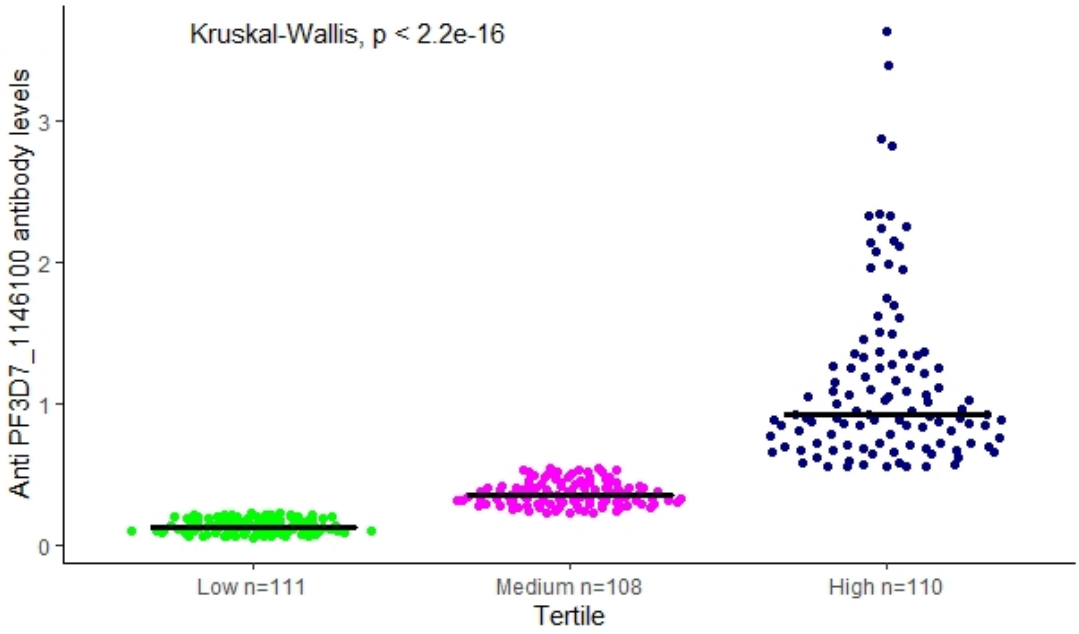


Figure 4.9: Classification of participants into the low, medium and high tertile based on their anti-PF3D7_1146100 antibody levels.

The low tertile was made up of 111 individuals, while the medium and high tertile had 108 and 110 participants respectively. The median differences in the categories was statistically significant ($p<2.2e-16$) through Kruskal-Wallis test.

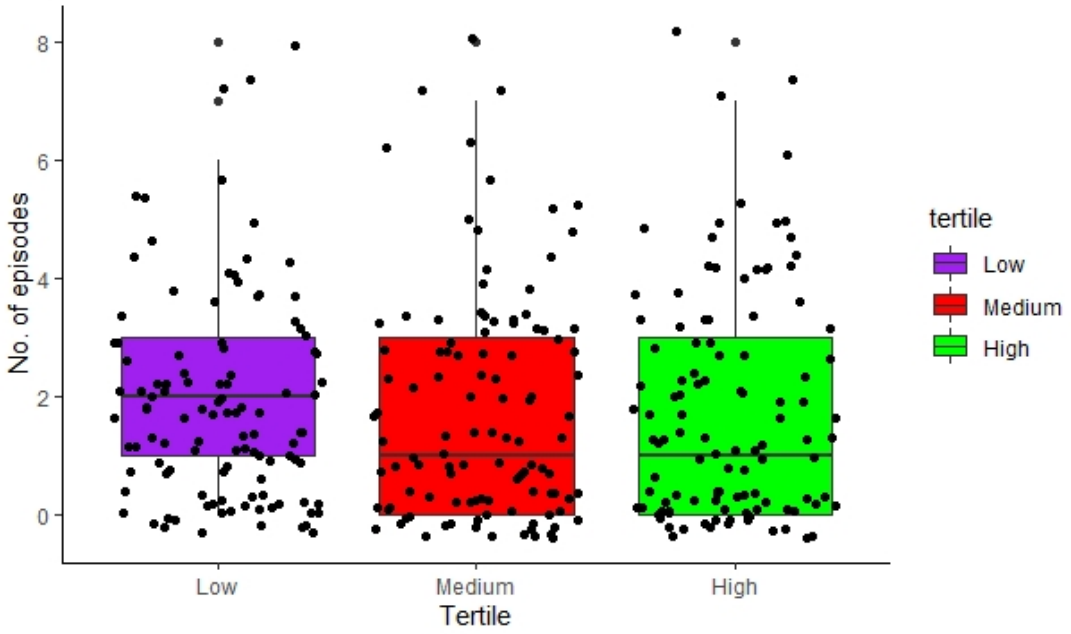


Figure 4.10: Influence of tertile classification on the count of malaria clinical episodes an individual had throughout the follow up.
The low tertile was characterized by many episodes compared to both the medium and high tertile.

In the first model, the obtained odds of being malaria positive at the end of the follow up was 0.711(0.47-1.07) in the low versus medium responders at 95% confidence interval. However, this was a chance occurrence as indicated by the p-value (p=0.107). As such, further analysis for this group was not carried out. For the medium verses high model, the obtained odds ratio was 0.89 and within 0.60-1.31 range at 95% confidence interval and with a p-value of 0.547. Again, being statistically insignificant, this group was dropped. Modelling the low verses high responders and malaria positivity yielded an odds ratio of 0.63(0.42-0.95) at 95% confidence interval. The obtained p-value was 0.0272 indicating that this association was statistically significant and therefore further analysis was done.

When correlating antibody responses against a *P. falciparum* antigen to anti-disease immunity, age and exposure are usually confounders (Doolan et al., 2009). After adjusting the model for age, the odds of being malaria positive was 0.73 in the presence of anti-PF3D7_1146100 antibodies at a 95% CI of 0.48-1.12. This was however not statistically significant as shown by the p-value (p=0.146) indicating that age was a major contributor to this outcome (p-value =0.003). Adjusting further for cumulative exposure using schizont OD values, the odds ratio was still 0.73 (0.44-1.16) at 95% CI. Still, this was not statistically significant as the p-value obtained from the model was 0.176. The p-value obtained for cumulative exposure (p=0.89), showed it had a negligible effect in the model (Table 2).

Table 2: A Logistic regression analysis summary.

Model 1= Low v/s Medium, Model 2=Medium v/s High, Model 3= Low v/s High, Model 4= Model 3 adjusted for age and Model 5= Model 4 adjusted for exposure. Adjusting for age is shown by [a] and exposure by [b].*shows that the p-value is statistically significant ($p < 0.05$)

Model	Intercept	β coeff	Odds ratio	p-value	95% CI
Model 1	0.893	-0.342	0.71	0.107	0.47-1.07
Model 2	0.567	-0.120	0.89	0.547	0.60-1.31
Model 3	**0.808**	**-0.462**	**0.63**	**0.027***	**0.42-0.95**
Model 4	1.806	-0.316	0.73[a]	0.146	(0.48-1.12)[a]
Model 5	1.767	-0.333	0.73[a,b]	0.176	(0.44-1.16)[a, b]

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Evidence from passive transfer studies (Cohen et al., 1961; McGregor, 1964) give an implication that antibodies could be targeting conserved antigens, essential for parasite's growth or survival. Most of these antigens are yet to be identified. There are a number of studies that have applied reverse vaccinology to identify novel antigens. In most cases, presence of signal peptides and transmembrane domains, *P. falciparum* export signals and subcellular localisation has been the main criteria for antigen selection. This is because humoral immune responses have been shown to target antigens found on the surface of or secreted by the parasite. Using this strategy, there is a likelihood of discovering antigens that are conserved, highly polymorphic or have antigenic variation. These variations and polymorphisms observed in blood stage antigens have been a bottleneck in malaria vaccine development. Most of the erythrocytic stage vaccine candidates induce a strain specific immune response shown by the clinical trial results (Takala and Plowe, 2009; Thera et al., 2011; Bailey et al., 2020) preventing them from advancing into malaria vaccines.

In this study, a unique approach in antigen discovery was exploited. Reverse vaccinology was applied on functional genomics data of genes shown to be essential for *P. falciparum* survival through saturation mutagenesis. PF3D7_1146100 was among the antigens discovered using this strategy. It is a conserved protein whose function in *P. falciparum* is yet to be established. This gene is 1432 bp long with introns that when spliced, a predicted mRNA sequence of 528 bp is obtained. Its translated product is 175 amino acids long with the first 19 amino acids constituting a signal peptide (Figure 3.3) hence its selection as a probable target for antibodies against *P. falciparum.*

Studies on heterologous expression of *P. falciparum* antigens have implicated the presence of parasite's signal peptides, transmembrane domains, transit peptides and PEXEL in the target antigen, in slowing down their expression and secretion (Aguiar et

al., 2004; Mehlin et al., 2006; Vedadi et al., 2007). For this reason, the PF3D7_1146100 signal peptide was cleaved. The mouse IgG kappa signal peptide present on the pTT28 vector was used instead. Crosnier and colleagues showed that using a mouse IgG kappa signal peptide increased the expression of RH 5 approximately 3.5 fold in HEK 293 cells, in comparison to using the native *P. falciparum* peptide (Crosnier et al., 2013). This confirmed findings of Reymond and colleagues in which the signal peptide of *Dictyostelium* was required for expression of CSP in *Dictyostelium* (Reymond et al., 1995).

The high AT content and frequency of arginine and lysine repeats in *P. falciparum* antigens has been reported to impact negatively on their recombinant protein production (Aguiar et al., 2004; Mehlin et al., 2006; Vedadi et al., 2007). This is due to the incompatibility in codon preference between *P. falciparum* and the non-natural host. As a result, the most preferred and frequently used tRNAs during translation of *P. falciparum* proteins, abundant in the parasite, are mostly rare in heterologous expression systems (Yadava and Ockenhouse, 2003; Flick et al., 2004; Birkholtz et al., 2008). The depletion of these limited tRNAs during protein translation, results in truncated proteins due to ribosomal unit drop-off from the mRNA sequence (Flick et al., 2004). Codon optimization increases the GC content and improves levels of the rare tRNA in the host, enhancing protein expression, solubility and correct folding (Narum et al., 2001).

On this basis, PF3D7_1146100 was codon optimized for expression in mammalian cells (Expi 293 F cells). Although Crosnier et al. (2013) reported that codon optimization did not on its own improve heterologous expression of RH 5, Narum et al. (2001)'s findings were >4 fold increase in the expression of $MSP1_{42}$ and EBA 175 region II of codon optimized genes expressed in mouse cells compared to the native ones. Improved expression levels of codon optimized EBA 175 F2 domain in *E. coli* and *P. pastoris* was also reported by Yadava and Ockenhouse (2003). Before Crosnier et al. (2013), Flick et al. (2004) had reported the same expression levels of codon optimized and native (GST-

Duffy Binding Like α domain of Erythrocyte Membrane Protein 1) GST-DBL α in *E.coli* cells.

A recombinant form of an antigen that is soluble and correctly folded is important when studying its role in naturally acquired immunity to clinical malaria. Thus, PF3D7_1146100 was successfully cloned and expressed in Expi 293 F cells, a mammalian expression system. The choice of this expression system was informed by previous studies in which HEK 293 cells were exploited in the production of recombinant *P. falciparum* antigens in their soluble and native structure (Zenonos et al., 2014; Crosnier et al., 2013).

Mammalian cells have a secretory pathway in which secreted and trans-membrane proteins are translated in an oxidizing environment enabling the formation of disulphide bonds between cysteine residues in the amino acid sequence, and other covalent bonds which result in protein folding (Wright, 2009). They also provide post translational modifications that are vital in protein localisation, solubility and antigenicity. As shown in Figures 4.2, the purified protein was resolved in SDS PAGE followed by transfer to PVDF membrane by western blotting, however, the bands obtained had a higher molecular weight than the predicted (20.3 KDa). This is possibly due to post translational modifications involving o-linked glycosylation as well as protein phosphorylation in the serine, threonine and tyrosine residues (Storey et al., 1998; Giersing et al., 2005). This possibility however should be validated by phosphorylation and glycan detection assays. In another study, a similar observation on the difference between the predicted and the experimental molecular mass of LSA3 antigen was made, and was attributed to the protein's physiological properties as it was expressed in a wheat germ cell free system (WGCF) (Morita et al., 2017).

The recognition of PF3D7_1146100 by pooled hyper immune sera from malaria immune adults showed that this antigen is immunogenic. Its immunoreactivity facilitated the

determination of coating concentration to be 1.5 µg/ml probably due to the fact that it is relatively conserved. This is in line with other studies that have established the coating concentration of antigens that are less polymorphic to be high (Kamuyu et al., 2018; Patel et al., 2013; Reiling et al., 2010). Antigens that are less polymorphic are under low immune pressure; as a result, a lot of the antigen is required to mount an immune response. It is also likely that the location of the antigen on the merozoite could have an impact on the amount of antibodies generated against it.

Formation of a protein complex that helps the parasite to invade the host RBCs or involvement in a signalling pathway could also result in low immunoreactivity. This could be due to the position of the antigen in the complex or in the signalling cascade, as some epitopes could be masked by the proteins it is associated with. This needs to be validated as the localisation of PF3D7_1146100 is still unknown however; it has a PH-domain like, a characteristic of protein kinase family. Another explanation is its relatively modest expression during the seven time points of asexual intra-erythrocytic development cycle (IDC) in comparison to other antigens (Otto et al., 2010), despite its accessibility for transcription throughout IDC (Toenhake et al., 2018).

The conformation of a protein is a key determinant factor for its interaction with antibodies (Stowers et al., 2001; Yadava and Ockenhouse, 2003; Birkholtz et al., 2008; Fernandez-Robledo and Vasta, 2010) and other antigens (Wright, 2009). As such, a protein in its native or correctly folded structure interacts effectively with other proteins and in case of antibodies, conformational epitopes are well displayed. PF3D7_1146100 could be having linear epitopes as indicated by the slight change in the OD values of MIG to the heat-treated and the native protein. This could be due to the low percentage of cysteine residues present in the antigen (3.2%). The oxidation of thiol groups of cysteine residues in an amino acid sequence, results in the rapid formation of disulphide bonds making a protein to fold and display epitopes or regions for forming protein complexes (Wright, 2009; Festa et al., 2013). This approach was preferred as *P.*

falciparum antigens that could be interacting with PF3D7_1146100 are still unknown, an area that can be further explored.

Prospective cohorts provide a platform for assessing the longevity of antibody responses in subjects to an antigen(s) and their role in protection against clinical malaria (Marsh, 1992; Perraut et al., 2003). In this case, participants constituted children aged 1 to 14 years from a malaria endemic area. Children are most affected as they succumb to clinical malaria and therefore identifying targets that will induce a protective immune response for prioritization as vaccine candidates, necessitates using such a cohort. The seroprevalence of anti-PF3D7_1146100 was 34.04%. The conserved nature of this protein, its modest expression, essentiality and accessibility to the immune system could be responsible for the low seroprevalence. This is in line with other studies that have reported low seroprevalence for conserved antigens in comparison to other polymorphic antigens (Douglas et al., 2011; Kamuyu et al., 2018; Patel et al., 2013). Antibody levels to PF3D7_114600 in the children were lower in comparison to PHIS and MIG, possibly due to the difference in levels of exposure and the maturity of the acquired immune system.

Anti-PF3D7_1146100 antibodies were observed to increase with age, their concentration being lowest in the 0-3-year age category and highest in the 10-12-year category. This is in agreement with studies done on longitudinal cohorts of children (Chelimo et al., 2005; Akpogheneta et al., 2008; Dodoo et al., 2008; Nebie et al., 2008a; Nebie et al., 2008b; Pinkevych et al., 2012; Cherif et al., 2017; Murungi et al., 2013; Seck et al., 2020; Kyei-Baafour et al., 2021) . Long lived antibodies are acquired through repeated exposure to *P. falciparum* and host related factors like the extent to which the acquired immune system is developed (Baird, 1995; Cherif et al., 2017) . According to Yman et al. (2019), the half-life of antibodies secreted by both long-lived and short-lived antibody secreting cells was approximately 1.8 to 3.7 years and 10-16 days respectively in a cohort of

travellers in the absence of re-exposure. In another study, long-lived antibodies were shown to persist for 5-10 years (Akpogheneta et al., 2008).

Exposure to *P. falciparum* resulted in production of short-lived antibodies accompanied by low levels of long-lived antibodies which were boosted by repeated exposure. The low levels of antibodies in the 0-3 year category observed in this study can be attributed to the low *P. falciparum* exposure, probably due to having very few cases of infection (Yman et al., 2019). Another reason could be due to the poorly developed acquired immune system as the development of long-lived plasma cells increases with age (Baird, 1995). Hence, as the immune system develops, boosting of long-lived plasma cells takes place resulting in their increased secretion (Akpogheneta et al., 2008). Participants were bled prior to the onset of a malaria transmission season enabling the assessment of long-lived antibodies that persisted in the absence of exposure during the dry season. Consequently, long-lived anti-PF3D7_1146100 antibodies followed the age-dependent pattern in their acquisition indicating that they were malaria specific.

In assessing the level of antibodies acquired and malaria outcome, individuals who had no malaria at the end of six months follow-up had high anti-PF3D7_1146100 antibodies. This was an indication of a possible relationship between anti-PF3D7_1146100 antibodies and onset of the disease. The same was observed for antibodies against schizont extract, possibly portraying the role of cumulative exposure to acquisition of antibodies, which could have a protective effect against clinical malaria and the overall status of the asexual blood stage immunity (McCallum et al., 2008).

Focussing on the possibility of protective effect of anti-PF3D7_1146100 antibody levels, in a univariate logistic regression analysis, anti-PF3D7_1146100 antibodies were significantly associated with anti-disease immunity in the high responders compared to low responders. However, after adjusting for age and exposure, the association was not significant. A previous study carried out by Murungi et al. (2013) in the same area

showed that antibody concentration in the cohort was significantly low to all the antigens evaluated (Murungi et al., 2013). They attributed their observation to Junju being an area of intermediate malaria transmission based on the parasite prevalence rate of children aged 2-10 years ($PfPR_{2-10}$) which was 29%. A lower parasite prevalence rate was obtained from this study (14%) indicating a reduction in transmission in Junju, which could possibly be a better explanation for the lack of significant association of anti-PF3D7_1146100 antibodies with protection against clinical malaria. Since the protective effect of antibodies to their specific antigens against malaria must meet a certain threshold (Murungi et al., 2013), this study shows that for the threshold of anti-PF3D7_1146100 antibodies to be met, there should be repeated exposure coupled with a well-developed acquired immune system. Using a cohort of children from a high transmission area and adult cohorts could best explain this association (Baird, 1995; Yman et al., 2019).

Other studies were unable to find the correlation between antibodies against a specific antigen to malaria positivity; however, some of their IgG subclasses (especially cytophilic IgG1 and IgG3) were significantly associated with protection from malaria (Nebie et al., 2008a; Cherif et al., 2017; Metzger et al., 2003). The same could be done for this antigen as our study did not assay for IgG subclasses of anti-PF3D7_1146100 antibodies. Naturally acquired antibodies against cysteine rich protective antigen (CyRPA), a protein that is part of the CyRPA-Ripr-RH5 complex vital for RBC invasion by the parasite, have been found not to be significantly associated with protection; however, their vaccine-induced antibodies were able to significantly prevent parasites growth *in vitro* (Healer et al., 2019). For PF3D7_1146100, this could also lay a basis for the evaluation of its vaccine induced antibodies against parasite growth inhibition having in mind that this antigen is vital for *P. falciparum* survival and is moderately expressed in all the stages of the blood stage cycle.

5.2 Conclusion

The combination of functional genomics and reverse vaccinology provides a unique platform for identifying novel essential *P. falciparum* antigens that could be targeted by antibodies, as evidenced by this study. Furthermore, this is the first study to show that expression of PF3D7_1146100 in a heterologous system is possible. The evaluation of the association between antibodies against this protein and protection from clinical malaria is being reported for the first time here using a prospective cohort of children between the ages of 1-14 years.

The findings of this study suggest that the longevity of antibodies against a conserved antigen is acquired at a very slow rate and is therefore age dependent. There is need for validation of these findings in other malaria endemic settings and cohorts. The essentiality and immunoreactivity of this antigen should not be ignored. This is despite the fact that its naturally acquired antibodies lack significant association in protection against clinical malaria in a multivariate analysis. It could be a possible vaccine candidate when evaluated in functional assays like growth inhibition assay (GIA), opsonic phagocytosis assay (OPA), antibody dependent cellular cytotoxicity (ADCC) among others.

5.3 Recommendations

The findings of this study present numerous knowledge gaps that could be important to look into.

- PF3D7_1146100 is a conserved protein whose function and subcellular localization are unknown. Establishing its role and localization in the parasite could help inform its application in malaria eradication and other parasitic diseases. Gene knock out studies could help inform its function in *P. falciparum* and other Apicomplexa organisms.

- This antigen should also be studied using a prospective cohort of children from a high malaria transmission intensity setting.
- Cohorts of adults from both High and intermediate transmission settings should also be exploited due to their fully developed naturally acquired immunity.
- Establishing a protective threshold of antibodies to this antigen could standardize its evaluation in the association to protection against clinical malaria in areas of different malaria endemicity.
- PF3D7_1146100 is immunogenic, the growth inhibitory activity of antibodies to it, induced by natural infection can be evaluated to determine its prioritization as a vaccine candidate.
- Growth inhibitory function of anti- PF3D7_1146100 antibodies raised in animal models could help inform the presence of other epitopes other than those accessible to NAI.
- Mapping of PF3D7_1146100 epitopes could help in the generation of synthetic peptides.
- It is also important to assess NAI of this antigen in field isolates.
- PF3D7_1146100 transcriptomic data shows that it is also expressed in gametocyte stages and ookinete. Evaluating this antigen in the context of transmission blocking immunity is key, as it could be a potential multistage vaccine candidate.
- Modelling the 3-Dimensional structure and protein crystallization of this antigen will aid in monoclonal antibody and drug targeting.
- Establishing whether PF3D7_1146100 interacts with other proteins could result in the identification of other novel antigens or shade more light on protein complexes exploited in *P. falciparum* signalling pathways.

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APPENDICES

Appendix 1: The genomic sequence of PF3D7_1146100

1432 base pairs

ATGAAGTGTTTAATTTTTTGTTTCTCCATTTTTTTACTCTTGACCCTCTCCCTC
TGCTCAACACACAAGGAGCAGAAATATTGTAACAGCACACACAGAGGGATT
TTGAAATATCATAAAAAAAAAgtcctcataaaaaataaaatatacactatatatatgtattcatatatatatatat
atatatataaatatatatgtgattattttattgtatgacaagataatatatttatatataattaacatatatttagGACAATAATG
AATATGTCACCGTATCAGCTATATTAAAAAATAATGCCTTAGAATTATTTAA
gtaaaaagttataaacataaatatagattttaaaatatctactctttctcataatatagtaatcacatatatgttatacaattatacatata
tatatatatatatatatatatatatatatagGGGATCTAAATTCTTTGAAAAATTTTCTTTATATGA
TATCATAACTCCCATTGTAATTTTATCAACAGACGTGGAATGTTTGACATTA
AACTTTGgtatgttaaagaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaggaaaaagagctagttataataaatttggta
ttatatatatatatatattttctttttttttttccttgaacagTTAATGATGATTCAGTTATACTTTGgtaaaaatt
caaaaatgtatatttgtaaattgaaggttatacactttaatttgaatttgaaatatatatttttttttttttttctttttctggaaatgtagT
TGCGATACGGAGGAATCTATAAATAATTGGgtaacaaaaaaaaaaaaaaggaaatttaaataaag
catatatatatatatatatatatatatatatatatatatgtgtatgaccttatgataactataatttttattacctccttaatcatataataaata
acaatatatatgttttatttctttatttttattatttttttttttttttacagTGGTTATATTTAACCAAACAAATTCT
TTGTCTCCATAAGGGTGAACTAAGAAACGAAAACGACAACAAAACATTGGA
AGAGGAACAGAATAACgtaaaaggaataatacgataaatgaacaaataaataaatatatatatacatatatgtata
tttatatatcttatatattgataaactatatatttttaacatctatcaaattttatagATTATAAACAATAATAATTTG
AATGAAGTTTCGATTAACATAACTGAGGATGACCTCGACAATATCCCAAACG
gttataattaataaaaaataaaaagtaaaaaaaaacaaaaggtatatatgtaaacatatacatatataaatatatatatatatatatatt
catttatttatttacttattaatttcttctttccagTCCTTATAAAGACTGAATCATAA

The highlighted bases constitute introns.

Appendix 2: The cDNA sequence of PF3D7_1146100

528 base pairs

ATGAAGTGTTTAATTTTTTGTTTCTCCATTTTTTTACTCTTGACCCTCTCCCTC
TGCTCAACACACAAGGAGCAGAAATATTGTAACAGCACACACAGAGGGATT
TTGAAATATCATAAAAAAAAAGACAATAATGAATATGTCACCGTATCAGCT
ATATTAAAAAATAATGCCTTAGAATTATTTAAGGGATCTAAATTCTTTGAAA
AATTTTCTTTATATGATATCATAACTCCCATTGTAATTTTATCAACAGACGTG
GAATGTTTGACATTAAACTTTGTTAATGATGATTCAGTTATACTTTGTTGCGA
TACGGAGGAATCTATAAATAATTGGTGGTTATATTTAACCAAACAAATTCTT
TGTCTCCATAAGGGTGAACTAAGAAACGAAAACGACAACAAAACATTGGAA
GAGGAACAGAATAACATTATAAACAATAATAATTTGAATGAAGTTTCGATT
AACATAACTGAGGATGACCTCGACAATATCCCAAACGTCCTTATAAAGACTG
AATCATAA

Appendix 3: The predicted protein sequence of PF3D7_1146100

175 amino acids

MKCLIFCFSIFLLLTLSLCSTHKEQKYCNSTHRGILKYHKKKDNNEYVTVSAILK
NNALELFKGSKFFEKFSLYDIITPIVILSTDVECLTLNFVNDDSVILCCDTEESINN
WWLYLTKQILCLHKGELRNENDNKTLEEEQNNIINNNNLNEVSINITEDDLDNIP
NVLIKTES

The predicted signal peptide made up 19 amino acids is highlighted in pink.

Appendix 4: The R code used for generation of the titration curve

#Titration curve

```
library(ggplot2)

setwd("C:/Users/Faima/Desktop/EGP 15")

titdata<-read.csv("egp15_titration.csv")

str(titdata)
```

```
titdata$conc.ug.ml.<-round(titdata$conc.ug.ml.,digits = 2)

titdata$conc.ug.ml.<-as.factor(titdata$conc.ug.ml.)

str(titdata$conc.ug.ml.)

summary(titdata$conc.ug.ml.)

titdata$od<-(titdata$od-0.1605)

variable.names(titdata)

titdata$sera<-titdata$type

str(titdata)

titdata1<-subset(titdata,type=="phis")

tidata<-titdata1[1:13,]

str(tidata)

titdata2<-titdata[23:44,]

tidata1<-titdata2[1:13,]

str(tidata1)

tiddata<-rbind(tidata,tidata1)

str(tiddata)

titdata$sera<-titdata$type

str(titdata$sera)

ggplot(tiddata,aes(conc.ug.ml.,od,group=sera))+

  geom_line(aes(color=sera,linetype=sera), size=1)+

geom_point(aes(color=sera,shape=sera),alpha=0.5,size=2.5,xlim=c(0,1.50),ylim=c(0,1.5
))+

     scale_shape_manual(values=c(20,18)) +
```

```
scale_color_manual(values=c("royal blue", "magenta"))+

labs(x="Concentration (ug/ml)",y="Optical density",caption = "EGP10 Titration curve",colour="sera")+

theme_classic()
```

Appendix 5: R code used for analysis of recombinant PF3D7_1146100 epitope data

```
#Epitope analysis

library(ggplot2)

library(ggpubr)

setwd("C:/Users/Faima/Desktop/EGP 15")

Epitopes<-read.csv("Epitope.csv")

str(Epitopes$Sera)

summary(Epitopes$Sera)

Epitopes$Sera<-factor(Epitopes$Sera, ordered = TRUE, levels = c("MIG","Non immune","Blank"))

Epitopes$Antigen<-factor(Epitopes$Antigen, ordered = TRUE, levels = c("Untreated","Heat-treated"))

e<-ggplot(data=Epitopes,aes(Sera,OD, fill= Antigen))

e+geom_bar(stat = "identity", width=0.7,position=position_dodge(width=0.8))+

  geom_errorbar(aes(ymax=OD.max, ymin=OD.min),width=0.3,

                position=position_dodge(.8), group="Sera")+theme_classic()+

  labs(x="Sera type",y="Anti-PF3D7_1146100 antibody levels",title = "Linear or Conformational epitopes?")+

  theme(plot.title = element_text(hjust = 0.5))+
```

```
scale_fill_manual(values = c("Heat-treated"="orange","Untreated"="magenta"))

theme(plot.title = element_text(hjust = 0.5))

 summary(Epitopes)
```

Appendix 6: R code used for analysis of the antibody responses for the Junju 2014 cohort of children.

```
library(ggplot2)

library(ggbeeswarm)

library(ggpubr)

library(ggiraphExtra)

setwd("C:/Users/Faima/Desktop/EGP 15")

labdata<- read.csv("egp15_2014.csv")

head(labdata)
```

Recognition of recombinant PF3D7_1146100 antigen by sera from a prospective cohort of children from Kilifi

```
chilabdata<-subset(labdata,type!="adult")

cld<-ggplot(chilabdata,aes(type,od))

cld+geom_quasirandom(aes(col=type))+

 stat_summary(fun.y= median, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.5, color="black")+

 theme_classic()+

 labs(x="Sera",y="Anti-PF3D7_1146100 antibody levels",colour="Sera\n")+

 scale_colour_hue(labels=c("blank n=4","child n=333","MIG n=1","naive n=26","PHIS n=2"))
```

#**reading in schizont data, epidata and merging of labdata and epidata to form mergeddata**

summary(labdata$type)

schizont<-read.csv("shizont_ELISA_labdata.csv")

head(schizont)

epidata<- read.csv("j2014m.csv")

head(epidata)

str(labdata)

str(epidata)

mergeddata<-merge(labdata,epidata,by="studyno")

subsetting mergeddata to childdata

childdata<-subset(mergeddata,type.x=="child")

str(childdata)

#merging schizont data and childdata to form clndata

clndata<-merge(schizont,childdata,by="studyno")

str(clndata)

str(clndata$sex)

summary(clndata$sex)

summary(clndata$agecat)

#antibody responses to PF3D7_1146100 increase with age

summary(clndata$agecat)

clndata$agecat <- as.character(clndata$agecat)

str(clndata$agecat)

```
summary(clndata$agecat)

clndata$agecat<-factor(clndata$agecat,ordered = TRUE,levels = c("0-3 years","4-6 years","7-9 years","10-12 years",">12 years"))

pagecat<-ggplot(clndata,aes(agecat,od))

pagecat+geom_quasirandom(aes(col=agecat))+

 stat_compare_means()+

 stat_summary(fun.y= median, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.65, color="black")+

 theme_classic()+

 labs(x="Age category",y="Anti-PF3D7_1146100 antibody levels",colour="Age category\n")+

 scale_colour_hue(labels = c("0-3 years  n=64", "4-6 years  n=64", "7-9 years n=55","10-12 years  n=88",">12 years  n=58"))
```

#antibody responses to schizont extract increase with age

```
agecats<-ggplot(clndata,aes(agecat,schizont))

agecats+geom_quasirandom(aes(col=agecat))+

 stat_compare_means()+

 stat_summary(fun.y= median, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.65, color="black")+

 theme_classic()+

 labs(x="Age category",y="Anti-schizont antibody levels",colour="Age category\n")+

 scale_colour_hue(labels = c("0-3 years  n=64", "4-6 years  n=64", "7-9 years n=55","10-12 years  n=88",">12 years  n=58"))
```

#individuals with no malaria during the follow up had high anti-PF3D7_1146100 responses

```
str(clndata$od)

clndata$malpos<-as.factor(clndata$malpos)

clndata$malpos <- ifelse(clndata$malpos == 1, "Positive", "Negative")

str(clndata$malpos)

clndata$malpos<-as.factor(clndata$malpos)

b<-ggplot(clndata,aes(malpos,od))

b+geom_quasirandom(aes(col=malpos))+theme_classic()+

  stat_compare_means()+

  stat_summary(fun.y= median, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.63, color="black")+

  labs(x="Malaria outcome",y="Anti-PF3D7_1146100 antibody levels",colour="Malaria outcome at the end of the follow up")+

  scale_colour_hue( labels = c(" Negative n=106", "Positive  n=223"))

  theme(plot.title = element_text(hjust = 0.5))
```

#individuals with no malaria during the follow up had high anti-schizont antibody responses

```
  clndata$malpos <- ifelse(clndata$malpos == 1, "Positive", "Negative")

  str(clndata$malpos)

  clndata$malpos<-as.factor(clndata$malpos)

  schimal<-ggplot(clndata,aes(malpos,schizont))

  schimal+geom_quasirandom(aes(col=malpos))+theme_classic()+

    stat_compare_means()+

    stat_summary(fun.y= median, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.63, color="black")+
```

```
  labs(x="Malaria outcome",y="Anti-schizont antibody levels",colour="Malaria outcome at the end of the follow up")+
   scale_colour_hue( labels = c(" Negative n=106", "Positive  n=223"))
 theme(plot.title = element_text(hjust = 0.5))
```

#effect of increase in anti-PF3D7_1146100 antibodies and age on malaria outcome

```
clndata$malpos<-as.factor(clndata$malpos)
aom<-ggplot(data=clndata,aes(agecat,od, col= malpos))
aom+geom_quasirandom()+theme_classic()+
 stat_summary(fun.y=, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.5, color="black")+
 labs(x="Age category",y="Anti-PF3D7_1146100 antibody level")+
 theme(plot.title = element_text(hjust = 0.5))
```

#distribution of episode number levels in each age category

```
 clndata$episodesno<-
factor(clndata$episodes,ordered=TRUE,levels=c("8","7","6","5","4","3","2","1","0"))
clndata$agecat<-factor(clndata$agecat,ordered=TRUE,levels=c("0-3years","4-6 years","7-9 years","10-12 years",">12 years"))
str(clndata$agecat)
mce<-ggplot(data=clndata,aes(agecat,episodes, fill= episodesno))
mce+geom_bar(stat = "identity", width=0.6,position=position_dodge(width=1))+theme_classic()+
 labs(x="Age category",y="No. of episodes")+
```

```
  theme(plot.title = element_text(hjust = 0.5))
```

#Tertiles

```
cutN <- function(X , n = 4){
  cut(
    X ,
    include.lowest = TRUE ,
    breaks = quantile(
      X ,
      probs = (0:n)/n ,
      na.rm = TRUE ))}
clndata$tertile<-cutN( clndata$od, n = 3)
clndata$tertile<-ordered(clndata$tertile,labels=c("Low  n=111","Medium  n=108","High n=110"))
tertile.summary <- summary(clndata)
u<-ggplot(clndata,aes(tertile,od))
u+geom_quasirandom(aes(col=tertile),show.legend = F)+
  stat_compare_means()+
  stat_summary(fun.y= median, fun.ymin=median, fun.ymax=median, geom="crossbar", width=0.5, color="black")+
labs(x="Tertile",y="anti EGP 10 antibody levels" )+theme_classic()+
  scale_colour_manual(values = c("green", "magenta","navy blue"))
  theme(plot.title = element_text(hjust = 0.5))
str(clndata$episodes)
```

```
summary(clndata$episodes)
clndata$episodes<-as.integer(clndata$episodes)
str(clndata$tertile)
summary(clndata$tertile)
clndata$tertile<-ordered(clndata$tertile,labels=c("Low","Medium","High"))
v<-ggplot(clndata,aes(tertile,episodes))
v+geom_boxplot(aes(fill=tertile))+
geom_jitter()+theme_classic()+
labs(x="Tertile",y="No. of episodes",colour="Tertile\n" )+
  scale_fill_manual(values=c("green","magenta","navy blue"))
#logistic regression
clndata$tertile<-ordered(clndata$tertile,labels=c("Low","Medium","High"))

low<-subset(clndata,tertile=="Low")
medium<-subset(clndata,tertile=="Medium")
high<-subset(clndata,tertile=="High")
lomed<-subset(clndata,tertile!="High")

#univarites analysis
#model 1 - Low v/s Medium
summary(lomed)
modell<-glm(lomed$malpos~lomed$tertile,family = "binomial")
```

```
summary(modell)

exp(coef(modell))

confint.default(modell)

#model 2 - Medium v/s High

medhi<-subset(clndata,tertile!="Low")

summary(medhi)

modelm<-glm(medhi$malpos~medhi$tertile,family = "binomial")

summary(modelm)

exp(coef(modelm))

mean(medium$episodes)-mean(low$episodes)

confint.default(modelm)

#model 3 - High v/s Low

hilow<-subset(clndata,tertile!="Medium")

summary(hilow)

modelh<-glm(hilow$malpos~hilow$tertile,family = "binomial")

summary(modelh)

confint.default(modelh)

exp(coef(modelh))



#model 4 - Adjusting for exposure

modelh<-glm(hilow$malpos~hilow$tertile+hilow$schizont,family = "binomial")
```

```
summary(modelh)

confint.default(modelh)

exp(coef(modelh))
```

#model 5 - Adjusting for age

```
modelh<-glm(hilow$malpos~hilow$tertile+hilow$age_init+hilow$schizont,family = "binomial")

summary(modelh)

confint.default(modelh)

exp(coef(modelh))
```

Appendix 7: Logistic regression model

Logistic regression was used to describe the factors that impacted malaria outcome in a cohort of children living in a malaria endemic area. This statistical technique was chosen due to the binary nature of the response variable which was absence or presence of malaria at the end of the 6 months follow up. In statistics, logistic regression analysis is a tool exploited in describing the relationship between an independent or predictor variable and a binary dependent or response variable (presence of malaria is represented by 1 and its absence by 0). The independent variable(s) can be continuous or categorical. Logistic regression measures the effect of change of the independent variable on the dependent variable whose relationship has been made linear through logit-transformation. When describing the relationship between one predictor variable and the binary response variable, the logistic regression analysis is referred to as univariate. When adjusting for confounding effects, the analysis involves more than one predictor variable and is termed multivariate.

$\text{logit } y = \beta_0 + \beta_1 x_1$ (Univariate model)

$\text{logit } y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots\ldots \beta_n x_n$ (multivariate model)

y= dependent variable (malaria outcome) β= regression coefficient x= independent variable

The regression coefficient, β shows the degree to which a change in any of the predictor variables influences the change in the response variable. It gives an estimation of the extent of change in the response variable due to a unit change in the predictor variable (s). A larger regression coefficient is a reflection of how more a unit change in the independent variable impacts the dependent variable. A negative β coefficient value denotes an inverse relationship between the dependent variable and the independent variable and the opposite is true. The significance of the influence of the predictor variable on the response variable is shown by low p-values.

As mentioned earlier, a univariate model consists of only one predictor variable that does not fully explain its influence on the response variable. To assess the influence, the other independent variables have to be included in the model in order to reduce bias, which is strength of this statistical tool. In this multivariate model, the β coefficient value denotes the impact of the changes in the predictor variables on the response variable after adjusting for the effect of all other predictor variables in the model.

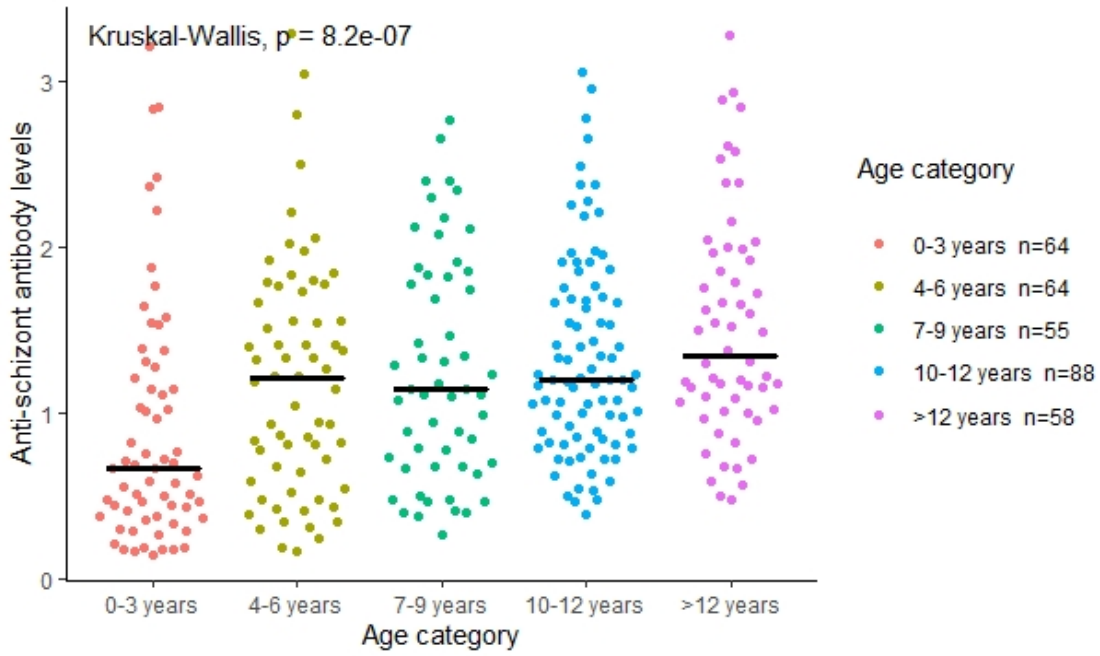
In this study, the forward selection method of building a model was explored, that is, predictor variables were added one after another and their impact on the model assessed.

Assumptions of the models

1. Independence of errors that is, separation of sample group outcomes
2. There is a linear relationship between continuous independent variables and their respective logit-transformed outcomes

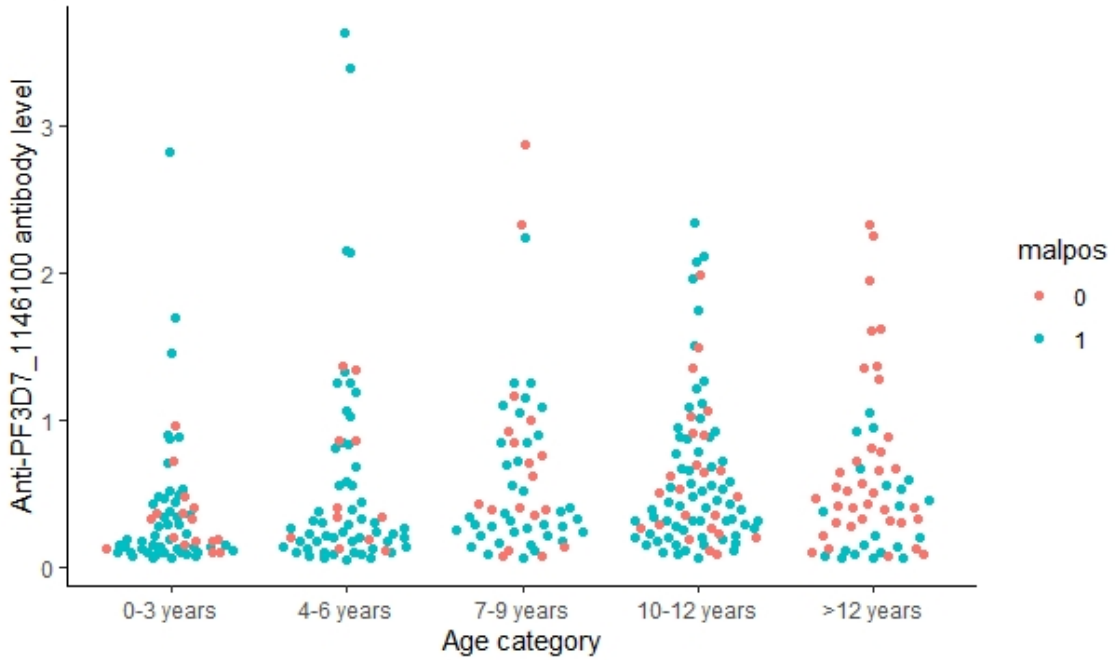
3. There is no multicollinearity or redundancy between independent variables
4. The predicted outcome differs slightly from the actual outcome of a sample member

Appendix 8: Anti-schizont antibodies increase with age



Antibodies against schizont extract are observed to increase with age with the children above 12 years recording the highest median levels in the cohort. This shows that the children in the 0-3 years were less exposed to *P. falciparum* when compared to the >12 years category.

Appendix 9: Anti-disease immunity increases with age



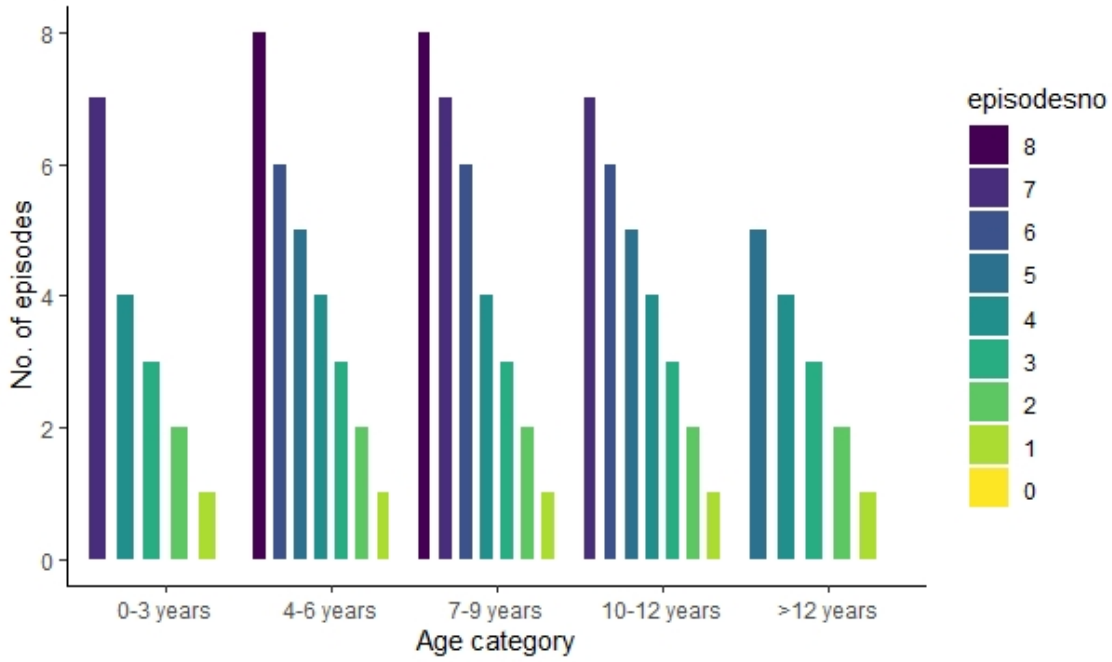
The number of malaria negative children and anti-PF3D7_1146100 antibodies are observed to increase with age. Fewer malaria negative children characterized with low anti-PF3D7_1146100 antibodies are observed in the 0-3 years category and the number increases up to the children above 12 years who have high anti-PF3D7_1146100 antibodies.

Malpos represents malaria outcome at the end of the follow up.

Red dots represent **0** in the graph indicating **malaria negative children**

Blue dots represent **1** in the graph indicating **malaria positive children**

Appendix 10: Distribution of malaria episode levels in each age category



Five levels of episode numbers are observed in the 0-3 years and >12 years age categories with the highest number of episodes being 8 and 5 respectively. This could be attributed to the low exposure to *P. falciparum* in the young children and acquisition of anti-disease immunity in the older children. The other age categories have 7 levels each with the highest number of episodes being 8 in the 4-6 years and 7-9 years age categories and 7 in the remaining category. A maturing immune system and increased P. falciparum could be the probable explanation.

Appendix 11: Ethical approval document

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **October 15, 2018**

TO: **PROF. FAITH OSIER,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGMR-C**
KILIFI.

Received: 19/10/18

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE
RESEARCH, COAST

Dear Madam,

RE: **SERU 3139 (*REQUEST FOR ANNUAL RENEWAL*): *DEFINING* THE MEROZOITE TARGETS OF PROTECTIVE IMMUNITY AGAINST PLASMODIUM FALCIPARUM MALARIA THROUGH MULTI-CENTRE COHORT STUDIES.**

Thank you for the continuing review report for the period **October 29, 2017** to **September 14, 2018.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **October 30, 2018** through to **October 29, 2019**. Please note that authorization to conduct this study will automatically expire on **October 29, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **September 17, 2019.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

ENOCK KEBENEI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT