# The expression of recombinant *Plasmodium falciparum* sexual-stage proteins and assessment of naturally acquired transmission-blocking immunity

**KIRIMA CHRISTINE KINYA**

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## DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work has been used, this has been properly acknowledged and referenced according to the University of Nairobi's requirements.

**Candidate**: Kirima Christine Kinya

Signature……………………………… Date……………………………..…

This thesis has been submitted with our approval as supervisors

Signature……………………………… Date……………………………..…
Dr David Odongo
School of Biological Sciences
University of Nairobi

Signature……………………………… Date……………………………..…
Dr Melissa Kapulu
KEMRI-Wellcome Trust Research Programme
Kilifi

Signature……………………………… Date……………………………..…
Prof Philip Bejon
KEMRI-Wellcome Trust Research Programme
Kilifi

## DEDICATION

To my ever supportive family: Mr and Mrs Kirima and my sweet sister Faith Kirima. Thank you for walking this journey with me. I am blessed to have you all in my corner!

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFIRM | Assessment of the Infectious Reservoir of Malaria |
| AMA-1 | Apical membrane antigen-1 |
| cDNA | Complementary DNA |
| DNA | Deoxyribonucleic acid |
| $H_20$ | Water |
| HEK | Human embryonic kidney |
| IDeAL | Initiative to develop African research leaders |
| IRS | Indoor residual sprays |
| KEMRI | Kenya medical research institute |
| KWTRP | Kemri-Wellcome trust research programme |
| LAMB | Longitudinal Assessment of Malaria Transmissibility |
| LLIN | Long-lasting insecticidal net |
| MSc | Master of Science |
| MSP-1 | Merozoite surface protein-1 |
| PCR | Polymerase chain reaction |
| PhD | Doctor of Philosophy |
| PgD | Postgraduate diploma |
| TBE | Tris-borate-EDTA |
| TBV | Transmission blocking vaccine |
| RNA | Ribonucleic acid |
| UV | Ultraviolet |
| WHO | World Health Organization |
| WGCFS | Wheat germ cell free system |

## ABSTRACT

Persons residing in regions endemic to malaria have been shown to develop antibodies against gametocytes, which could subsequently prevent the progression of the parasite within the mosquito vector. The acquired immune ability to affect gametocyte infectivity within the mosquito vector and in-turn block transmission of parasites to the vector is termed as transmission-blocking immunity. Various gametocyte-specific antigens of *Plasmodium falciparum* have been characterised and evaluated as candidates for a possible transmission-blocking vaccine. This study aimed to express gametocyte-specific surface antigens with the cell -free wheat germ system and mammalian HEK293 systems of expression and assess the ability of antibodies from sera of malaria-exposed persons to recognize the recombinant proteins.

The most highly expressed surface localized proteins of mature (Stage V) gametocytes according to published proteome data (Lasonder *et al.,* 2016), were amplified and expressed for immunoprofiling by ELISA. Seven gametocyte-specific surface antigens were

expressed using the WGCFS to express the proteins MDV-1, PSOP25_3D7, PSOP25_10668 and CPP4, while the mammalian cells HEK293 expression system was used to express the proteins CVMPPP, PEB-P and PSOP1. The plasma samples used in this analysis were those that were collected during two previous studies, that is, the Longitudinal assessment of malaria transmissibility (LAMB, N = 284) and the cross-sectional study, Assessment of the infectious reservoir of malaria (AFIRM = 413).

The observations presented in this thesis show the ability to not only produce recombinant gametocyte-specific proteins using various expression systems, but to produce proteins that are recognized by the sera of persons exposed to malaria. The findings show that host responses to gametocyte proteins are influenced by the participant's age, as well as the density and presence of parasites. Antibody responses among individuals older than 15-years were consistently higher for all antigens when compared the younger individuals. Gametocyte-positive individuals had higher responses ($p<0.05$) as opposed to the gametocyte-negative individuals, for all the antigens. The results also reveal significant cross-correlations between gametocyte density ($p<0.001$) and the responses to all the recombinant gametocyte antigens evaluated. Furthermore, for the AFIRM study, a model was fitted to predict the odds of one presenting with gametocytes. The following predictors were included in the logistic model: age category, gametocyte status, season, parasite density (as measured by qPCR18S, NASBA18s and NASBAPfs25), responses to AMA-1, sickle gene and α-thalassemia.

The model shows that individuals in whom parasites were detected by qPCR18s, had 1% ($p<0.05$) higher odds of being gametocyte positive compared to those in whom no parasites could be detected. On the other hand, participants who were under 5 years of age had 99% ($p<0.05$) odds of being gametocyte negative. Individuals who responded to the antigens MDV1 and AMA1(a marker for exposure to parasites), had higher odds of being gametocyte positive, in that for every one-unit increase in the mean response to the two recombinant proteins, an increase of 160% and 93% in the odds of gametocyte positivity was seen and this was statistically significant ($p < 0.05$). Participants who were sampled during the dry season had 2% ($p < 0.05$) higher odds of being gametocyte positive compared to those sampled within a malaria season, while adjusted for parasite density as measured by qPCR18s.

Antibody responses among individuals older than 15-year were consistently higher for all antigens in comparison to responses for the other two age groups. A similar trend was observed with AMA1, which serves as a marker for exposure to asexual parasites. When assessing for the role of season in influencing responses, there was no difference in responses among those samples during peak malaria season and those sampled after the malaria season had passed. This can be attributed to the maintenance of the parasite during the dry season by undetectable sub-microscopic or low-density gametocytes. An increase in responses to AMA-1, as revealed by the model, resulted in an increase in responses to recombinant proteins when adjusted for all other predictors.

The longitudinal LAMB study enabled evaluation of responses over six different time points.  A multivariable logistic regression model to predict parasitaemia, showed that one-unit increase in the mean response to AMA1, increased the odds of parasitaemia by 56% ($p<0.05$). An increase in age increased odds of being negative for parasites by 96% ($p<0.05$).

This study is unique as it offers a look at responses based on both a cross-sectional as well as longitudinal study. In future, it would be important to further add on to the observations by measuring transmission-blocking activity using of mosquito feeding assay. Moreover, understanding the mechanism underlying the recognition of gametocyte-specific antigens, and further finding a highly immunogenic target(s) will go a long way in advancing the development of a vaccine to block transmission.

# CHAPTER ONE

# 1.0 INTRODUCTION

## 1.1 General introduction

Malaria is still one of the world's leading causes of mortality and morbidity. A recent report by WHO indicated at least 219 million malaria cases in the year 2017 alone (WHO, 2018) which saw an increase from the 216 million cases in 2016 (WHO, 2017). Sub-Saharan Africa has majority of the cases; out of 435,000 deaths reported in 2017, 93% occurred in the Africa region with a majority of the deaths attributable to infection with *P. falciparum*, a parasite that is predominantly found in the tropics of Africa (WHO, 2018). In addition to numerous efforts put forth to control and prevent malaria infection, strategies towards possible elimination will go a long way in putting an end to the social, economic and health burden that results from the disease.

This study aimed to express gametocyte-specific surface antigens and to assess their recognition using sera obtained from malaria-exposed individuals. It was conducted at the Kemri-Wellcome Trust Research Programme (KWTRP) laboratories, using archived plasma samples from individuals who had been enrolled in either a cross-sectional study (from 2 years and above) or a longitudinal study (from 18 years and above) to assess mosquito infectivity using membrane-feeding assays. Recombinant proteins for the sexual-stage antigens were expressed with either the mammalian HEK293 system or the wheat germ system to enable characterisation of antibody responses to antigens presented by the parasites. The properties of the proteins that were expressed, are published in proteome data (Lasonder *et al.,* 2016) and includes their suspected role in gametocyte/gamete development, their antigenicity scores and their sizes. They include the proteins CVMPPP, PEBP, PSOP1, PSOP25_3D7, PSOP25_10668, MDV1 and CPP4.

The mammalian expression system is a highly efficient system that is able to yield large amounts of properly folded and appropriately folded recombinant proteins (Almo and Love, 2014). It has been used in producing recombinant malaria proteins. In this case mammalian Human Embryonic Kidney cells (HEK293 cells), were used for transfection to enable protein expression. The two types of HEK293 cells that were used for this study were the HEK293E cells which are derived from the original cell line and been optimized for expression with mammalian cells. The HEK293F cells that have been adapted for expression using a commercial medium.

The wheat-germ system, like the mammalian, has been used with success in the production of recombinant gametocyte surface antigens. It is a high-throughput system that produces correctly folded and functional proteins. Its ability to produce complex proteins made it an appropriate choice of protein expression for this study.

This study highlights the significance of probing further and understanding the numerous factors that are involved in developing immunity to block falciparum malaria transmission.

### 1.2 Statement of research problem

The last ten years have seen an increase in efforts to control and prevent malaria. Strategies such as mosquito control interventions and the use of anti-malarials have contributed significantly towards decreasing the clinical cases and deaths attributable to malaria but have yet to lead to the eradication and/or elimination of malaria. Furthermore, anti-malarials including ACTs are threatened by the rise of parasites resistant to drugs. Malaria vaccines, particularly those that interrupt the transmission cycle would enhance efforts aimed at malaria control and elimination.

## 1.4 Justification

Transmission-blocking vaccines (TBV) aim to prevent transmission of malaria from one individual to another and offer new hope towards eliminating malaria. To develop these vaccines, studies on the candidate antigens to be targeted in the form of sexual-stage proteins expressed by the parasites are important. Previous studies have shown evidence of gametocyte-specific antibodies capable of rendering the parasites non-infectious to their mosquito vectors. However, other studies into the lead candidate antigens for TBV development such as Pfs25 have shown them to be poorly immunogenic or as in the case of Pfs230 and Pfs48/45, their recognition does not always correlate with transmission-reducing immunity. For this reason, studies into novel immunogenic antigens which are capable of inducing functional antibody responses are required in order to find new candidate antigens to be used in the progression of a transmission blocking vaccine.

## 1.3 Objectives

### 1.3.1 General objective

To characterise antibody responses to *P. falciparum* gametocyte surface antigens and evaluate factors influencing transmission-blocking immunity.

### 1.3.2 Specific objectives

1. To express *P. falciparum* gametocyte surface antigens using a cell-free or mammalian protein expression system
2. To determine antigen-specific sero-reactivity of antibodies to gametocyte-specific surface antigens by ELISA

# CHAPTER TWO

## 2.0 LITREATURE REVIEW

### 2.1 Malaria

Malaria is a mosquito-borne disease caused by the parasite *Plasmodium*. Five species infect humans, including *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium falciparum, Plasmodium knowlesi* and *Plasmodium vivax*. Of these, infection with *P. falciparum* is termed as most serious (Gardner *et al*., 2002). Malaria inflicts a significant burden on human health, resulting in major effects of morbidity and mortality. In 2017, 219 million cases, majority of which occurred in the African region and 93% of the 435,000 deaths were reported that year, also occurred in the African region (WHO, 2018).

*Plasmodium* parasites, which were discovered by Charles Louis Alphonse (Cox, 2010), are transmitted to hosts by female *Anopheline* mosquitoes. Infection with malaria presents as prodromal symptoms that are common to all the different species of malaria. These symptoms include malaise, dizziness, lassitude, anorexia, head and back aches, myalgias, nausea and fever (Bartoloni and Zammarchi, 2012). Aside from the obvious health complications imposed by the disease, there are also social and economic burdens that result, as discussed by Sachs and Malaney (2002) including negatively impacting on worker productivity, population growth, premature mortality, and increase in medical costs incurred by families of those infected.

## 2.2 *P. falciparum* parasite

The *Plasmodium* parasite appears to have a worldwide distribution but is usually found in the tropics of Africa (Autino *et al.,* 2012). The report by Gething *et al*., 2011, indicated that by 2010 there were an average 2.57 billion people worldwide living in areas at risk of infection with *P. falciparum*, be it through stable or unstable transmission. Out of these, those that lived in regions with stable *P. falciparum* transmission were 1.44 billion, 52% of whom were in Africa. Furthermore, in 2016 alone, 91% of the 445,000 malaria-related deaths in Africa were due to infection with *P. falciparum* (WHO, 2017).

*Plasmodium* parasites are variably distributed worldwide. *P. ovale* is predominantly found in parts of Africa and Asia (Collins and Jeffery, 2005). Various species of female *Anopheles* mosquitoes that have been found to be susceptible to infection with *P. ovale* include among others, *A. gambiae*, *A. funestus*, *A. stephensi* and *A. freeborni*. *P. falciparum* is predominantly found in the tropical regions of Africa where coinfection with *P. malariae* may sometimes occur. *Plasmodium vivax* is found dominating south-eastern Asian regions as well as in South America (Autino *et al*., 2012).

*Plasmodium* parasites are transmitted to humans through being bitten by an infected female *Anopheles* mosquito. Aside from prodromal symptoms that are common upon infection with any of the *Plasmodium* parasites (Bartoloni and Zammarchi, 2012), *P. falciparum* can result in severe malaria, that is characterised by among others, cerebral malaria, renal failure, severe anaemia, hypoglycaemia, as well as death (Gomes *et al*., 2011).

### 2.2.1 The life cycle of *Plasmodium falciparum*

The life cycle of *P. falciparum* is illustrated in Figure 2.2(a) below. Human infection occurs following when a female Anopheles mosquito injects sporozoites from its salivary glands, into the host's blood. Within about 45 minutes (Bartoloni and Zammarchi, 2012) of the bite, the sporozoites move to the liver, invade hepatocytes and mature into schizonts that contain merozoites. Once schizonts rupture, they release merozoites into the bloodstream. The *P. falciparum* pre-erythrocytic liver stage lasts an average of about 5.5 days.

The merozoites then attack red blood cells, go through a sequence of asexual multiplication (erythrocytic shizogony) and develop into trophozoites. During the erythrocytic multiplication stage, that clinical manifestations begin to present (Kuehn and Pradel, 2010). The trophozoites may either mature into schizonts that rupture to release more merozoites capable of invading new red blood cells, or into schizonts containing merozoites that form gametocytes.

Gametocytes cause transmission of infection from hosts to vectors (Baker, 2010). While immature gametocytes are found within the bone marrow, mature ones are found circulating in peripheral blood thus easily accessible to the mosquito vector during a blood meal. Once taken up by the vector, each gametocyte becomes either 8 male gametes or one female macrogamete. After fertilization of the gametes in the mosquito midgut, a zygote is formed which becomes a motile ookinete motile to breach the midgut wall, forming oocysts. The oocyts enlarge and burst to release sporozoites which move to the mosquito salivary gland and are infectious to the host upon their next blood meal (Josling and Llinás, 2015).

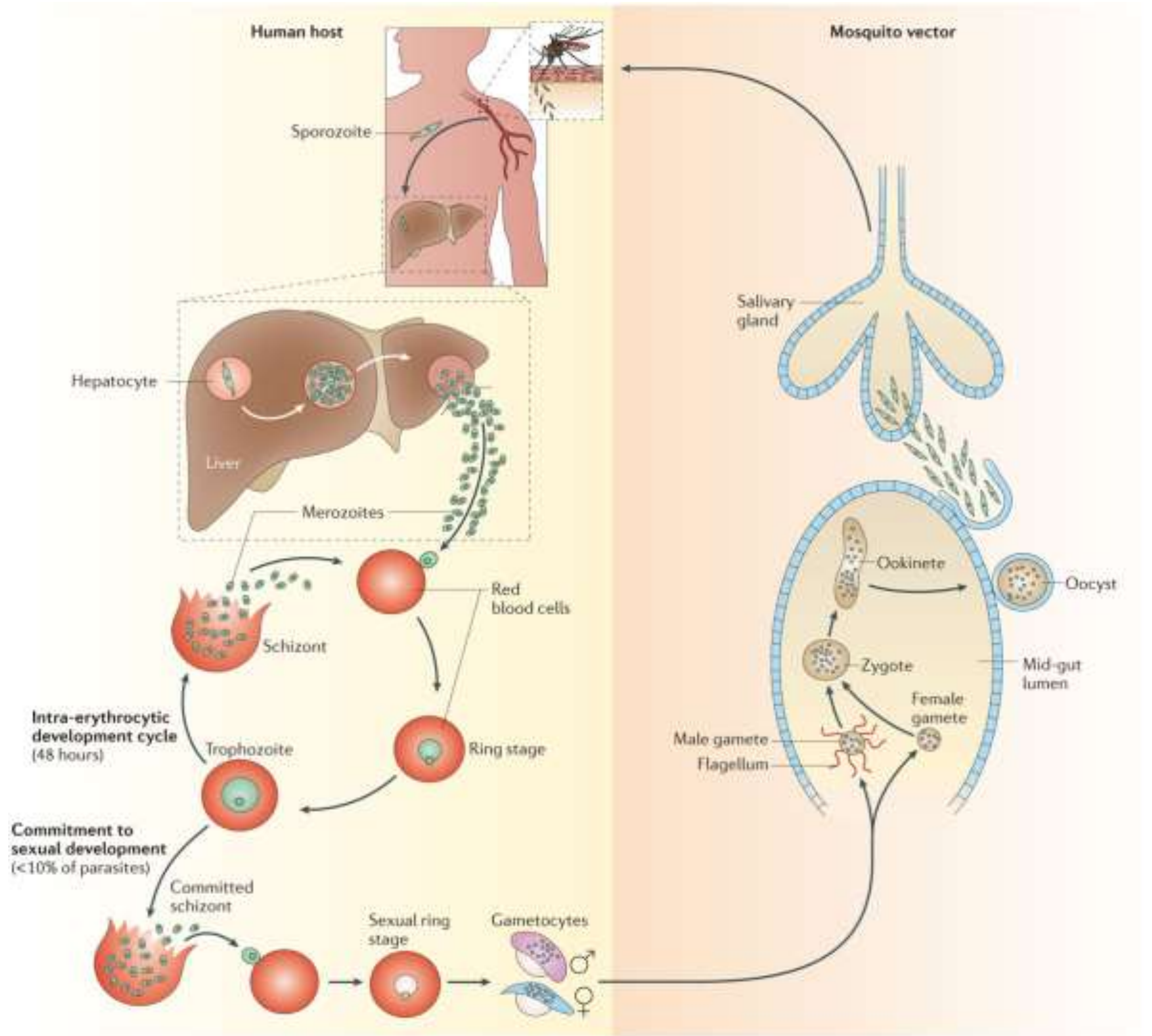



**Figure 2.2(a): Life cycle of *Plasmodium falciparum*. (Adopted from Josling and Llinás, 2015)**

## 2.3 Gametocyte stages

The banana-shaped gametocytes have a direct role in the transmission of infection from an infected host to the vector (Nacher, 2004). Their development and differentiation in red blood cells mark the initial step in the sexual phase of malaria (Kuehn and Pradel, 2010).

There are five morphologically distinct stages (Stages I to V) that they go through during formation and maturation (Bousema and Drakeley, 2011). While Stage I gametocytes may not be distinguished from their asexual trophozoite counterparts, Stage II become more distinct as they elongate and begin to form subpellicular microtubules. The ends of gametocytes in Stage III become more rounded (Josling and Llinás, 2015).

It is during stage IV that the distinction between male and female can be made, in that, while the males have a larger nucleus and a more diffused pigment, the female gametocyte nucleus is smaller and has a more concentrated pigment (Bousema and Drakeley, 2011). The process by which gametocytes develop from the asexual counterparts is termed as gametocytogenesis and takes about 7-10 days (Bousema and Drakeley, 2011).

Stage V crescent-shaped gametocytes are the mature gametocytes. Here, the females appear more curved than the males (Josling and Llinás, 2015). Gametocytes are the transmissible stage, capable of infecting the insect host (Baker, 2010). Their transmission is dependent on their presence in the peripheral circulation (Bousema *et al.,* 2011). Factors such as maturity, density, as well as human host and mosquito immune system influence the infectiousness of gametocytes (Bousema *et al.,* 2006). Targeting gametocytes would assist in the malaria elimination efforts by eliminating the asymptomatic and low-density gametocyte reservoirs that provide mosquitoes with sources of acquiring infection (Bousema *et al*., 2011).

## 2.4 Malaria elimination strategy

Numerous strategies to prevent and control malaria have been put in place (Gachelin *et al.*, 2018, Hemingway *et al.*, 2016) with many of them accompanied by challenges, that limit their effectiveness (Kokwaro, 2009, Guyant *et al.*, 2015). There is a need for interventions that would not only prevent and control malaria, but would also contribute to its eradication. With looming challenges such as the emergence of drug resistant *P. falciparum* parasites, and evidence that a sub-microscopic reservoir of gametocytes can indeed maintain malaria transmission even in areas of low endemicity (Karl *et al.,* 2011), innovative ways to curb infections, such as the use of vaccines, have become imperative.

Recent studies into the development of vaccines against malaria are focusing on targeting proteins expressed by the parasite sexual stage, in a bid to curb further transmission of the parasite from hosts to an uninfected mosquito, thus altering the life cycle of the parasite. Efforts put forth by the international community towards the control, prevention and elimination of *P. falciparum* infection, have made great strides over the years although much remains to be done. The death rates associated with *P. falciparum* infection still remain significantly high (WHO, 2017). Challenges such as the emergence of ACT resistance

of *P. falciparum* in Southeast Asia (Lover *et al.,* 2016), may set back all the strides made thus far towards the control of the infection.

### 2.4.1 Vector control strategies

Several measures to control mosquitoes for the control and prevention of malaria have been employed over the years, as reviewed in Gachelin *et al.,* 2018. The control of mosquito vector populations has been cited as the reason for success in areas where malaria has been eradicated (Karunamoorthi, 2011).

These strategies include the use of bed nets (LLINs) and the application of intradomicile residual insecticides or indoor spraying (Childs *et al.,* 2016), both of which target the feeding behaviour and reduce the lifespan of the vector thereby reducing transmission (Shiff, 2002). Other strategies include targeting the larval stages of the mosquito vector such as using larvivorous fish and biological (bacteria and fungi) agents which are applied to mosquito breeding sites (Ramirez *et al.,* 2009). While success has been reported using these measures, none are able to directly contribute to already circulating active asexual and gametocyte infections in host populations.

### 2.4.2 Anti-malarial drugs

Drug treatment against malaria has plays a role in controlling infections with malaria (Greenwood, 2010), especially in areas of low transmission (White, 2008). This is because drugs are able to minimize the incidence and prevalence of malaria. Limitations such as the rise of drug-resistant parasites (Blasco *et al*., 2017), curb the effectiveness of these drugs. Moreover, gametocytes are less susceptible to anti-malarial drugs with only a few agents being able to clear this transmissible stage of the parasite (reviewed in, White *et al*., 2014). One such agent is primaquine which effectively kills gametocytes (Graves *et al*., 2018) but whose use is contraindicated in people with deficiencies such as glucose-6-phosphate dehydrogenase (Recht *et al.,* 2018) and methaemoglobin reductase which are common in malaria endemic regions (Carter *et al.,* 2011).

Integrated efforts that combine anti-malarial drugs as well as IRS and LLINs have further reduced the disease burden (Shiff, 2002). Alternative approaches that could supplement existing strategies by preventing transmission, would assist in decreasing the reservoir of parasites for the vector, thus further reducing the burden of disease.

### 2.4.3 Vaccines for malaria elimination

Recently, attention to the advancement of a vaccine for malaria has been renewed worldwide, with groups such as the Malaria Vaccine Roadmap, funded by WHO setting an ambitious goal of having a licensed vaccine with 80% protection by the year 2020 (*A Research Agenda for Malaria Eradication: Vaccines, 2011*). There is need for interventions that would not only prevent and control malaria, but would also contribute to its eradication. One such intervention is in the form of vaccines. Currently, there is no malaria vaccine with most of the vaccine candidates at various stages of testing (Coelho *et al.*, 2017).

Malaria vaccines are grouped according to targets in the parasite life cycle (Greenwood and Targett, 2011). The first group of vaccines are the pre-erythrocytic that aim at invasive sporozoites that are inoculated into the human host via the bite of a mosquito. The number of parasites a mosquito inoculates presents a bottleneck (Sinden, 2010), hence the logic behind these vaccines.

RTS, S/AS01 is currently the most advanced malaria vaccine. It is based on the surface antigen dominantly expressed on sporozoites, has faced numerous challenges on the safety and logistics front (Ballou, 2009), as well as challenges on its receptivity among the public, on it and concerns surrounding the associated side effects (Dimala *et al.*, 2018). Upon its development, RTS, S was considered one of the most efficacious malaria vaccines. However, in children (5-17 months), the vaccine did show protective effects against severe malaria and a vaccine boost increased its efficacy but in infants, it did not seem to provide any significant protection against severe malaria even after administering a booster dose (Bejon *et al.,* 2011). Research into a more effective vaccine and understanding the factors influencing the development of responses to malaria antigens is required.

Another group of vaccines are the blood-stage, targeting the parasite's erythrocytic stage leading to a reduction in parasite densities as well as preventing the advancement of the infection to a severe form of disease (Osier *et al.,* 2008). Examples of such vaccines are those based on the MSP-1 as well as the AMA 1 (Longley *et al*., 2015). Specific antibodies against these antigens present on infected RBCs and merozoites, prevent the invasion and infection of new RBCs (Bull *et al*., 1998).

The other group of vaccines are those that interfere with malaria transmission (VIMT) (Alonso *et al*., 2011), also referred to as transmission blocking vaccines (TBVs). The antigens presented by sexual stages of the parasite, elicit immune responses that block fertilization in the mosquito midgut when the parasite is ingested by the mosquito. TBV candidate antigens include P25 and P28 that are ookinete antigens (Saul, 2007).

Antibodies against these antigens interrupt the ookinete maturation and the formation of oocysts (Tomas, 2001). An alternative approach is to target gametocytes within the human host that would render them non-infectious to mosquitoes. A vaccine combining TBVs with blood-stage and/or pre-erythrocytic antigens would serve to alleviate clinical malaria as well as prevent transmission (Sauerwein, 2007).

## 2.5 Immunity to malaria

Persons who are repeatedly exposed to malaria, tend to develop resistance to clinical disease over time and this is termed as naturally acquired immunity (Langhorne *et al.*, 2008). During a malaria episode, naturally acquired immunity has been found to be capable of reducing the density of asexual parasites and by extension affecting the production of gametocytes, (Meibalan and Marti, 2016). In endemic areas, reduction in both the rates of disease as well as parasitization with increase in age is an indicative sign of immunity to infection (Langhorne *et al.*, 2008). Malaria immunity develops slowly and is never complete as evidenced by asymptomatic carriers, usually among adults in endemic regions (Doolan *et al.*, 2009).

### 2.5.1 Transmission Blocking Immunity

Evidence of transmission blocking immunity (TBI) stems from studies showing that individuals infected with malaria can develop antibodies which when taken up by a mosquito together with gametocytes, can block the successful progression of the parasite within the insect host (Sutherland, 2009, Bousema *et al.,* 2011, Gebru *et al.,* 2017).

Malaria transmission depends on the presence of mature gametocytes in the blood of the human host (Bousema *et al*., 2011). Factors such as gametocyte sex ratio, gametocyte density and prevalence, influence the potential of transmission and infectiousness of gametocytes (Bousema and Drakeley, 2011). In the human host, cellular and humoral immune responses elicited upon infection are capable of targeting gametocytes at their various stages of development including during formation or maturation (Girard *et al*., 2007).

During a mosquito blood meal components of host immune system, including components of the complement system, cytokines as well as sexual-stage specific antibodies are taken up (Arévalo-Herrera *et al*., 2011), all of which impede the progression of the parasite within the insect vector. Of these, antibodies have been widely studied with reference to transmission blocking immunity and shown to recognize specific antigenic epitopes on gametocyte infected erythrocytes (Stone *et al*., 2016), as well as antigens such as Pfs 48/45 and Pfs230 that are initially expressed by gametocytes and only appear on the surface of gametes upon ingestion by the mosquito vector (Carter *et al*., 2000).

Antibodies specific to gametocytes as well as factors such as host and mosquito factors, influence transmission blocking immunity (Arévalo-Herrera *et al*., 2011). Human immune responses elicited upon infection with *P. falciparum*, target amongst others, antigens presented on the surface of red blood cells infected with gametocyte. The human immune responses elicited by sexual antigens impact human to mosquito to human transmission, by inhibiting the growth of the parasite in the mosquito (Targett and Greenwood, 2008).

The vector ingests these antibodies during blood feeding, which are can interfere with the parasite within the mosquito, rendering the parasite non-infectious to humans (Bousema *et al*., 2010). There are also antibodies that are against the asexual stage of the parasite which according to Ouedraogo *et al*., (2011), can also indirectly impact on gametocyte carriage by decreasing the number of asexual parasites that go on to become gametocytes. These immune responses are attributed to protection presented by natural immunity against infection with *falciparum* parasites in malaria prevalent areas (Ouedraogo *et al*., 2011). A study done in Tanzania by Bousema *et al*., (2010) focusing on the antigens Pfs48/45 and Pfs230, and showed that the concentration as well as the prevalence of the antibodies to these antigens played a major role in reducing transmission.

The sexual stage immune responses among the investigated cohort, increased with their age and were related with duration of gametocyte exposure. This study also reported high prevalence of antibodies in adults and suggested that the finding may be due to an overall higher exposure to gametocytes (Bousema *et al*., 2010).

### 2.6 Sexual stage antigens as targets for transmission-blocking immunity

Immunity in humans serves to decrease infectivity of the parasite to the vector. Upon the mosquito ingesting a blood meal, it takes in gametocytes, which are responsible for transmission, as well as immune factors that are present in the blood. These immune factors are capable of blocking further development of the gamete, zygote and ookinete stages (Boudin *et al*., 2005).

Transmission-blocking immunity was first demonstrated by Carter and Chen (1976), using a *P. gallinaceum*-chicken model, in which chickens were vaccinated with gametes and infected with the parasite. The models were able to inhibit parasite infectivity to mosquitoes by inducing immune responses that significantly reduced oocyst development (Carter and Chen, 1976). In the human host, immune responses elicited following infection with

*P. falciparum* are capable of targeting gametocytes at the various stages of development including during formation or maturation. Further, these antibodies can recognize specific epitopes presented on the surface of erythrocytes infected with gametocytes (Stone *et al*., 2016).

Some of the antigens on the surface of gametocytes include Pfg27 and Pfs16, which are some of the earliest sexual stage antigens to be expressed; and Pfs230 and Pfs48/45 proteins that are expressed by gametocytes and gametes (Kongkasuriyachai and Kumar, 2002). Each of these antigens plays a different role in the various parasite stages. Gene-knockout studies have revealed that these proteins are involved in parasite development and in some cases, a loss of infectivity to mosquitoes has been demonstrated (Tomas, 2001).

For example, Pfs48/45 has been found to play a role in fertilization of gametes, where the knock out parasite was shown to develop normally into gametocytes and gametes but there was a reduction in zygote formation resulting from the impaired ability of the male gametes to penetrate the female gametes (Tomas, 2001).

Sexual stage antigens can either be pre- or post-fertilization antigens and some may be involved in the development of the parasite. The pre-fertilization proteins are expressed on gametocytes and gametes. They include Pfs48/45, Pfs47 and Pfs230 while the post-fertilization proteins are present on the stages found in the mosquito vector, such as Pfs25 (Singh *et al*., 2016) and HAP2 that have a role in gamete fertilization.

### 2.6.1 Pfs48/45 and Pfs230

There is confirmation that individuals living in malaria endemic regions have transmission blocking activity (TBA) induced by antibodies against gametocyte-infected red blood cells (Kapulu *et al.,* 2015). Pfs230 and Pfs48/45 are pre-fertilization antigens that contribute to the formation of exflagellation centres that are important for male microgamete fertility for Pfs230 (Eksi *et al*., 2006) and Pfs48/45 in fertilisation of macrogametes (van Dijk *et al*., 2001). Both these proteins have been studied to determine their efficacy as TBV candidates.

One such study, Bompard *et al*., 2017, showed blocking efficacy of antibodies raised in mice against region C-terminal end of Pfs230 and concluded that transmission blocking activity (TBA) in this case was dependent on the level of exposure to the parasite as well as the titres of antibodies raised. Pfs48/45, which also plays a role in the development of macrogametes was shown to exhibit TBA by inducing antibodies that inhibit zygote formation in the mosquito vector (Roeffen *et al*., 1996).

### 2.6.2 HAP2

HAP2 is another pre-fertilisation protein, whose function has been shown to be essential in the fusion of gametes (Blagborough and Sinden, 2009). Transmission of *P. falciparum* was reported to decline when a critical site of the protein was targeted with species-specific antibodies (Angrisano *et al*., 2017). Furthermore, *in vitro* studies with antibodies against HAP2 conducted by Blagborough and Sinden (2009), reported a reduction of up to 81% in the formation of ookinetes, making it a viable candidate antigen for vaccine development.

## 2.7 Limitations of TBVs and Transmission blocking immunity

In Kilifi county, where this study took take place, there has been a variation in malaria transmission over the last 25 years. This variation as reported by Mogeni *et al.,* (2016) indicates that while there was a period of marked decrease of malaria transmission between years 2003 and 2008, there was a "post-decline" period between the years 2009-2014, which saw an increased number of older children who were susceptible to malaria.

The study concluded that a possible explanation for this finding was the previously low levels of transmission, thus older children who were not exposed to infection became more susceptible to malaria. The increasing proportion of malaria susceptible individuals poses a great risk and is an indicator that health costs would be much higher should malaria rebound in the area. Alternative interventions such as TBVs would play a role in reducing the intensity of transmission and thereby contributing to malaria control. The prospect posed by the development of a TBV are quite promising but there are many challenges hindering its advancement.

One such challenge is the fact that antibody titres to TBV antigens have been shown to be much lower than antibodies against the asexual blood-stage. Antibody titres have also been shown to depend on recent gametocytes exposure, and therefore are short-lived (Bousema *et al*., 2007). Another limitation is in the production of recombinant sexual-stage proteins, particularly those whose ability to elicit functional antibodies is dependent on their confirmation (Kapulu *et al*., 2015). Different protein recombinant expression systems have been used to attempt the production of conformational *P. falciparum* proteins, including prokaryotic systems such as *E.coli* (Guerra *et al.,* 2016) as well as a eukaryotic systems such as the cell-free wheat germ system (Tsuboi *et al*., 2008).

Moreover, given that most TBVs would not be able to induce complete TBA (Zheng *et al.*, 2016) and offer no direct benefit to the individual to whom it is administered (Arama and Troye-Blomberg, 2014), this then poses a limitation to their use and hence their development. So far, the gametocyte-specific antigens studied have not been able to elucidate fully the impact of transmission-blocking immunity. For instance, there are studies that have reported the presence of antibody responses that are gametocyte-specific even in the absence of Pfs48/45 and Pfs230 (Stone *et al.*, 2018). This could therefore indicate that there are more antigenic targets whose identification and characterisation, could improve our understanding on the dynamics of TBI.

# CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Study area and background

This study was conducted within the precepts of the KWTRP. This thesis made use of data and samples collected from two studies that were a cross-sectional survey and a longitudinal cohort study. The cross-sectional study, assessment of the infectious reservoir of malaria (AFIRM) (Gonçalves *et al*., 2017) was carried out between 2013 and 2014, and involved the measurement of malaria transmissibility in children and adults in the south of Kilifi County. This study described the prevalence of gametocytaemia, in a cross-section of the population (N = 413) as well as determined the frequency of transmission of malaria using mosquito membrane feeding and molecular assays. Participants were included in the study irrespective of asymptomatic parasite carriage status and were grouped into three age categories, less than 5-year olds, 5 to 15 year olds and those above 15 years of age. Individuals were enrolled and sampled during both dry and wet seasons.

The longitudinal cohort study, longitudinal assessment of malaria transmissibility (LAMB) study [Kapulu *et al.,* unpublished] was conducted from 2014-2015 and included participants 18 years and older residing in the south of Kilifi County. In this longitudinal study, participants were followed up for 4 months and samples collected at various time points (Days 0, 14, 28, 56, 84 and 112), from each individual. The study aimed to assess the infectiousness of asymptomatic infected individuals and estimate the duration of infectiousness over the study period.

This thesis study produced sexual-stage antigens as full-length recombinant proteins by use of the wheat germ (Kapulu *et al.*, 2015) and mammalian protein expression systems (Nikolaeva *et al.,* 2017) and characterised immune responses to these antigens using samples collected from the two studies described. Immune responses to gametocyte antigens were assessed in a subset of plasma samples collected from participants of Kilifi County who took part in the AFIRM and LAMB study and whose blood samples were tested for mosquito infectivity by membrane feeding assays. All the 413 and 284 plasma samples from the AFIRM and LAMB study respectively were used in this study.

### 3.2. Identification of gametocyte-specific antigens

So as to identify highly expressed surface localised proteins of mature (stage V) gametocytes, the published proteome data (Lasonder *et al.*, 2016) was used. Expression values of known TBV antigens served as the basis for selecting antigens that are highly expressed in stage V of gametocytes. The PlasmoDB, GeneDB, Tropical Diseases Research (TDR) Targets Database and the rodent malaria genetically modified parasites databases (RMgmDB) were used to gather data on genes encoding surface antigens.

Information on the presence of transmembrane domains, predictive signal peptides, protein export motifs, predicted antigenicity (TDR Targets), glycosylphosphatidylinositol (GPI) anchors, and possible evidence for a role in gametocyte/gamete development or fertilisation based on rodent malaria gene disruption studies were recorded for each antigen. The data were then filtered based on the occurrence of a signal peptide and predicted antigenicity score and only gene sequences with a predicted signal peptide were considered for expression.

### 3.3 Protein Production by the Wheat germ expression system

The system which was employed in this study is an appropriate method as it is a high-throughput system capable of expressing rather complex proteins that are correctly folded and functional (Tsuboi *et al*., 2008). Moreover, the method has previously used with success, to express recombinant malaria proteins by Miura *et al.,* (2014) and Kapulu *et al.,* (2015).

#### 3.3.1 Amplification of genes encoding sexual stage antigens

The *P. falciparum* sexual-stage antigens were PCR amplified using either cDNA or genomic DNA (gDNA) as template DNA. PCR reaction in a final volume of 50 µl was prepared, consisting of the gene's primers, 2x PCR master mix, nuclease-free water and the template DNA. The amplification reactions were then placed in a thermocycler (Veriti 96-Well Thermal Cycler).

#### 3.3.2 Purification of fragments

The amplified PCR fragments were resolved in agarose gel using Tris-borate-EDTA (TBE) as running buffer. To prepare the gel, 0.75 g agarose was dissolved in 1X TBE buffer (100 ml) and heated to dissolve under a microwave then allowed to cool before adding dye, 5 µl of redsafe (RedSafe™ Nucleic Acid Staining Solution 20,000x), that emits fluorescence when bound to DNA or RNA thus enabling visualization under UV. The gel was then poured into a tray and left to polymerize for 1 hour.

Five μl of DNA loading dye (ThermoFisher Scientific) was added to 25 μl of the PCR products before loading to the gel, along with 3.5 μl of DNA hyperladder (HyperLadder$^{TM}$ 1Kb, Bioline). The gel was run for 1 hour at 110V and visualised under UV light using a trans-illuminator. The expected band-size of antigen was cut from the gel using a sterile scalpel blade and placed in an Eppendorf tube and weighed. The QIAquick Gel Extraction Kit (*Qiagen,* Germany) was used to purify the DNA from gel according to the manufacturer's instructions. DNA was eluted in 100 μl of elution buffer and the concentration measured using a spectrophotometer (NanoDrop 1000).

### 3.3.3 Ligation and cell transformation

The purified PCR products were ligated into a blunt TOPO vector (ThermoFisher Scientific) by setting up a 6 μl reaction consisting of 1 μl PCR Blunt II- TOPO vector, PCR product (3 μl), salt solution (1 μl) and nuclease-free $H_20$ (1 μl). The reaction was then left at room temperature for 30 minutes and 2 μl of the ligation mix then used to transform 25 μl TOP 10 (Invitrogen) competent cells by first incubating 30 minutes on ice followed by heat-shocking at 42˚C for 30 seconds. The tubes were put back on ice for 30 minutes and 160 μl of warm SOC media (Invitrogen) added to the cells and the reaction kept an incubator for 1 hour with agitation at 250 rpm. The mixture (150 μl) was plated on pre-warmed Lubria Base agar petri dishes with Kanamycin antibiotic and left overnight at 37˚C overnight.

### 3.3.4 Colony Screening

Colonies were screened by PCR with M13 primers. Single colonies *were* picked and streaked on a pre-warmed, Lubria Base agar culture plate containing Kanamycin and the tip washed off in tubes containing 10 µl of nuclease-free $H_2O$. The PCR reaction was setup by adding 6.25 µl of KAPA 2G HS master mix, 0.625 µl of M13 primers, 0.5 µl of the diluted bacterial colony and 4.5 µl of nuclease-free $H_2O$.

The PCR conditions included a denaturation step of 3 minutes at 95˚C, 35 annealing cycles at 95˚C for 15 seconds, 55˚C for 15 seconds and 72˚C for 1 minute per kb of DNA to be amplified. The extension stage was carried out primers at 72˚C for 10 minutes and the heating block allowed to cool for 5 minutes at 15˚C before resolving samples on a 1% agarose for 1 hour at 110V and envisioned with a chemiluminescence imager (ChemiDoc™ XRS+ System). For the positive bands, their corresponding colonies, were inoculated in 3 ml LB plus Kanamycin broth with a sterile pipette tip and left overnight at 37˚C in a shacking chamber at about 250 rpm.

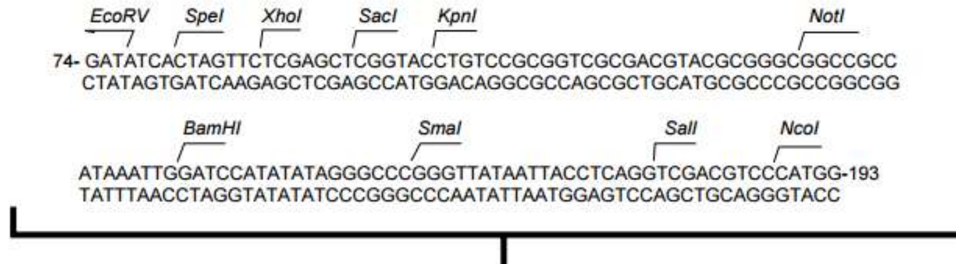
### 3.3.5 Cloning of antigens into expression vector

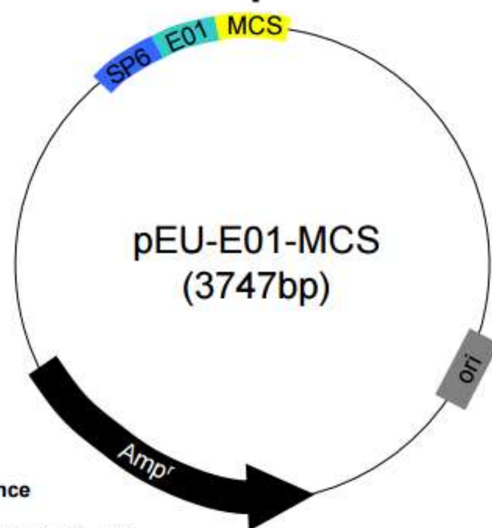
Plasmid DNA from the overnight culture was extracted with the *Qiagen Miniprep kit* (Qiagen, Germany) as per manufacturer's instructions and the DNA concentration measured using NanoDrop 1000 Spectrophotometer. In order to create restriction sites, the plasmids were then digested using Xho1 and Not1 restriction enzymes in a volume of 25 µl containing 10x CutSmart reaction buffer (New England BioLabs, Inc), 15 µl plasmid DNA, 0.5 µl of each xho1 and Not1 restriction enzymes (New England BioLabs, Inc) and 6.5 µl of nuclease-free $H_2O$.

The reaction was then kept for 1 hour at 37˚C and the digested product resolved in agarose gel (0.75%) at 110V for 1 hour. The insert was then purified from gel as described in section 3.3.2. The DNA was ligated into the linearized pEU-E01-MCS (*CellFree Sciences*) vector, using the Ligation High reagent (Toyobo). The volume of vector and insert to use, was determined with the New England BioLabs (NEB) ligation calculator tool with the aim of using 1:2 or 1:3 ratio of vector to insert.

Once this ratio was calculated, an equal volume of the ligation high reagent as the volume of vector plus insert, was added to the reaction. The vector contains a SP6 promoter, E01 translation enhancer and ampicillin resistance gene as shown on Figure 3.2.5 below. Therefore, the ligation reaction containing the linearized vector, the insert and the ligation high reagent was incubated for 1 hour at 16˚C and used to transform TOP 10 (Invitrogen) competent cells, as previously described, and 70 µl then plated on LB agar plates (with Ampicillin) and left to grow at 37˚C.

*Multiple Cloning Site*




**pEU-E01-MCS sequence**
SP6 promoter:-17~ 1
Translational enhancer(E01): 16~ 72
Multiple Cloning Site:74~193
Origin:1190~1830
Ampicillin resistance gene:1974~2838

Position 1 is located at the final G (underlined in the following sequence) of SP6 promoter:
ATTTAGGTGACACTATA<u>G</u>

**Figure 3.3(a): Map of the pEU-E01-MCS vector (Adapted from the WEPRO 7240H Kit, CellFree Sciences)**

Recombinant colonies were screened using wheat germ flank-to-flank PCR and the reaction set up with 6.25 µl 2X KAPA 2G, 0.625 µl wheat germ forward and reverse primers, 0.5 µl of the template and 4.5 µl of nuclease-free water. Positive colonies were grown in 3 ml of LB broth having ampicillin, overnight at 37˚C. To inoculate a 100 ml LB broth with ampicillin the 3 ml overnight culture was used, left overnight at 37˚C and the plasmid purified using the Qiagen Plasmid extraction kit. A spectrophotometer was used to determine the concentration of the DNA (NanoDrop 1000) at 260 nm and at 280 nm for purity.

### 3.4 Protein Expression

Protein synthesis with (WGCFS), was carried out as per manufacturer's manual of WEPRO7240H expression kit (ENDEXT® Technology). Briefly, a transcription reaction was set up as displayed in the table below:

| Reagents | Small-scale expression | Middle-scale expression | Large-scale expression |
|---|---|---|---|
| Nuclease-free $H_2O$ | 11.5µl | 28.75µl | 143.75µl |
| 5X Transcription buffer LM | 4µl | 10µl | 50µl |
| 25mM dNTP mix | 2µl | 5µl | 25µl |
| RNase Inhibitor | 0.25µl | 0.625µl | 3.125µl |
| SP6 RNA Polymerase | 0.25µl | 0.625µl | 3.125µl |
| Plasmid | 2µl | 5µl | 25µl |
| Total | 20µl | 50µl | 250µl |

The mixture was incubated at 37˚C for 6 hours, and the products run on a 1% agarose gel at 110V for 30 minutes to detect the quality of mRNA products. The mRNA was allowed to cool to room temperature, while the wheat germ extract was thawed under running water and immediately placed on ice. A translation reaction adding up to 20.8 µl was then set-up as follows:

| **Reagents** | **Small-scale expression** | **Middle-scale expression** | **Large-scale expression** |
|---|---|---|---|
| mRNA | 10 µl | 50µl | 250µl |
| Creatine Kinase (1mg/ml) | 0.8 µl | 0.2µl | 1µl |
| WEPRO®7240H (OD/ml) | 10 µl | 50µl | 250µl |
| Total | 20.8µl | 100.2µl | 501µl |

One tube of 1X SUB-AMIX® was thawed, mixed by pipetting and 206 µl added into the well of a 96-well flat bottom plate. A bilayer reaction was then carried out by carefully transferring the translation mixture into the bottom of the well containing 1X SUB-AMIX® to form two layers, with the translation mixture in the lower layer and the 1X SUB-AMIX® in the upper layer. The plate was then sealed around the edges with Para film to avoid evaporation and incubated at 15˚C for 20 hours.

### 3.4.1 Detection of proteins

To detect the presence of expressed proteins the translation mix was recovered after the 20-hour incubation period and prepared accordingly to run on a protein gel, SDS-PAGE (sodium-dodecyl sulphate-Polyacrylamide gel electrophoresis) gel. The bilayer reaction was mixed by pipetting gently. Ten µl of Laemmli sample buffer was added to 10 µl of the translation mix and heated at 70˚C for 10 minutes. The samples were then loaded into the SDS gel alongside a colour protein standard ladder, at 150V for 60-75 minutes or until the dye reached the gel front. The gel was then visualised to assess that the protein migrated at the expected size.

### 3.4.2 Purification of WGCFS expressed proteins

The proteins were tagged with six histidine residues that allowed the use of immobilized metal affinity chromatography (IMAC) for their purification. The samples were prepared by diluting the protein with binding buffer in a 1:1 ratio, after which beads (Ni Sepharose® High Performance) coupled to a chelating group, in this case $Ni^{2+}$, were prepared and incubated together with the sample, on a rotating platform at 4˚C for 2 hours. The beads bind proteins with exposed histidine groups, making them appropriate for recovery of histidine tagged proteins. To prepare the beads the required volume of $Ni^{2+}$ beads was taken from a 20% ethanol slurry. The beads were washed with 10X the volume of the beads of distilled water and spun down for 2 minutes at 1400g.

Finally, an equal volume of the beads of the binding buffer was added and the beads re-suspended and added to the sample. The entire translation mix was then added into micro spin columns primed with binding buffer and spun down for 2 minutes at 1400 g. The column was washed 5X with 100 µl of binding buffer and the protein eluted from the column five times with 100 µl of elution buffer.

The recovered washes and elutions were then concentrated using Amicon Ultra 0.5 mL centrifugal filters (Sigma-Aldrich). Here, the concentration columns were placed in a collection tube and pre-rinsed using 100 µl of elution buffer by centrifuging at 14,000 g for 15 minutes. Up to 500 µl of the sample is loaded onto the column and span down at 14,000 g for 5 minutes or until desired concentration is reached. Once the sample was recovered it was placed in clean "Protein Lo Bind Tubes". 10 µl of the sample was boiled in Laemmli sample buffer, boiled for 10minutes at 70˚C and loaded onto SDS gel to run at 150V for 1 hour. A western blot was carried out using anti-his HRP to detect the presence of the histidine-tagged proteins.

### 3.5 Protein Production by the Mammalian Expression system HEK293

Constructs were codon optimised for expression in the mammalian system. After successful amplification, PCR fragments were gel purified as described in section 3.3.2. In fusion cloning was setup with 2 µl of 5 times In-fusion HD Enzyme premix, the linearized vector, the purified PCR product and an appropriate volume of water added to bring the total reaction volume to 10 µl. The reaction was placed at 50°C for 15 minutes before being put back on ice, after which transformation into TOP10 E.coli cells was carried out. Positive colonies were screened using 0.625 µl T7 forward and antigen reverse primers, 6.25 µl of 2X KAPA 2G, 0.5 µl of the template DNA and 4.5 µl of nuclease free water, plasmids harvested by maxiprep and transfection into mammalian cells done.

### 3.5.1 Protein expression using HEK293E cells

As for the mammalian HEK293 expression system, its biochemical machinery has been shown to efficiently produce and appropriately modify proteins post-translation (Thomas and Smart, 2005). It has also been used in the production of *P. falciparum* gametocyte proteins (Nikolaeva *et al*., 2017). A vial of 293E cells were thawed in 16 ml of pre-warmed F17 medium plus L-Glutamine and Kolliphor, into a disposable 125 ml plastic vented cap Erlenmeyer culture flask and placed in a humidified incubator at 37˚C, 130 rpm, with 5% $CO_2$. To determine cell density, a 100 µl aliquot of the culture was used from which 10 µl was added to 10 µl of trypan blue dye and counting done using an automated haemocytometer (Countess Automated Cell Counter by ThermoFisher Scientific).

A small-scale transfection was first carried out to assess if expression occurred before proceeding to large-scale production. To perform a small-scale transfection, F17 media and Opti-MEM® media were pre-warmed in at 37˚C, while Lipofectamine was left to thaw at room temperature. To prepare transfection for a single antigen, 1 ml of Opti-MEM® was added into two 15ml falcon tubes. Plasmid DNA corresponding to 30 µg was added into the first tube in addition to 2X the amount of DNA, of the P3000 293fectin™ reagent and the tube vortexed mildly. To the second tube already containing 1ml of Opti-MEM®, 30 µl of Lipofectamine 3000 reagent was added, the tube vortexed mildly and both tubes incubated at room temperature for 5 minutes.

After the 5 minute incubation, the contents in the tube containing Opti-MEM® plus Lipofectamine, were added into the tube containing the DNA mixture and left at room temperature for 20 minutes. The above mixture was added to the cells that were at the required density (1.8*10^6 cells/ml) and viability (not less than 97%) and the flask swirled immediately after the addition to transfect. The cells were then incubated in a shaking, humidified incubator set at 37˚C, 130 rpm and 3% $CO_2$. One day (24 hours) post-transfection, (Typtone NI) TN1 was added to a final concentration of 0.5% of the total culture volume. The culture was maintained for 4 days post-transfection and harvested for purification and detection of expression. A large-scale expression was set-up by increasing the volume of cells used, the amount of plasmid DNA, as well as all other reagents such as Opti-mem and Lipofectamine.

### 3.5.2 Protein expression using HEK293F cells

To prepare a small-scale 30 ml culture suing HEK293F cells, 1.5 ml of Opti-MEM® was added into two 15 ml falcon tubes. Into one of the tubes, a volume equivalent to 30 µg of DNA was added and to the other tube, 80 µl of expifectamine 293 reagent was added. Both tubes were incubated at room temperature for 5 minutes. The contents of the two tubes were mixed together, the tube vortexed mildly and incubated at room temperature for 20 minutes, before adding to the flask containing cells. The cells were incubated in a shaking, humidified incubator set at 37˚C, 130 rpm and 8% $CO_2$. Enhancers were added 16-18 hours post-transfection with 150 µl of Enhancer1 and 1.5 ml of Enhancer2. The cells were incubated for 3 days and harvested for detection and purification.

### 3.5.3 Purification of mammalian system expressed proteins

Cell cultures were transferred into 50 ml falcon tubes and centrifuged at 2000 rpm for *5* minutes to recover the supernatant. The supernatant was incubated for 2 hours by adding imidazole to a final concentration of 50 mM and 30 µl of Ni Sepharose High Performance beads. To purify the proteins, 5ml polypropylene columns were primed with wash buffer before adding the supernatant that was allowed to pass the column by gravity while collecting the flow-through. The column was then washed 5X with 100 µl of wash buffer for each wash and eluted with 100 µl of elution buffer 5X. The samples were then prepared to be run on SDS gel by boiling 10 µl of the protein in Laemmli sample buffer, boiled at 100˚C for 3 minutes and run at 110V for 1 hour alongside a protein ladder dye.

## 3.6 Western Blot Analysis

The purified protein was run on SDS PAGE gel alongside a stained protein ladder and anti-histidine ladder at 110V. A PVDF membrane cut to the size of the gel was activated for 30 seconds in methanol and rinsed in transfer buffer.

The proteins were then transferred from the SDS gel to the membrane after which the membrane was blocked for 1 hour with 25 ml of 4% blocking buffer. To wash the membrane, 1XTBS was used 3 times for 5 minutes each. Blocking buffer (25 ml) containing anti-his HRP antibody to a dilution of 1:5000 was added and the membrane incubated for 1 hour, at room temperature. The membrane was washed again as described above and left in 25 ml of distilled water while awaiting imaging. Into a 15 ml falcon tube, 2 ml of NOVEX® reagent A and 2 ml of NOVEX® reagent B was mixed and added to the membrane after draining off the water. Incubation was carried at for 1 minute in the dark and the membrane imaged using the ChemiDoc imager (ChemiDoc™ XRS+ System).

### 3.7 Protein quantification

Proteins were quantified using Coomassie Plus (Bradford) assay kit (Thermo Scientific™ Coomassie Plus™ Kit), as per the manufacturer's instructions. The Coomassie plus Reagent was equilibrated at room temperature for about one hour while preparing the protein standards by diluting the contents of one albumin standard (BSA) ampule. 10 µl of the protein standards were then added into a 96-well plate in triplicate while 10 µl of the sample was added to the plate in duplicate. 300 µl of room temperature-equilibrated Coomassie reagent was added to each well and the plate placed on a mixer to shake for about 30 seconds, before reading at 595 nm on a plate reader and the concentration of the protein determined from the read-out.

### 3.8 ELISA

Immunoprofiling for responses to the different antigens was done by ELISAs. To determine the optimal concentration of antigen and antibody to use for the assays, a checkerboard titration was carried out. To do so, pooled hyper immune and non-immune serum was used. The antigens (CVMPPP, PEBP, PSOP1, PSOP25_3D7, PSOP25_10668, MDV1 and CPP4) were coated on a 96-well plate at different concentrations, from 0.5 µg/ml to 4 µg/ml and the serum diluted from 1:100 to 1:1000 dilutions. The optimal concentrations used were those that showed a clear difference between non-immune and hyper immune individuals and these were determined separately for all the antigens used.

A 3-day indirect ELISA protocol was utilized to perform the immunoprofiling assays. Once the optimal coating concentration of antigen and antibody was determined, a plate was coated with 100 µl /well of the antigen of interest (CVMPPP, PEBP, PSOP1, PSOP25_3D7, PSOP25_10668, MDV1 and CPP4) diluted in carbonate-bicarbonate buffer. The plate was left overnight at 4˚C. On the second day, the plate was washed 4X using an automated plate washer (ELx405™ Microplate Washer). The wash buffer was prepared with 1X Phosphate Buffer Saline and 0.05% Tween (1XPBS-T). 200 µl of blocking buffer (4% skimmed milk in 1XPBS-T), was added to the wells of the plate and kept for 5 hours. Serum was diluted in the blocking buffer, according to the optimal concentration for the different antigens.

After the 5 hour incubation period, diluted serum (100 µl) was added to the well and the plated left overnight at 4˚C. The third day of the assay, involved washing the plate with wash buffer as described earlier, and incubating with the 100 µl of the secondary antibody Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP, diluted 1:5000 in blocking buffer. After incubating for 3 hours, the plate was washed and the substrate added to a final volume of 100 µl /well. The reaction was stopped by adding 2M H2SO4, and the plate read using at 492 nm using an automated spectrophotometer plate reader.

### 3.9 Statistical Analysis

Data was analysed using R version 3.4.1. Study participants from the AFIRM, cross-sectional study, were categorized into groups by age (<5 years, 5 to 15 years and >15 years). Spearman's rank correlation test was used to assess association between antibody levels of tested antigens. Regression models allowed for the adjustment of any confounding factors, to the responses elicited to the various antigens.

# CHAPTER FOUR

# 4.0 RESULTS

## 4.1 Protein production

This study began by expression of over twenty different stage V gametocyte proteins using both the wheat germ cell-free expression system and the mammalian HEK293 expression system. Once successfully amplified, expression was first attempted in the WGCFS at small scale to determine the yield (Low expresser, medium expresser or high expresser) and a decision based on this made on whether to move on to large scale expression.

Proteins that showed medium to high expression using WGCFS were then produced at large scale in the same system and are PSOP25_3D7, PSOP25_10668, CPP4 and MDV1. For those where expression failed in WGCFS or were deemed to be low expressers, were expressed using the mammalian system and are CVMPPP, PEBP and PSOP1. Those included in this study were those that were successfully amplified and expressed at a scale large enough to allow for downstream immunoprofiling by ELISA analysis. A summary of the characteristics of the antigens that were successfully expressed are included in Table 4.1.

**Table 4.1:** Summary characteristics of expressed antigens

| Antigen | Gene ID | Gene Description | M.Wt (kDa) | Transcript Length (bases) | Protein length (amino acids) | Signal peptide | GPI Anchor | TM Domain | PPED | TDR predicted antigenicity (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| CVMPPP | PF3D7_1314500 | Cop-coated vesicle membrane protein p24 precursor, putative | 17.7 | 621 | 206 | Yes | Yes | Yes | No | 73.9 |
| PEBP | PF3D7_0303900 | phosphatidylethanolamine-binding protein, putative | 23.15 | 594 | 197 | Yes | No | No | No | 92.3 |
| PSOP1 | PF3D7_0620000 | Secreted ookinete protein, putative (possible localization in apicoplast - GO) | 53.285 | 1404 | 467 | Yes | No | no | No | 90.6 |
| PSOP25 | PF3D7_1216500 | Ookinete surface-associated protein 8, putative | 59.6 | 1518 | 505 | Yes | No | Yes | No | 26.4 |
| MDV1 | PF3D7_0208800 | male development gene 1, protein of early gametocyte | 25.8 | 666 | 221 | Yes | No | No | No | 9.3 |
| CPP4 | PF3D7_0208800 | Conserved protein, unknown function | 28.2 | 708 | 235 | Yes | No | Yes | No | 21.6 |

### 4.1.1 Amplification of DNA material

Successfully amplified genes were run on agarose gel and images viewed under UV light using the ChemiDoc™ XRS+ System. PCR reactions were performed to generate amplicons and the products run on agarose gels. The successfully amplified *P. falciparum* gametocyte-specific genes are marked in the images above with a red arrow as well as the expected size of the product. The images also include a 1 kb DNA ladder (marked 'M' on the image) that was run in the gel alongside the products so as to confirm the size of amplicons by base pair (bp).

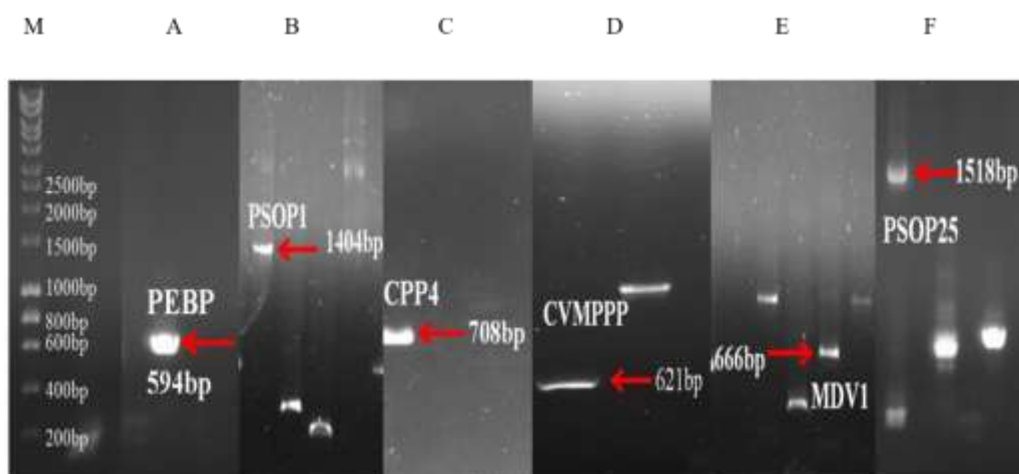



**Figure 4.1(a)** Gel images of successfully amplified genes by PCR

The expected sizes of the amplicons were 594bp - PEBP (Panel A), 1404bp - PSOP1 (Panel B), 708bp - CPP4 (Panel C), 621bp - CVMPPP (Panel D), 666bp - MDV1 (Panel E) and 1518bp - PSOP25 (Panel F). The expression of a number of genes was attempted as is seen by the presence of other unmarked bands but these were not included in further analysis as the amplicons were either not of the expected size of filed during other stages of expression.

### 4.1.2 Bacterial colony screening by PCR

Upon successful amplification, genes were ligated into the cloning vector and then ligated into the appropriate expression vector. The products of the reaction (Figure 4.1(b)) were then run on agarose gels to confirm the presence of expression, the reaction scaled up for harvesting of the plasmids (maxiprep) and DNA concentration determined for use in the different expression systems.

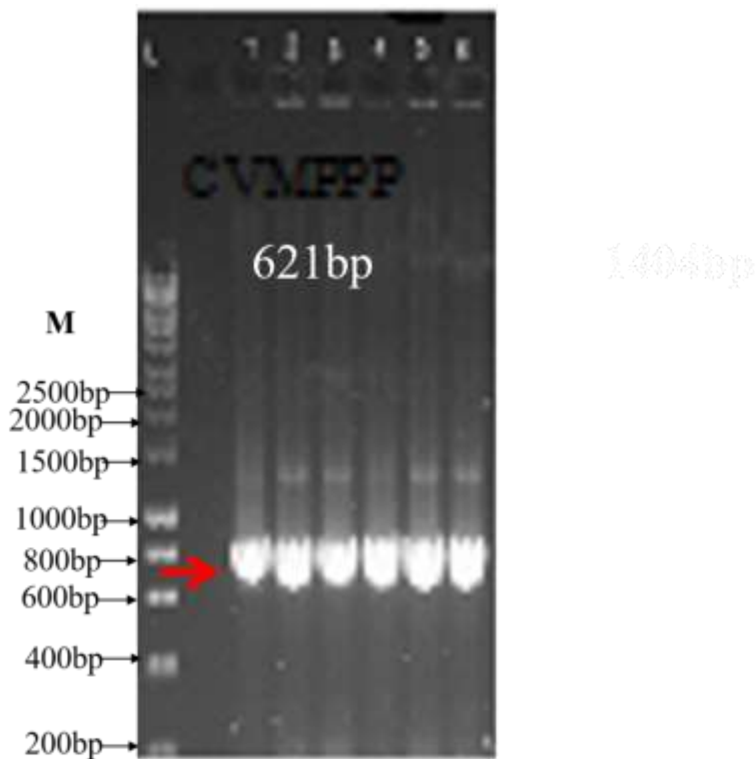



**Figure 4.1(b)** Image showing screened colonies

The gel image shows a representation of colony screening used to determine successful ligation into the expression vector. For each antigen, at least 10 colonies were screened to determine expression by setting up a PCR reaction. The image shows colony screened for the antigen CVMPPP and also includes a 1kb DNA ladder marked with an 'M'.

### 4.1.3 Protein detection

Proteins were detected by SDS-PAGE gels (Figure 4.1(c)). The gel images were taken upon purification of the proteins and includes the flow through (**FT**) which is was first product obtained after incubating the translation mix with beads. The washes (**W**) were obtained when binding buffer was passed through columns and the elutions (**E**) obtained upon passing of elution buffer through the columns. Once proteins were detected to migrate at the expected size they were quantified and used for immunoprofiling. The proteins PSOP25_3D7, PSOP25_10668, CPP4 and MDV1 were expressed using WGCFS while CVMPPP, PEBP and PSOP1 were expressed with the mammalian HEK293 system. The images show the successfully expressed recombinant proteins which were included in immunoprofiling of antibody responses. A protein colour broad standard ladder was run along with the proteins to confirm the sizes at which the proteins would migrate in kilo Daltons (kDa).

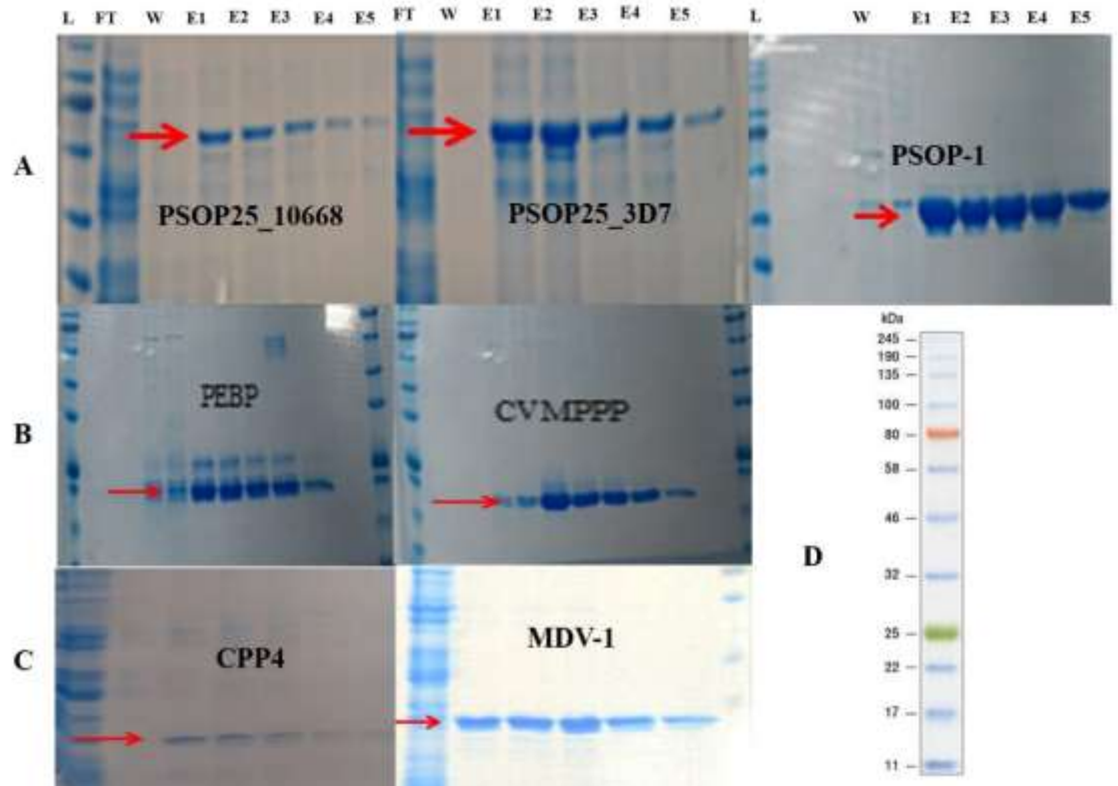



**Figure 4.1(c)** SDS-PAGE images showing detection of expressed proteins

The expected sizes of the respective proteins are shown by red arrows on the images containing the proteins: Panel A: 59.6kDa for PSOP25, 53.3kDa for PSOP1, Panel B: 23.1kDa for PEBP, 24.4kDa for CVMPPP, Panel C: 25.8kDa for MDV1 and 28.2kDa for CPP4. Panel D: Shows the coloured marker or observing protein separation during SDS-PAGE (Bio Labs. Inc.). **L**- Ladder, **FT**- Flow through, **W**- wash, **E**- elutions.

## 4.2 Description of AFIRM study

The AFIRM study was a cross-sectional study which recruited and enrolled individuals from the age of 2 years and above from the study site in Kilifi, Kenya which is considered to have low to moderate transmission to malaria. Plasma was collected from participants during the low intensity season from here on referred to as the 'dry season' and during peak intensity season from here on referred to as 'wet season'.

Table 4.2 is a summary of the characteristics of participants of the AFIRM study. There were N = 413 participants in total who were recruited and enrolled in to the study regardless of their parasitological status, with N = 235 sampled during the malaria season and N = 178 sampled after the malaria season. Those below 5 years of age were n = 82. Those between 5-15 years were N = 117 and those older than 15 years of age were N = 214. There were N = 262 females and N = 151 males.

**Table 4.2** Summary characteristics of participants of the AFIRM study

| | **Sex** | | **Asexual Positive** | | **Gametocyte Positive** | | **Season** | |
|---|---|---|---|---|---|---|---|---|
| **Age Group** | **Female (*n*)** | **Male (*n*)** | **Pos (*n*)** | **Neg (*n*)** | **Pos (*n*)** | **Neg (*n*)** | **Wet (*n*)** | **Dry (*n*)** |
| <5yrs | 42 | 40 | 44 | 38 | 14 | 68 | 64 | 18 |
| 5-15yrs | 73 | 44 | 71 | 46 | 47 | 70 | 73 | 44 |
| >15yrs | 147 | 67 | 115 | 99 | 61 | 153 | 98 | 116 |

### 4.2.2. Parasitaemia and gametocytaemia of AFIRM participants

Parasitaemia among participants was measured by qPCR for all parasites, NASBA18s to measure asexual parasites and NASBAPfs25 for sexual stages of the parasites there is also 18s qPCR for asexual parasites. Gametocytes were detected in 29.5% (122/413) of the participants, with asexual parasites measured by NASBA18s detected in 55.7% (230/413) and those measured by 18s qPCR in 38% (157/413) of participants. 18s qPCR parasite densities were lower in adults compared to children living in the same study area. To show an overview of parasitaemia among participants, individuals were divided into those in whom any parasites were detected, denoted by '1' or '0' in those no parasites of any stages were detected.

There was no difference in parasite positivity or negativity based on age at the time of sampling with the median age of those who were negative was 20 years as compared to those positive for parasites whose median age is slightly below 20 years as determined by 18s qPCR (Figure 4.2(a)). The same is true for asexual parasitaemia by NASBA18s (Figure 4.2(b)). In the case of gametocyte positivity measured by NASBAPfs25, there was no difference based on age whether participants were either at sampling (Figure 4.2(c)).

More individuals under 5 years of age were gametocyte negative at sampling with more individuals between 5-15 years being gametocyte positive. Participants in the older than 15 year age bracket had the lowest proportion of gametocyte positive persons with only 61/413 having detectable gametocytes (Figure 4.2(a)).

The box plot (Figure 4.2(a)) shows a summary of asexual parasite status (qPCR18s) by age of AFIRM study participants. Those who were parasite negative are denoted by ‘0’, while those in whom parasites were detected by qPCR18S are denoted by ‘1’.

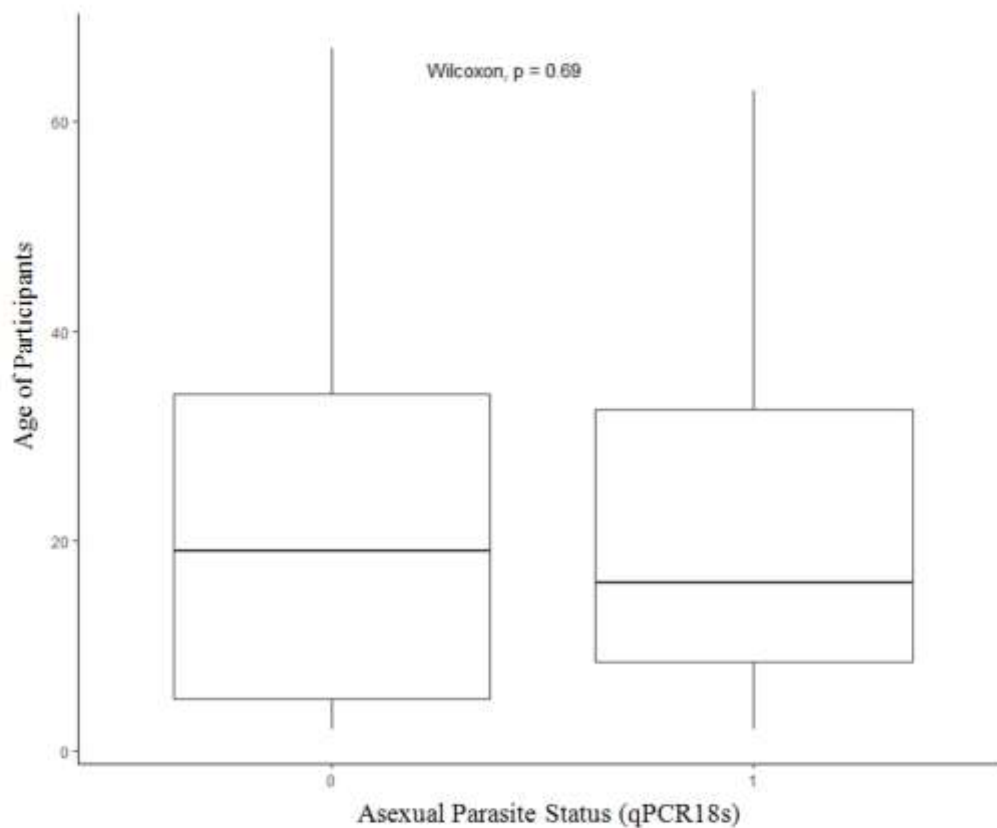



**Figure 4.2 (a)** Asexual Parasitaemia (qPCR18s) with age

Age of participants is shown on the y-axis while the gametocyte parasite status is represented on the x-axis. The box and whisker plots include 95% significance level and Wilcoxon p-values of significance.

The box plot (Figure 4.2(b)) shows a summary of asexual parasite status (NASBA18s) by age of AFIRM study participants. Those who were parasite negative are denoted by '0', while those in whom parasites were detected by qPCR18S are denoted by '1'.

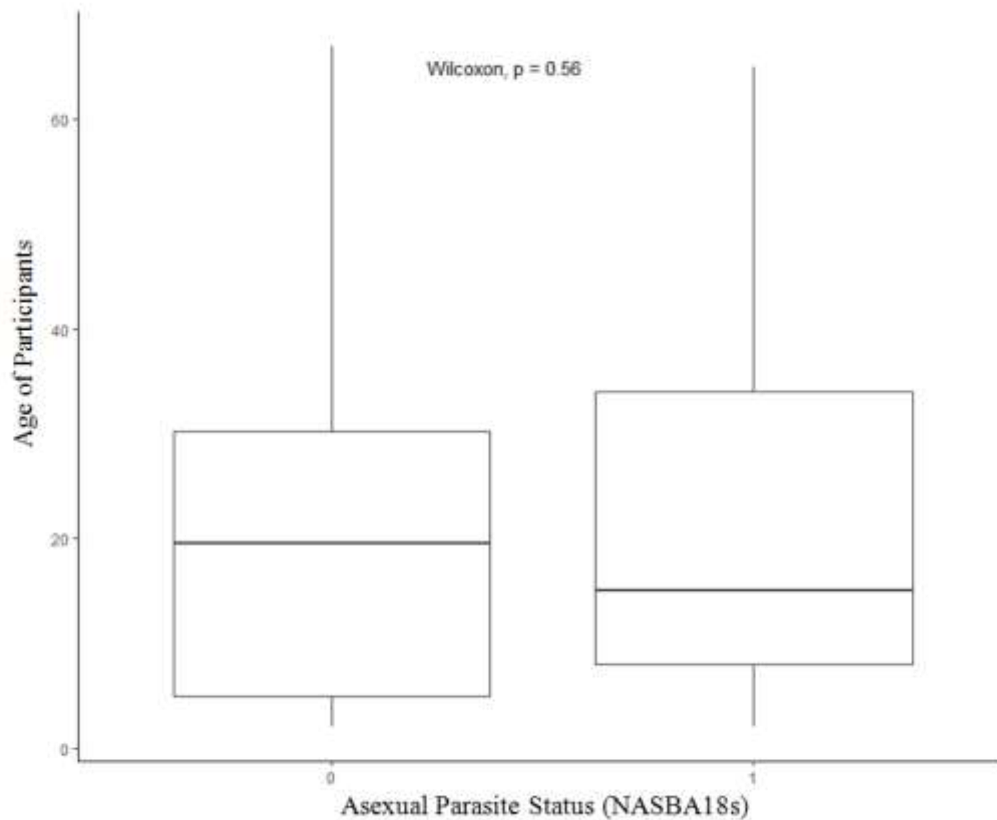



**Figure 4.2(b)** Asexual Parasitaemia (NASBA18s) with age

Age is shown on the y-axis while the gametocyte parasite status is represented on the x-axis. The box and whisker plots include 95% significance level and Wilcoxon p-values of significance.

The box plot (Figure 4.2(c)) shows a summary of gametocyte status by age of AFIRM study participants. Those who were gametocyte negative are denoted by '0', while those in whom gametocytes were detected by NASBAPfs25 are denoted by '1'.

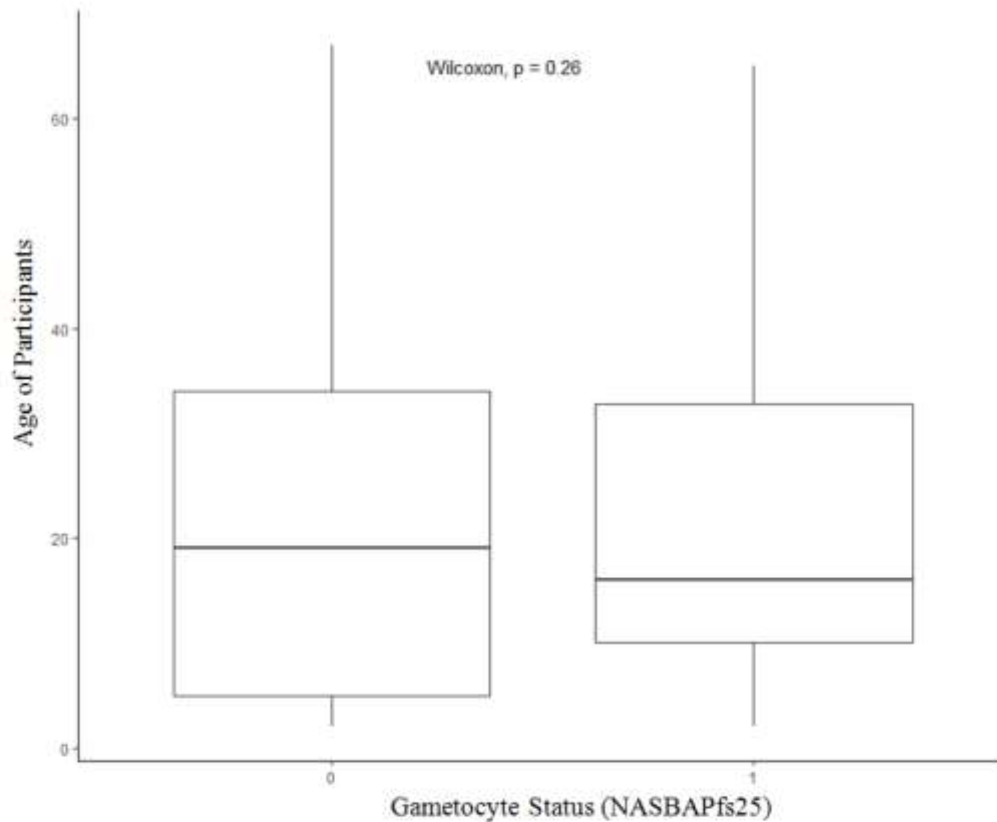



**Figure 4.2(c)** Gametocyte status by age

The age of participants is shown on the y-axis while the gametocyte status is represented on the x-axis. The box and whisker plots include 95% significance level and Wilcoxon p-values of significance.

The distribution among the age groups of asexual parasitaemia as measured by 18s qPCR (parasites densities quantified by DNA-based 18s qPCR), showed that densities were lower in adults compared to children (Figure 4.2(d)) in the study population. The box plot includes all 413 individuals who participated in the study. The results show that < 5 year olds had the highest densities in comparison to >15 year olds without significant difference between the <5 year olds and 5-15 year olds. There were more children (5-15 year olds) with high density parasitaemia but the highest density parasitaemia was in the younger age group albeit not statistically significant.

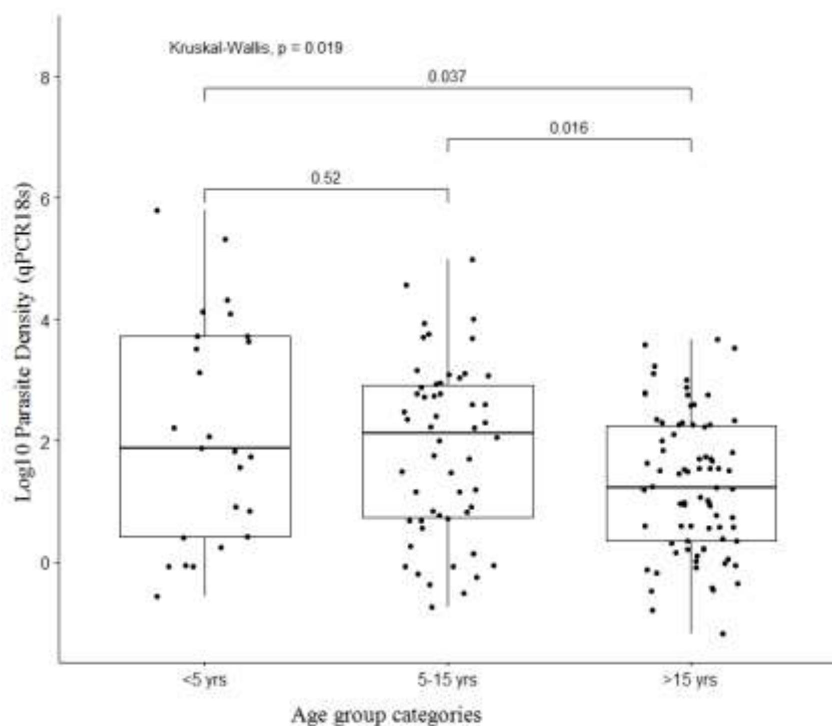



**Figure 4.2(d)** Asexual parasite densities measured by qPCR 18s, in relation to the age
Log transformed qPCR 18s densities shown on the y-axis with the age group categories shown on the x-axis. The box and whisker plots include 95% significance level and Kruskal-Wallis p-values of significance.

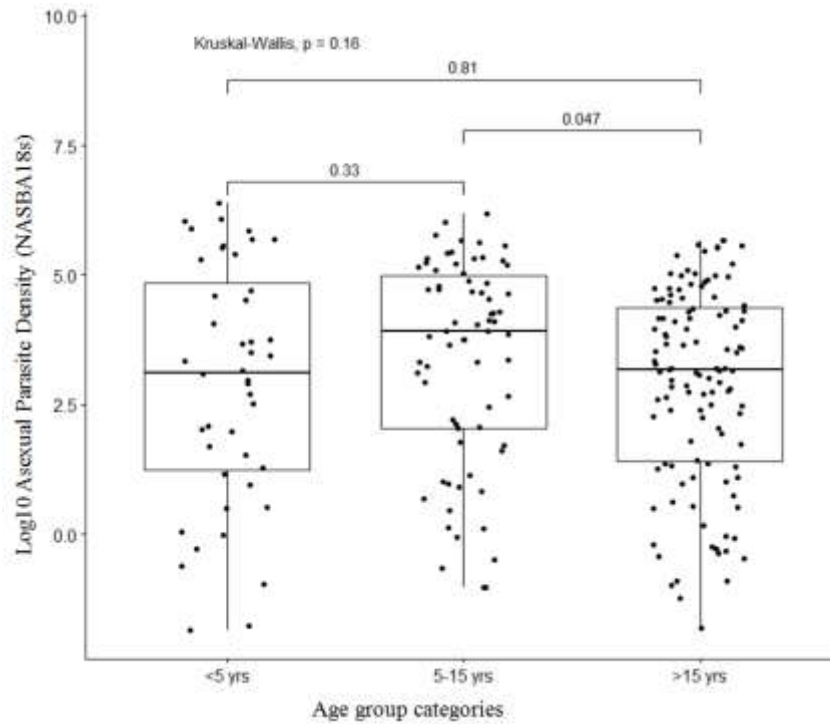



**Figure 4.2(e)** Asexual parasite densities measured by NASBA18s, in relation to the age

The y-axis shows the log transformed density of parasites as measured by NASBA18s, with the x-axis representing the age categories. The box and whisker plots include 95% significance level and Kruskal-Wallis p-values of significance.

The boxplot shows gametocyte density as measured by NASBAPfs25 in relation to age. The distribution of gametocyte density as measured by NASBAPfs25, in relation to age shows that participants age <5 years have higher densities in comparison to those aged 5-15 years albeit not statistically significant (Figure 4.2(f)). However, they have a significantly higher density than older participants (>15 year-olds) gametocytes/ml vs for the <5year olds. There was no difference in densities between the 5-15 year olds and >15 year olds.

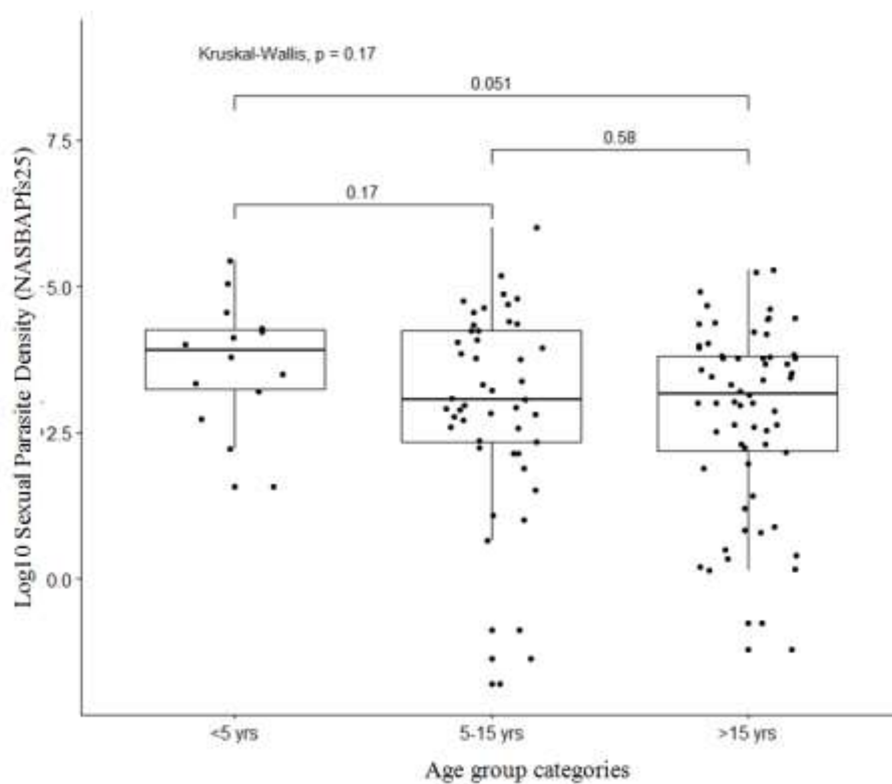



**Figure 4.2(f)** Gametocytaemia density as measured by NASBAPfs25, in relation to age

Participants as categorized in the three age groups, less 5 year-olds, 5-15 year-old and older than 15 years are as shown on the x-axis while the log transformed density of gametocytes as measured by NASBAPfs25, is on the y-axis. The box and whisker plots include 95% significance level and Kruskal-Wallis p-values of significance.

In order to determine an association between parasite densities, asexual and sexual correlation analysis was undertaken. There was a strong correlation of asexual and sexual parasite densities regardless of the assay of measurement (Figure 4.2(g)). This indicates that an increase in asexual parasite density also results in an increase in sexual parasite density as the former serves as the precursor for the latter. Correlation of parasite densities observed were: NASBAPfs25 and NASBA18S ($r=0.71$, $p<0.001$); NASBAPfs25 and qPCR ($r= 0.68$, $p<0.001$); and NASBA18S and qPCR ($r = 0.81$, $p<0.001$).

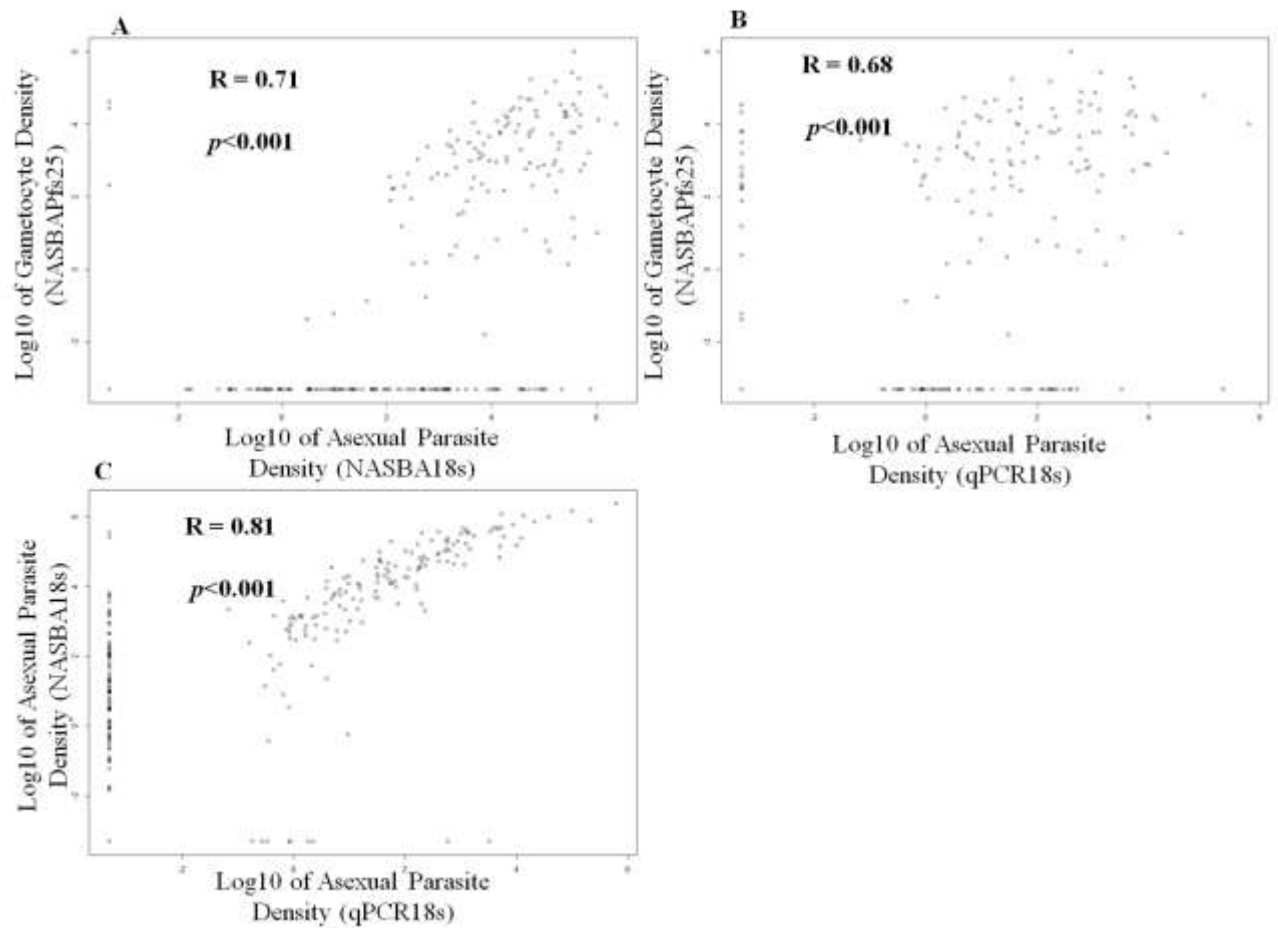



**Figure 4.2(g)** Correlation of parasite densities

Correlation of the asexual parasitaemia as measured by (NASBA18s) on the x-axis and gametocytaemia on the y-axis as measured by (NASBAPfs25) as shown on the scatterplot labelled **A**. Scatter plot **B** shows the correlation between asexual parasite densities as measured by qPCR 18s as shown on the x-axis and gametocyte density as measured by NASBAPfs25. Scatter plot **C** shows the correlation between asexual parasite densities by NASBA18s on the *y-axis*, and by qPCR 18s on the *x-axis*.

### 4.3 Profile of Antibody Responses- AFIRM study

To measure antibody responses to the recombinant proteins, plasma samples collected from participants of the study were used. ELISA was used to determine antibody responses which was given as ELISA optical density (O.D) values read at 495nm. The participant's characteristics were used to explore associations to the responses against the recombinant proteins.

#### 4.3.1 Profile of antibody responses with age

To determine the association between ages with responses to the recombinant gametocyte specific proteins antibody responses were measured and participants categorised into the three age groups for analysis. Antibody responses to recombinant proteins were measured using ELISA and are given as optical density (OD) values read at (495nm). The box plots show antibody responses to recombinant proteins CVMPPP (A), PEBP (B), PSOP1 (C), PSOP25 (3D7 and 10668, D and E respectively), MDV1 (F) and AMA1 (G). H shows a side-by-side comparison of responses to PSOP25-3D7 and 10668 with age. Those below the cut off line have antibody responses lower than a naïve donor and thus negative whilst those above the line are considered to have higher responses and therefore seropositive. Regardless of the antigen, individuals older than 15 years have significantly higher responses than those between 5-15 years as well as those younger than 5 years (Figure 4.3(a)).

Those between 5-15 years also have significantly higher responses than those younger than 5 years, and this trend was similar with all the antigens investigated. To determine any differences in responses between the variant antigens, PSOP25-3D7 and PSOP25-10668, a side-by-side comparison of responses with age was analysed. PSOP25-3D7 represents a laboratory-adapted isolate from *P. falciparum*, while PSOP25-10668 version represents a field isolate from Kilifi. There was no difference in responses regardless of age group, between the two antigens (<5years $p = 0.31$, 5-15years $p = 0.42$ and >15years $p = 0.11$).

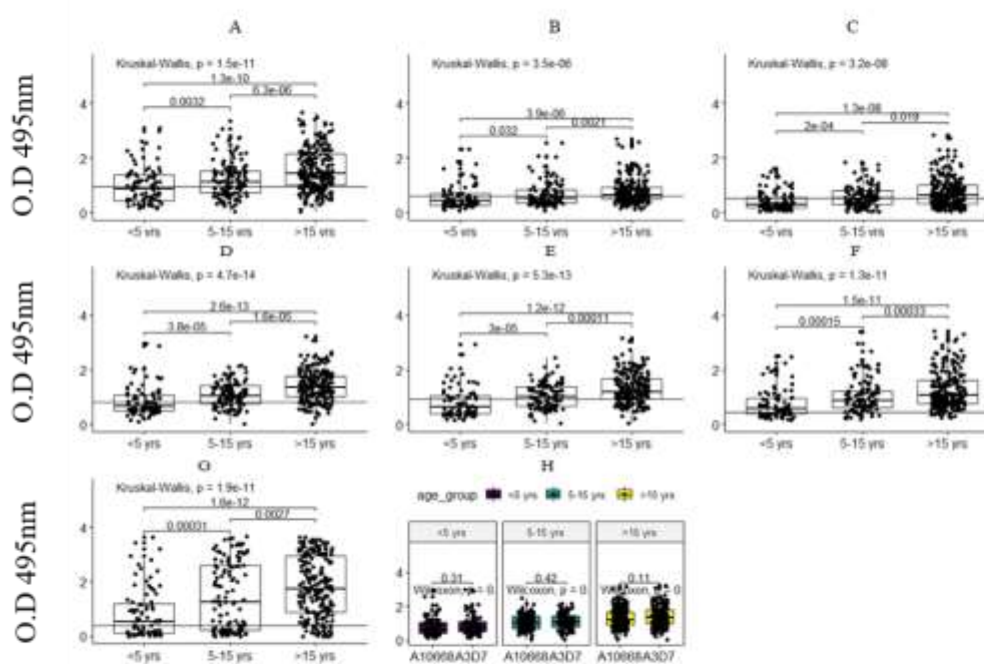



**Figure 4.3(a)** Antibody responses to recombinant gametocyte proteins with age
The x-axis shows the three age categories and the y-axis, the optical density (OD) values. The boxplots with 95% confidence intervals show the responses to the antigens within the given groups, with Kruskal Wallis p-values of significance.

### 4.3.2 Responses to recombinant proteins by gametocyte status

To assess differences in responses among those with detectable and undetectable gametocytes, results obtained by NASBA_Pfs25 were used. Antibody responses to recombinant proteins CVMPPP, PEBP, PSOP1, PSOP25 (3D7 and 10668), MDV1 and AMA1, were measured by way of ELISA given as optical density (OD) values read at (495nm). The box plots show antibody responses to recombinant proteins CVMPPP (A), PEBP (B), PSOP1 (C), PSOP25 (3D7 and 10668 D and E respectively), MDV1 (F) and AMA1 (G) with respect to gametocyte status. The boxplot 'H' shows a side-by-side comparison of responses to PSOP25-3D7 and 10668 with gametocyte status. Here, individuals were categorised as '0' if they had no detectable gametocytes, or as '1' if gametocytes were detected. Antibody responses were significantly higher responses ($p<0.05$), among those with detectable gametocytes regardless of the recombinant proteins included in the analysis. Being gametocyte positive did not influence the response to the variant antigens with there being no difference observed whether gametocyte negative or positive at sampling with responses to either PSOP25-3D7 or PSOP25-10668 (Figure 4.3(b) panel H). For the two antigens, the analysis does not reveal any significant difference in responses to either of the antigens between the two groups, that is gametocyte-positive ($p = 0.69$) and gametocyte-negative ($p = 0.11$).

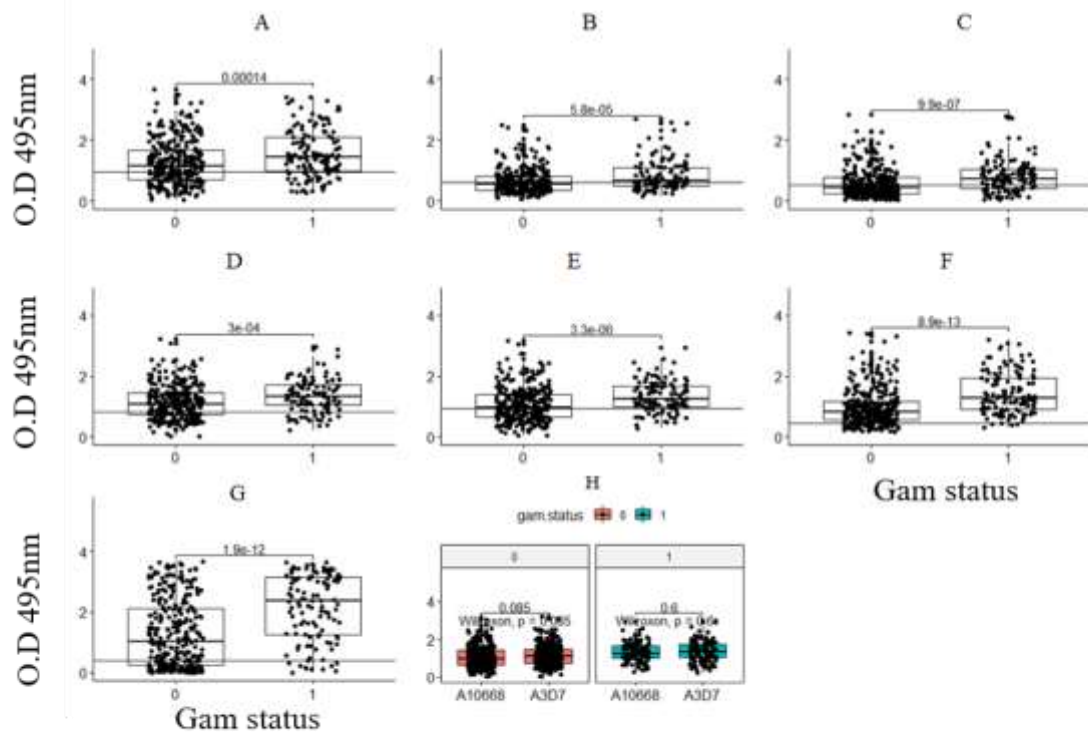



**Figure 4.3(b)** Antibody responses in relation with gametocyte status

The x-axis shows the presence ("1") or absence ("0") of gametocytes and the y-axis shows the optical density (OD) values. The cut off is presented by the solid blackline running across graphs arising from the y-axis. The boxplots show the responses to the antigens within the given groups, with Wilcoxon test p-values of significance and 95% confidence intervals.

### 4.3.3 Responses to recombinant antigens with season

To assess the impact of season on antibody responses, ELISA was used to measure antibody intensity to the recombinant proteins. Season was categorised into two: wet season, for those sampled during peak malaria season and dry season for those sampled after the peak season. To do so, the Wilcoxon test was used to determine the differences between the two seasons. All the box plots show malaria-exposed individuals from Kilifi. The UK donors that served as the negative control, were used to calculate the seropositivity cut off and excluded from the graphs. There was no significant difference in the responses for all antigens with regards to season [(CVMPPP, $p = 0.76$), (PEBP, $p = 0.098$), (PSOP1, $p = 0.25$), (PSOP25_3D7, $p = 0.67$), (PSOP25_10668, $p = 0.97$), (MDV1, $p = 0.52$), (AMA1, $p = 0.64$)] (Figure 4.3(c)) Boxplot 'H' from shows a side-by-side comparison of responses between PSOP25-3D7 and PSOP25-10668 with respect to season. For the two antigens, the analysis does not reveal any significant difference in responses to either of the antigens between the two seasons, that is dry season ($p = 0.19$) and wet season ($p = 0.29$). The responses are similar during both the dry and wet season across all the antigens.

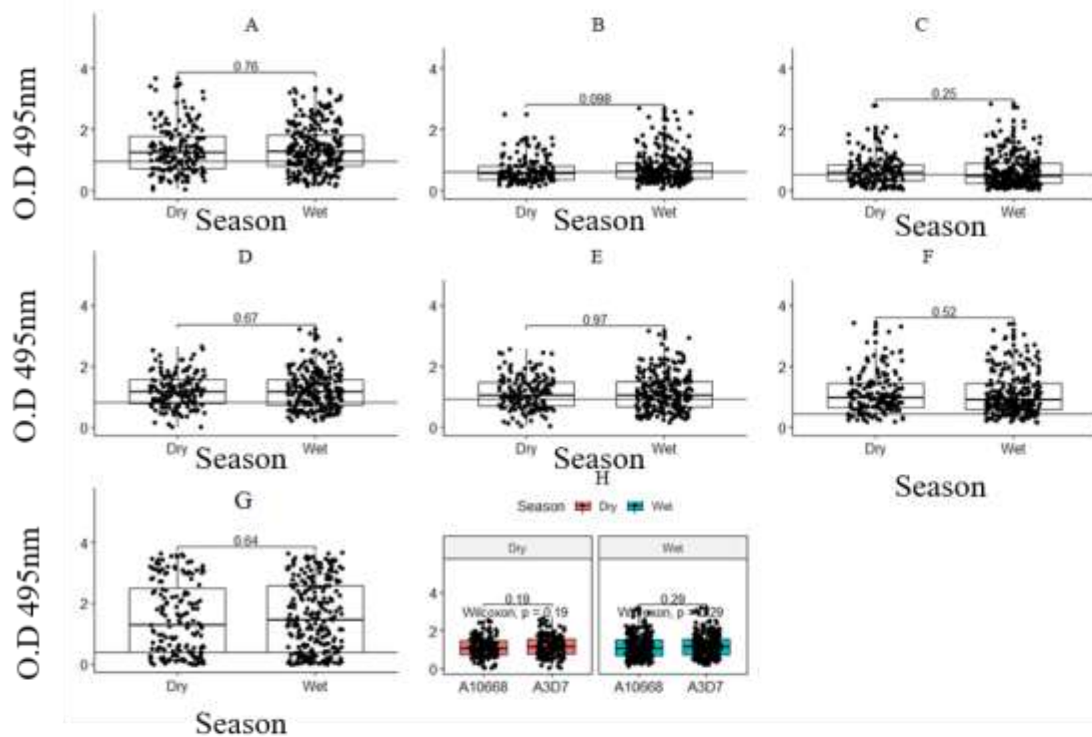



**Figure 4.3(c)** Antibody responses to recombinant gametocyte proteins by season

The x-axis shows the season, either dry or wet season, and the y-axis is the intensity of antibody responses represented as the optical density (OD) values read at 495nm. The cut off is presented by the solid blackline running across graphs arising from the y-axis. The boxplots show the responses to the antigens within the given groups, with Wilcoxon test p-values of significance and 95% confidence intervals.

## 4.4 Correlation Analysis

To determine association with other parameters important for antibody responses, correlation analysis was performed: (1) relationship with asexual parasite antigen responses; and (2) relationship with asexual parasites and gametocyte densities as measured by sensitive NASBAPfs18 and NASBAPfs25 as well as densities as measured by qPCR18s.

### 4.4.1 Correlation of responses to recombinant proteins with responses to AMA-1

To analyse the correlation of responses to the recombinant gametocyte proteins with responses to AMA-1, the mean responses of the two, as measured by ELISA O.D (at 495nm), were plotted against each other. Responses were correlated with mean responses to a non-gametocyte protein, AMA-1 which is an essential membrane protein of merozoites (asexual stage of *P. falciparum*). The presence of AMA-1 indicates infection with the parasites that could go on to the sexual stages. There was a significant correlation between responses to the recombinant gametocyte (sexual) proteins with responses to AMA1: [(cvmppp $r = 0.4$, $p<0.01$), (pebp $r = 0.32$, $p<0.01$), (psop1 $r = 0.35$, $p<0.01$), (psop25_3d7 $r = 0.41$, $p<0.01$), (psop25_10668 $r = 0.38$, $p<0.01$), (mdv1 $r = 0.39$, $p<0.01$)] (Figure 4.4.1).

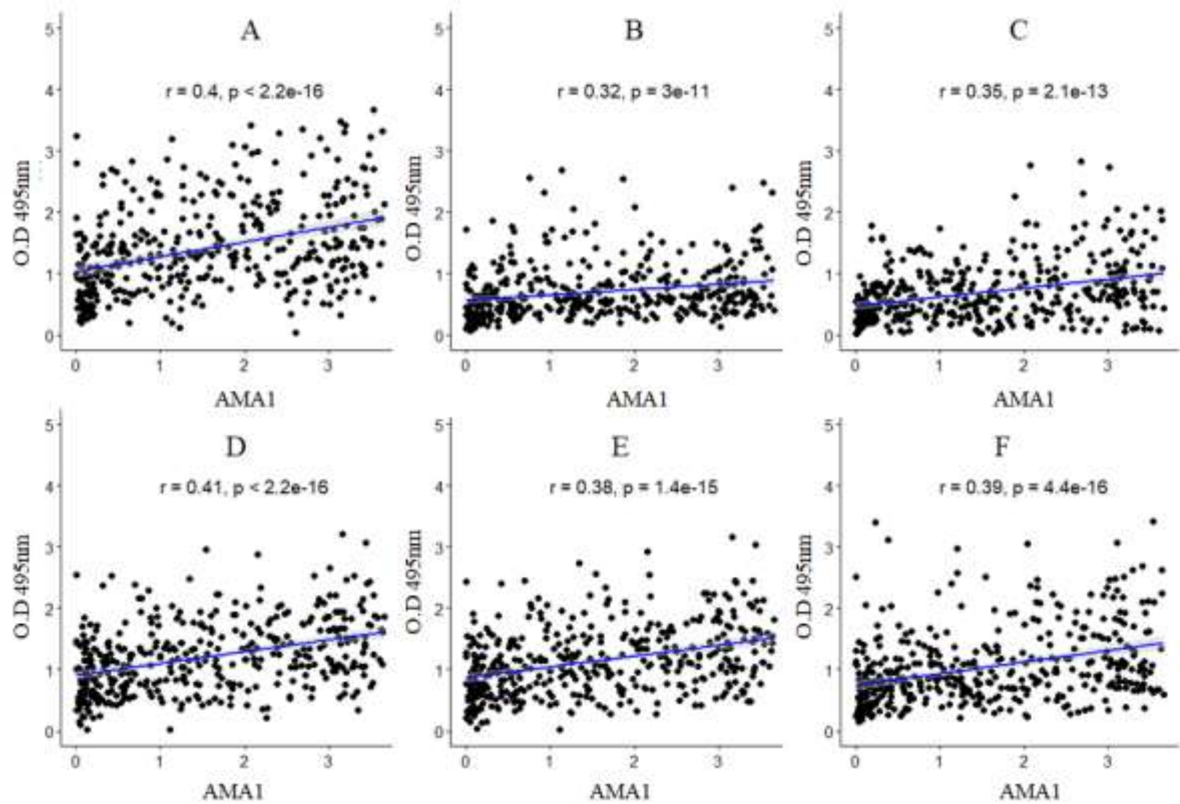



**Figure 4.4(a)** Correlation between responses to recombinant gametocyte antigens and AMA-1

The x-axis shows the mean response to AMA-1, while the y-axis shows the mean responses to the recombinant proteins. The scatter plots show correlation of responses between the recombinant gametocyte-specific antigens and responses to AMA1. The recombinant proteins shown in the figure are as follows: CVMPPP (A), PEBP (B), PSOP1 (C), PSOP25 (3D7 and 10668 D and E respectively) and MDV1 (F).

### 4.4.2 Correlation of responses with parasite density (qPCR18s)

The association between parasite densities (qPCR18s) with the responses to the recombinant proteins was determined. To do so, a correlation between the two was done and found that each of the recombinant proteins had a significant ($p<0.001$) correlation with parasite densities as measured by qPCR18s.

The correlation between responses to the recombinant antigens and parasite density (qPCR18s) as shown in Figure 4.4(b) is significant ($p<0.001$) across all proteins: (CVMPPP $r=0.19$, $p<0.001$, PEBP $r=0.22$, $p<0.001$, PSOP1 $r=0.25$, $p<0.001$, PSOP25_3D7 $r=0.17$, $p<0.001$, PSOP25_10668 $r=0.23$, $p<0.001$, MDV1 $r=0.31$, $p<0.001$, AMA1 $r=0.33$, $p<0.001$). This positive correlation may indicate than an increase in parasite density may result in an increase in the mean responses to the antigens.

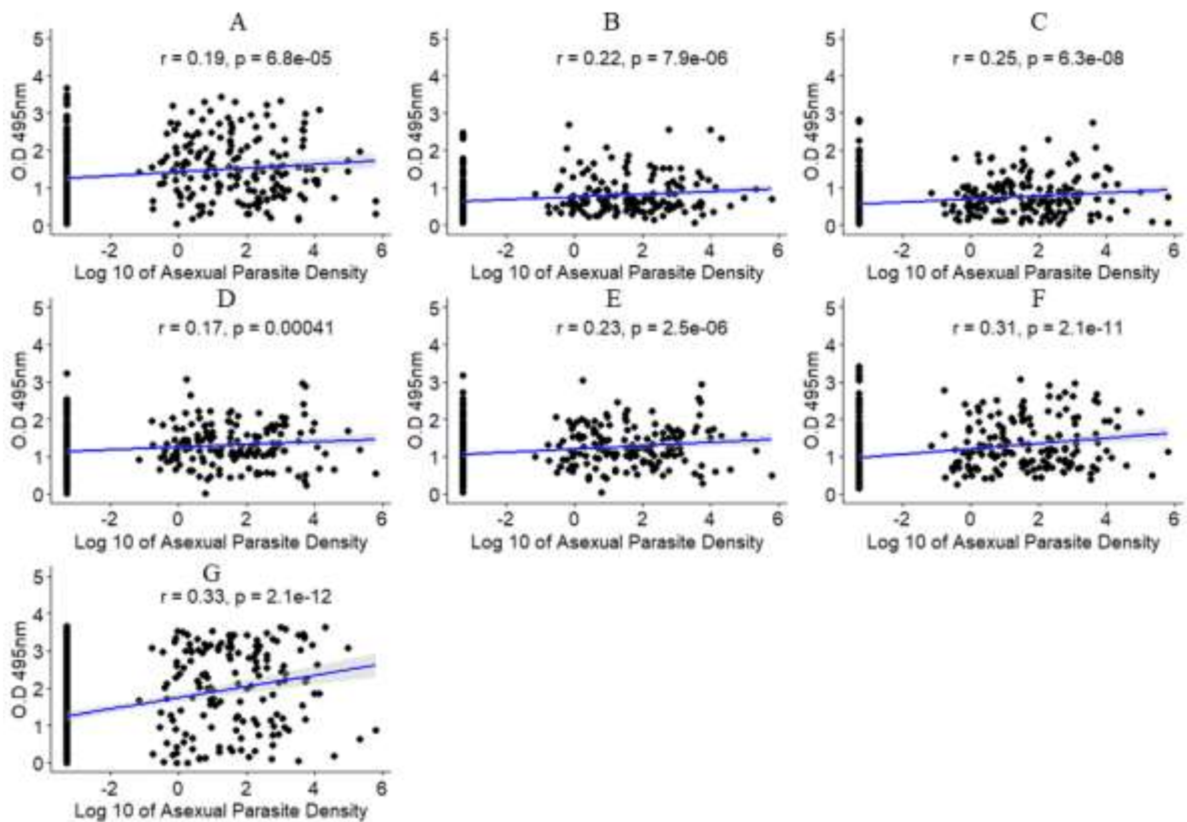



**Figure 4.4(b)** Correlation of responses with parasite density (qPCR18s)

The x-axis shows the log transformed density of parasites as measured by qPCR18s, while the y-axis represents the mean responses to each of the recombinant proteins. The recombinant proteins shown in the figure are as follows: CVMPPP (A), PEBP (B), PSOP1 (C), PSOP25 (3D7 and 10668 D and E respectively), MDV1 (F) and AMA-1 (G).

Responses to the recombinant gametocyte proteins were correlated with the density of asexual parasites as measured by NASBA18s. The responses were measured using ELISA and the antibody intensity given as ELISA optical density (O.D) values at 495nm. The recombinant proteins shown in the figure are as follows: CVMPPP, PEBP, PSOP1, PSOP25 (3D7 and 10668), MDV1 and AMA1 (A, B, C, D, E, F and G respectively). To determine a correlation between the responses to the density of parasites, the mean responses measured for each recombinant protein was plotted against the log transformed asexual parasite densities as measured by NASBA18s.

There was a significant correlation between the responses to the recombinant antigens and asexual parasite density across all the recombinant proteins (Figure 4.4(c)). (CVMPPP $r=0.22$, $p<0.001$, PEBP $r=0.28$, $p<0.001$, PSOP1 $r=0.23$, $p<0.01$, PSOP25_3D7 $r=0.22$, $p<0.001$, PSOP25_10668 $r=0.24$, $p<0.001$, MDV1 $r=0.35$, $p<0.001$, AMA1 $r=0.35$, $p<0.001$). The results suggest that an increase in asexual parasite density may result in an increase in the mean responses to the recombinant gametocyte specific proteins.

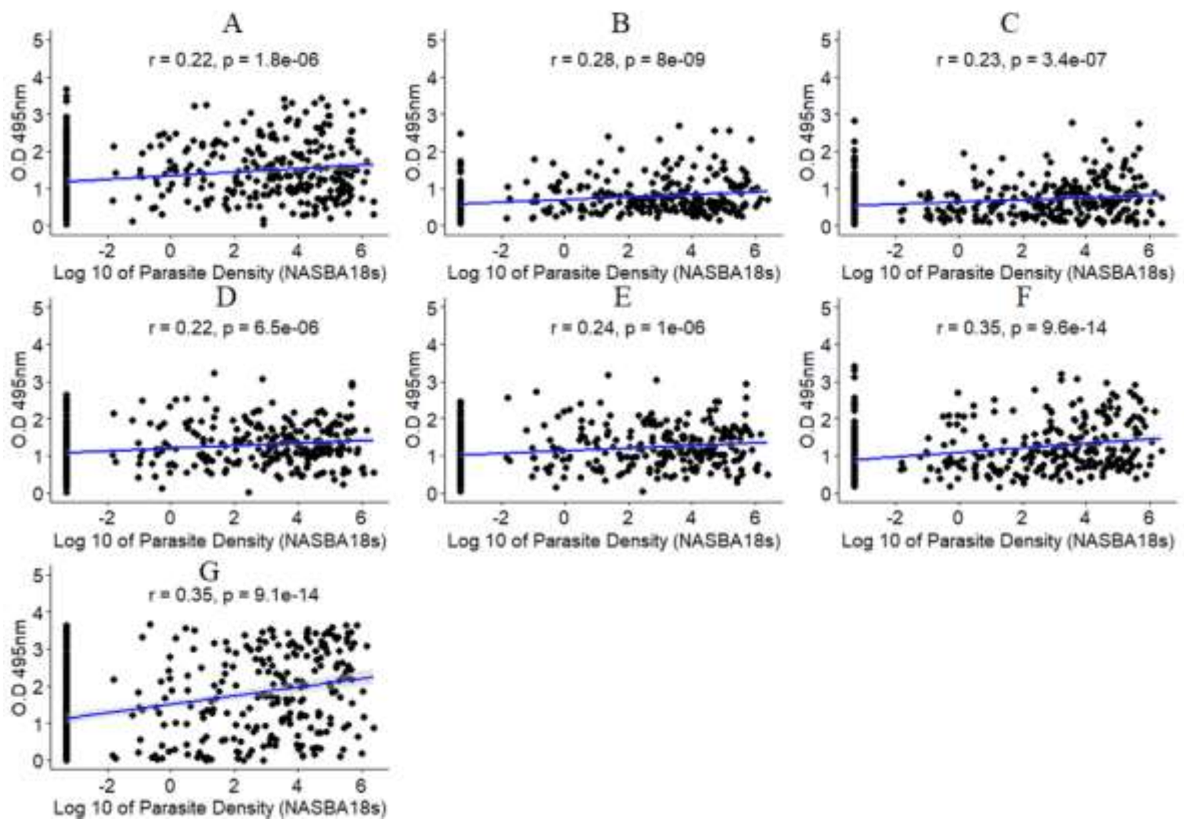



**Figure 4.4(c)** Correlation between responses and asexual parasite density (NASBA18s)

The x-axis shows the log transformed density of asexual parasitaemia while the y-axis represents the mean responses to each of the recombinant proteins, read at 495nm.

### 4.4.3 Correlation of responses to recombinant proteins with gametocyte density

Next, responses to the recombinant antigens were correlated with gametocyte density as measured by NASBAPfs25. Responses to the recombinant proteins were correlated with the density of sexual parasites as measured by NASBAPfs25. The responses to the recombinant proteins were measured using ELISA as given by optical density (O.D) values read at 495nm (Figure 4.4(d)). (CVMPPP $r=0.18$, $p<0.001$, PEBP $r=0.19$, $p<0.001$, PSOP1 $r=0.24$, $p<0.001$, PSOP25_3D7 $r=0.18$, $p<0.001$, PSOP25_10668 $r=0.24$, $p<0.001$, MDV1 $r=0.36$, $p<0.001$ and AMA1 $r=0.36$, $p<0.001$). A positive and significant correlation with gametocyte density was observed across all the recombinant proteins suggesting that an increase in the density of sexual parasites, may result in an increase in the observed mean responses to the gametocyte specific proteins.

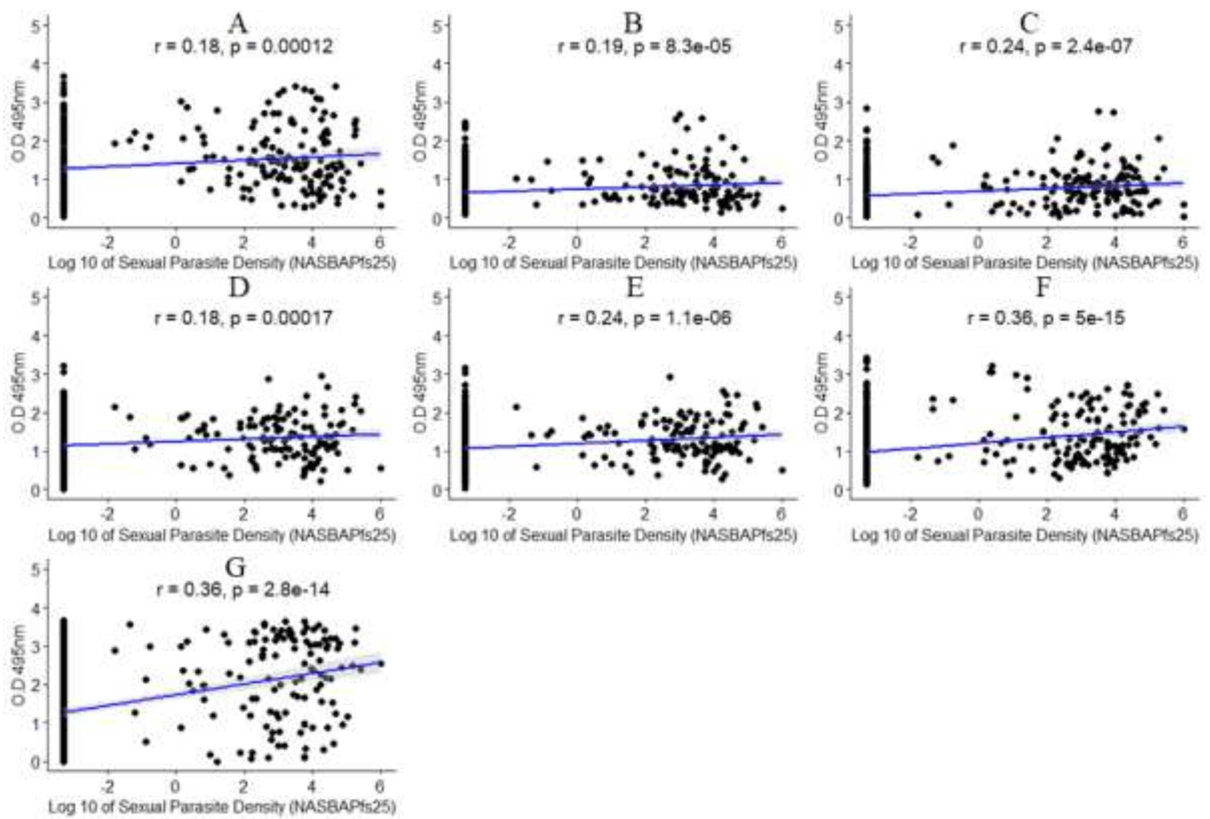



**Figure 4.4(d)** Correlation between responses and gametocyte density

The recombinant proteins shown in the figure are as follows: CVMPPP, PEBP, PSOP1, PSOP25 (3D7 and 10668), MDV1 and AMA1 (A, B, C, D, E, F and G respectively). The x-axis is the log transformed density of sexual parasites while the y-axis represents the mean responses for each of the recombinant gametocyte proteins.

### 4.4.5 Correlation of responses between the recombinant proteins

Furthermore, a Spearman's rank correlation test was performed to determine the correlation of responses between the recombinant proteins included in the analysis. The correlation matrix plot shown on Figure 4.4.5 shows a representation of the strength of association as denoted by the intensity of the colour as presented in the scale on the right of the plot and is a summary of the Spearman's rank correlation test. There is a significant correlation between responses to PSOP25_3D7 and PSOP25_10668 ($\rho = 0.89$) (Figure 4.4(d)). Responses to CVMPPP and PEBP, also showed a strong association. Overall there is a significantly high correlation between responses to all the recombinant gametocyte-specific proteins.

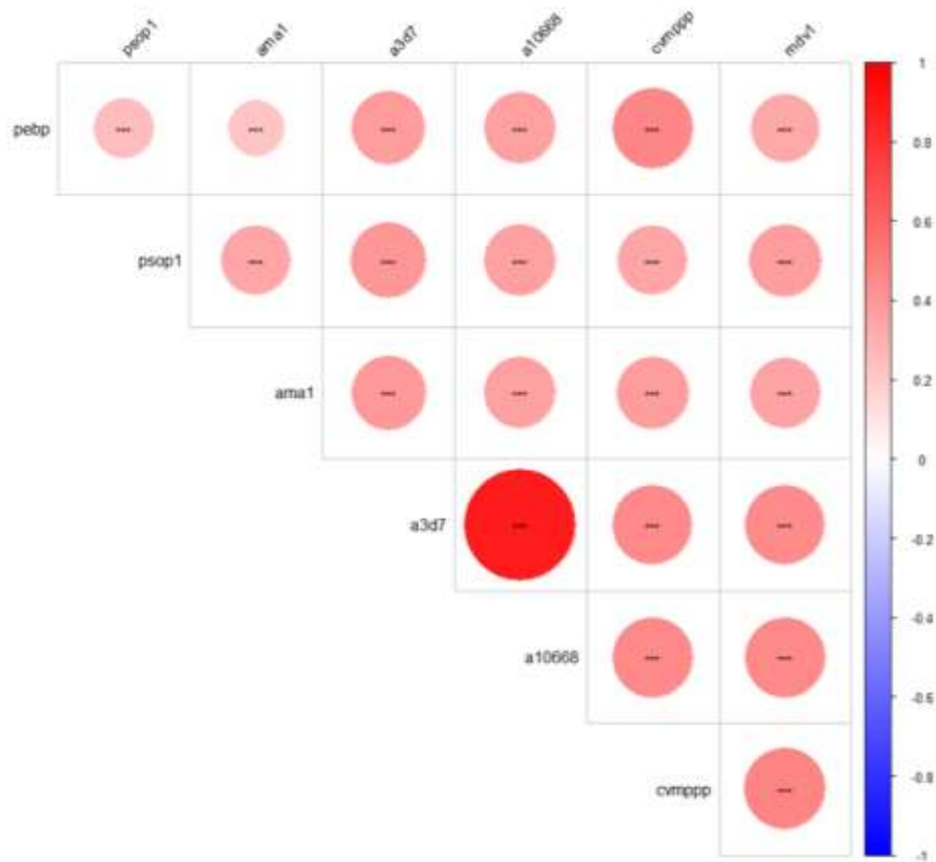



**Figure 4.4(e) Correlation of responses between the recombinant proteins.**

The correlation matrix plot (correlogram) shows a summary of the Spearman's correlation test. As shown by the asterisks, the correlation of responses between recombinant antigens is extremely significant (*p<0.05 ~* *, *p<0.01 ~* **, *p<0.001 ~* ***). Colour chart on the right and the size of the circle are proportional to the correlation coefficients.

### 4.5 AFIRM study Logistic regression: Multivariable Analysis for odds of gametocytaemia

Additionally, a model was fitted to determine if there would be any association between the status of gametocytaemia (whether positive or negative for gametocytes), to any confounding variables. The confounders that were added to the model are summarised in Table 4.5 (a) and are: (a) responses to recombinant gametocyte-specific proteins measured by OD; (b) age group in categories; (c) season of sampling; (d) parasite density as measured by qPCR, (e) sickle gene and (f) α-Thalassemia status. The variance inflation factors resulting from the covariates added to the model are shown on Table 4.5 (b). The model in Figure 4.5(a), shows the impact of interaction between predictor variables and the impact of this on the outcome, gametocyatemia. The model shows that individuals in whom parasites were detected by qPCR18s, had 1% higher odds of being gametocyte positive compared to those in whom no parasites could be detected, and this was statistically significant.

When adjusted for parasitaemia, participants who were under 5 years of age had 99% odds of being gametocyte negative compared to their counterparts from the other two age categories. Further, individuals who responded to the antigens MDV1 and AMA1, had higher odds of being gametocyte positive, in that for every one-unit increase in the mean response to the two recombinant proteins, an increase of 160% and 93% in the odds of gametocyte positivity was seen and this was significant ($p<0.05$). One-unit increase in the mean response to PSOP25_3D7 increased the odds of being gametocyte negative by 34% ($p<0.05$). Participants who were sampled during the dry season had 2% higher odds of being gametocyte positive compared to those sampled during the dry season, when adjusted for the parasite density as measured by qPCR18s.

**Table 4.5 (a)** Logistic regression for odds of gametocytaemia

| | **Multivariable analysis** | | | |
|---|---|---|---|---|
| | **Covariates** | **Odds Ratio** | **95% C.I** | **P-value** |
| Age Group Categories | Less than 5 years | 0.54 | -1.7, 4.3 | 0.27 |
| | Older than 15 years | 0.38 | -1.3, 0.2 | 0.16 |
| | Less than 5 years and parasitaemia (qPCR.18s) | 0.005 | -0.01, -0.0017 | **<0.05** |
| | Older than 15 years and parasitaemia (qPCR.18s) | 0.003 | -0.01, 0.002 | 0.2 |
| Season (Ref: Wet season) | Dry season | 0.33 | -0.7, 0.6 | 0.81 |
| | Dry season and parasitaemia (qPCR.18s) | 0.007 | 0.004, 0.03 | **<0.05** |
| Parasitaemia (qPCR18s) , Ref: Undetectable parasitaemia) | Detectable parasitaemia (qPCR18s) | 0.002 | 0.003,0.1 | **<0.01** |
| | Parasitaemia (qPCR18s) and Alpha Thalassemia Homozygotes | 0.52 | -0.002 - 0.00005 | 0.1 |
| | Parasitaemia (qPCR.18s)and Alpha Thalassemia Normal | 0.0009 | -0.002, 0.003 | 0.9 |
| | qPCR.18s:Sickle Heterozygotes (AS) | 0.0077 | 0.004, 0.02 | |
| Recombinant Proteins | CVMPPP | 0.24 | -0.5, 0.5 | 0.98 |
| | PEBP | 0.36 | -0.2, 1.3 | 0.13 |
| | PSOP1 | 0.32 | -0.4, 0.8 | 0.49 |
| | PSOP25_3D7 | 0.58 | -2.3,-0.03 | **<0.05** |
| | PSOP25_10668 | 0.56 | -0.4, 1.8 | 0.2 |
| | MDV1 | 0.27 | 0.4, 1.5 | **<0.001** |
| | AMA1 | 0.14 | 0.4, 1.5 | **<0.001** |
| Alpha-Thalassemia (Ref: Heterozygotes ) | Homozygotes | 0.47 | -1.4, 0.5 | 0.42 |
| | Normal (Hb) | 0.33 | -0.04,1.3 | 0.07 |
| Sickle gene (Ref: Normal AA) | Heterozygotes (AS) | 0.43 | -0.5, 1.2 | 0.43 |

**Table 4.5 (b)** Variance Inflation Factors calculated for the covariates included in the model

| **Co-variate** | **VIF (Variance Inflation Factor)** | **DF (Degrees of freedom)** |
|---|---|---|
| Age group categories | 1.12 | 2 |
| Season | 1.11 | 1 |
| qPCR18s | 65.03 | 1 |
| CVMPPP | 1.24 | 1 |
| PEBP | 1.19 | 1 |
| PSOP1 | 1.13 | 1 |
| PSOP25_3D7 | 2.18 | 1 |
| PSOP25_10668 | 2.08 | 1 |
| MDV1 | 1.16 | 1 |
| AMA1 | 1.13 | 1 |
| Alpha-Thalassemia gene | 1.04 | 2 |
| Sickle gene | 1.11 | 1 |
| Age group and parasitaemia by PCR.18s | 8.6 | 2 |
| Parasitaemia(qPCR.18s) and Alpha-Thalassemia gene | 4.08 | 2 |
| Season and parasitaemia (qPCR18s) | 1.08 | 1 |
| Parasitaemia (qPCR.18s) and Sickle gene | 1.14 | 1 |

The forest plot below shows a summary of the regression analysis to determine the association between the presence or absence of gametocytes, and predictor variables as well as includes interaction within the predictor variables. The confounders added to the model were as follows: Responses to recombinant gametocyte-specific proteins measured by OD, age group in categories, season of sampling, parasite density as measured by qPCR, sickle gene and α-Thalassemia.

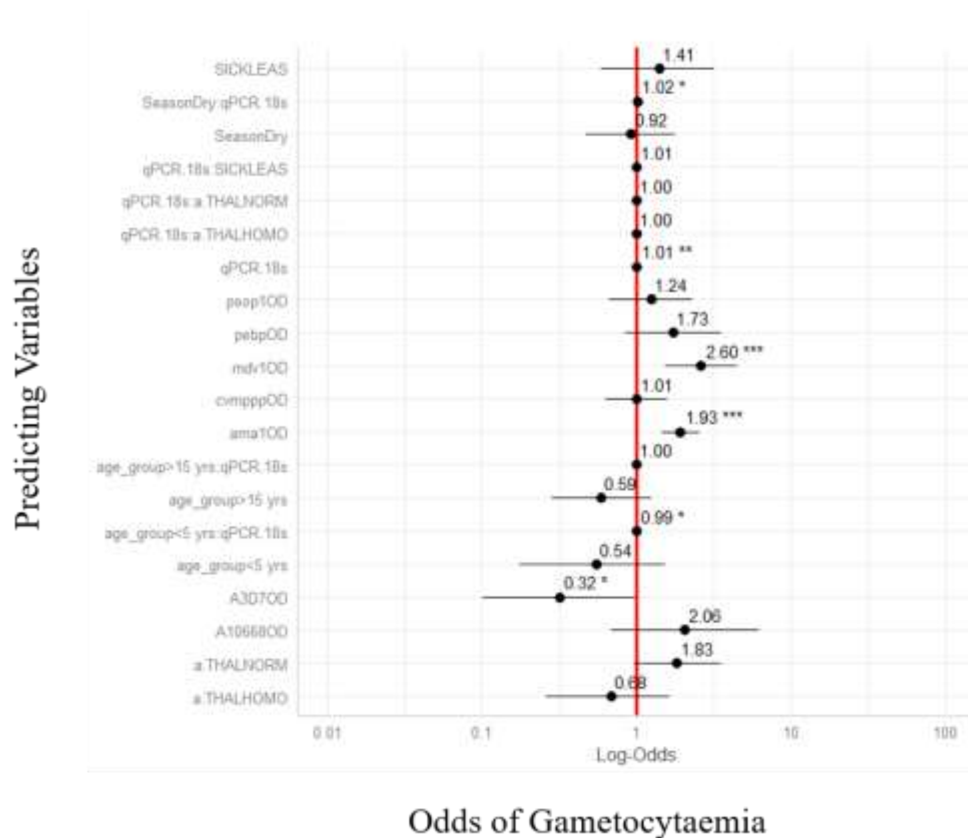



**Figure 4.5(a)** Forest plot showing summary of association between gametocytes status with confounding variables

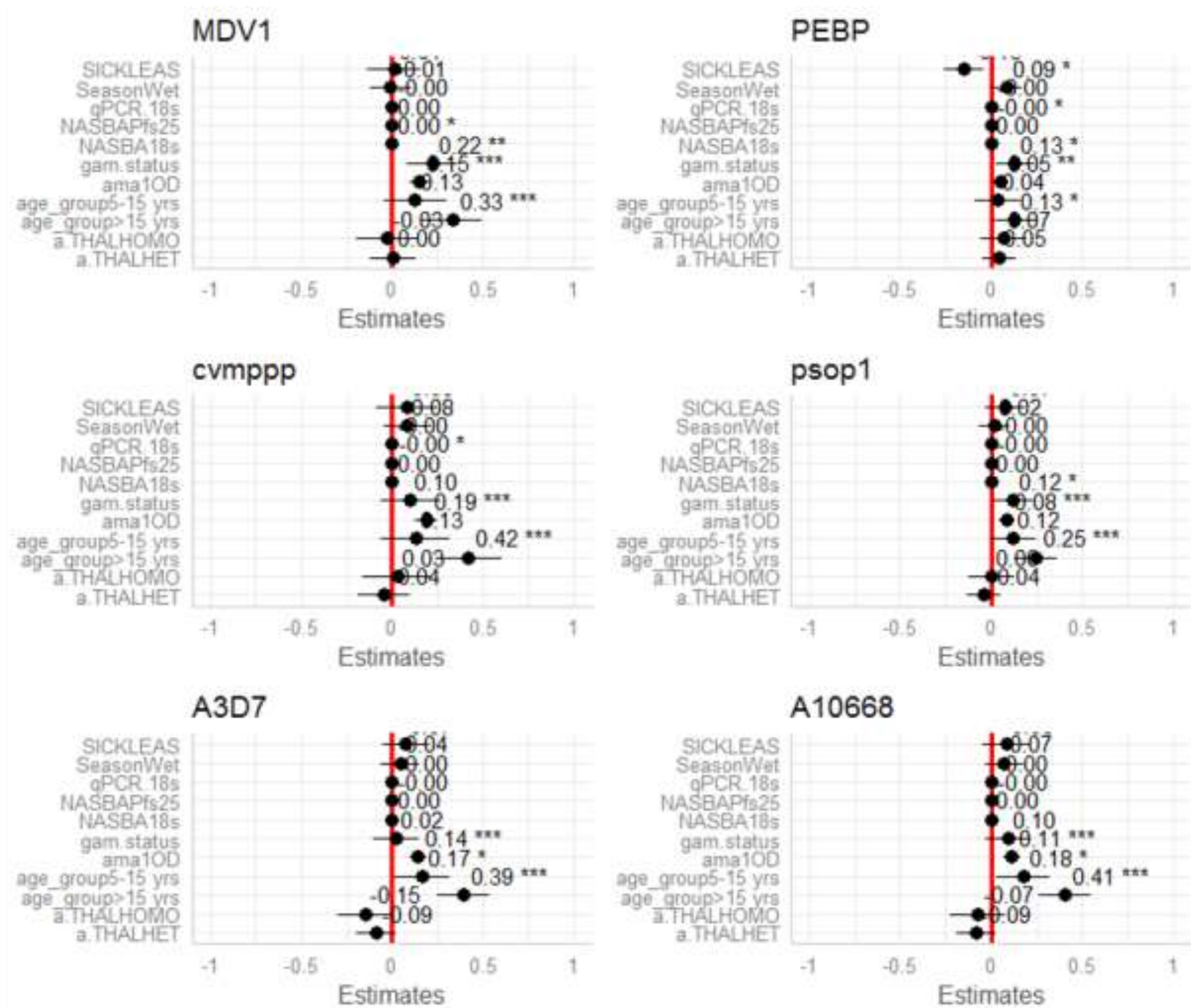
Key of forest plot from top to bottom: (a) sickle status, (b) season with parasite density, (c) season, (d) parasite density with sickle status, (e, f) parasite density with α-Thalassemia status, (g) parasite density, responses to antigens PSOP1 (h), PEBP (i), MDV1 (j), CVMPP (k), AMA1 (l), above 15 year-olds with parasite density (m), responses of those above 15 years (n), less than 5 year-olds with parasite density (o), less than 5 year olds (p), responses to antigens PSOP25-3D7 (q), PSOP25-10668 (r), α-Thalassemia normal individuals (s) and α-Thalassemia homozygous (t). Asterisks (*p<0.05 ~ *, p<0.01 ~ **, p<0.001 ~ ****).

An association between the mean responses to the recombinant gametocyte-specific proteins and a series of variables of interest was determined. To relate the outcome (in this case the responses to the different antigens) to a set of predictors of interest, a multivariable linear regression model was performed and shown Figure 4.5(b). The following predictors were included in the model: age in categories, gametocyte status, season, parasite density (as measured by qPCR18S, NASBA18s and NASBAPfs25), responses to AMA-1, sickle gene and α-thalassemia. The results indicate that parasite densities as measured by qPCR18S, NASBA18s and NASBAPfs25 do not appear to influence responses observed for the antigens. Gametocyte status on the other hand, appears to be a significant predictor of responses to the antigens MDV1, PEBP and PSOP1.

For these antigens, the presence of gametocytes results in an increase in the mean responses to the recombinant proteins by 0.22, 0.13 and 0.12 of one OD value, respectively with reference to individuals negative for gametocytes. For the other antigens, while gametocyte positivity influences responses, the increase does not appear to be statistically significant. Age as a predictor indicates that with reference to those between 5-15 years, individuals older than 15 years had higher mean responses to MDV1, CVMPPP, PSOP1, PSOP25-3D7 and PSOP25-10668 by 0.23, 0.30, 0.17, 0.28 and 0.29 of one OD value, respectively.

The model shows that with reference to the dry season, those sampled during the wet season had an increased mean response per one OD, of cvmppp = 0.08, psop1 = 0.02, psop25_3d7 = 0.04, psop25_10668 = 0.07, mdv1 -0.01. Only PEBP shows a significant increase in mean response among those sampled during the wet season (0.09, $p<0.05$). For the sickle trait, those heterozygote for the sickle gene (AS) did not significantly increase the mean responses to the recombinant antigens, with the sickle gene homozygotes (AA) as the reference. It was observed that individuals with the sickle trait had a lower mean responses to PEBP (-0.15, $p<0.01$). For α-thalassemia individuals, with heterozygotes as the reference, there was no influence to the antibody responses against the recombinant antigens (homozygotes 0.47, $p = 0.42$, normal 0.33, $p = 0.07$). A similar observation was made when parasite density was included as measured by qPCR18S, NASBA18s and NASBAPfs25.

Predicting Variables




Responses to Antigens

**Figure 4.5 (b)** Forest plots showing summary of multivariable linear regression analysis Key from top to bottom: (a) SICKLEAS-sickle trait status with 'AS' as reference, (b) SeasonWet-Season with the wet season as the reference, parasite densities as measures by qPCR18S (c), NASBA18s (d) and NASBAPfs25 (e), gam.status-gametocyte status (f), ama10D-responses to AMA1 (g), 5-15 year olds (h), older than 15 years (i), a.THALHOMO-α-thalassemia homozygotes (j), a.THALHET-α-thalassemia heterozygotes (k). Asterisks ($p<0.05$ ~ *, $p<0.01$ ~ **, $p<0.001$ ~ ***).

## 4.6 Immunoprofiling Analysis of the LAMB cohort

The LAMB study was longitudinal in design whereby participants were followed up over time, contributing six different time points over the period of follow up. The longitudinal design of the study hoped to overcome the limitations of the cross-sectional study by incorporating the temporal element of infection as immune responses vary during infection. Participants were recruited from Kilifi, Kenya and were aged between 18 to 65 years. Plasma was collected six times over the course of 4 months with the first sample taken at baseline, followed by twice fortnightly (every 2 weeks) and thereafter three times every 4 weeks.

Firstly, responses to all the antigens were analysed over the different time points. Antibody responses to the recombinant gametocyte-specific proteins over time. Participants of the LAMB study were followed up over time and samples collected over six different time points, that is, on the first day, denoted as *D0*, on day 14 (*D14*), day 28 (*D28*), day 56 (*D56*), day 84 (*D84*) and day 112 (*D112*). Sample size over the different time points are D0=52, D14=37, D28=50, D56=46, D84=47, and D112=52. Antibody responses to the recombinant proteins were measured using ELISA as given by optical density (OD) values read at 495nm. The recombinant proteins shown in the graphs are as follows: A-CVMPPP, B-PEBP, C-PSOP1, D-PSOP25_3D7, E-PSOP25_10668, F-MDV1, G-CPP4 and H-AMA1. The box plots in Figure 4.6(a), show the antibody responses against the recombinant gametocyte-specific proteins over the six time points. There was no significant difference in the mean responses to each of the recombinant proteins over the different days: [(CVMPPP $p$ = 0.87, PEBP $p$ = 0.38, PSOP1 $p$ = 0.91, PSOP25_3D7 $p$ = 0.91, PSOP25_10668 $p$ = 0.98, MDV1 $p$ = 0.097, CPP4 $p$ = 0.02, AMA1 $p$ = 0.87)]. All the box plots show only individuals from Kilifi. Samples from UK donors who served as the negative control were used to calculate the cut off for seropositivity (shown in the graphs as the solid black line running across from the y-axis), and are excluded from all the graphs.

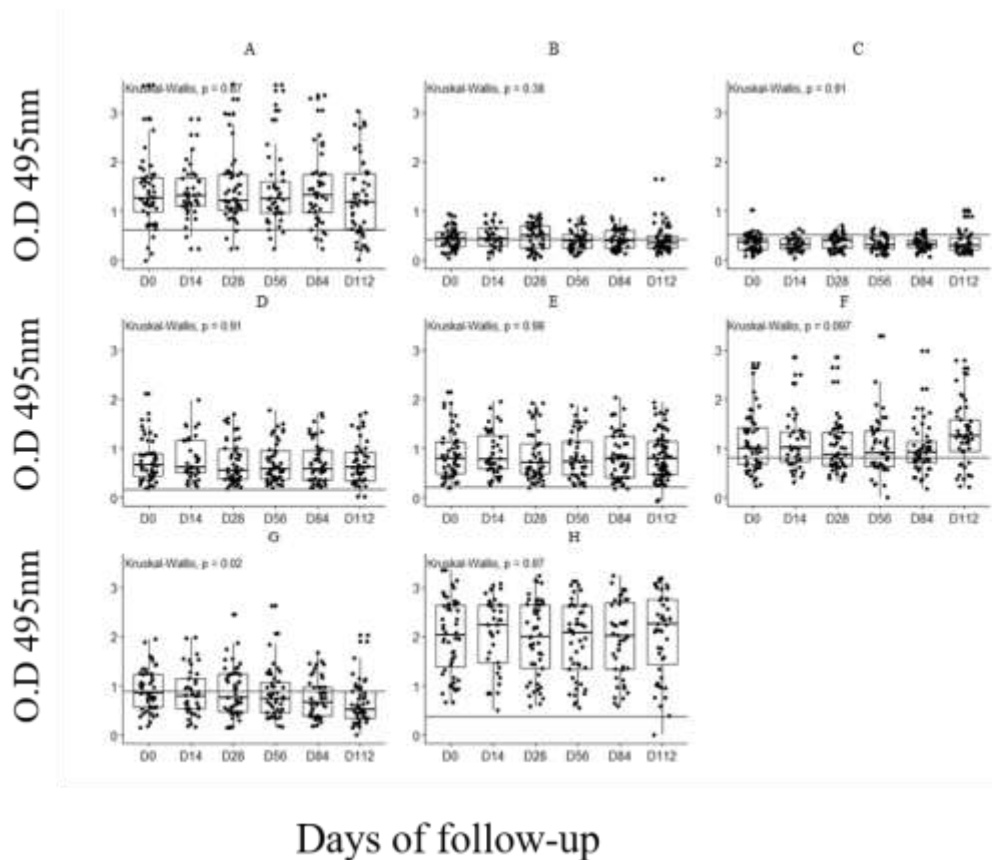



**Figure 4.6(a)** Antibody responses to recombinant gametocyte antigens over time

The mean responses to the recombinant antigens are given as the OD values read at 495nm and are represented on the *y*-axis for each of the proteins. The *x*-axis shows days of follow-up. The recombinant proteins shown in the graphs are as follows: A-CVMPPP, B-PEBP, C-PSOP1, D-PSOP25_3D7, E-PSOP25_10668, F-MDV1, G-CPP4 and H-AMA1.The box plots show the mean responses to the recombinant protein over the six time points and include a p value and 95% Kruskal-Wallis confidence intervals.

To determine if there was overall decline or increase in the antibody over time, a trend analysis was performed. The mean responses to the proteins were plotted for each of the days of follow-up. The scatter plots include the cut-off line represented by the black horizontal lines and the trend line represented by the red line. The cut-off for the different antigens was calculated as the mean plus 3 standard deviations of the non-immune controls. The graphs also show the cut off line for seropositivity, as the black solid line across each graph, arising from the y-axis. Therefore, those falling below the cut-off line are considered to elicit responses lower than those seen in malaria-naïve individuals, while those above the line are considered to have high responses to the proteins thus seropositive. The days of follow-up are day 0, 14, 28, 56, 84 and 112, with sample sizes of D0=52, D14=37, D28=50, D56=46, D84=47 and D112=52. There was no clear or consistent pattern of responses to the recombinant antigens over the time points analysed (Figure 4.6(b)).

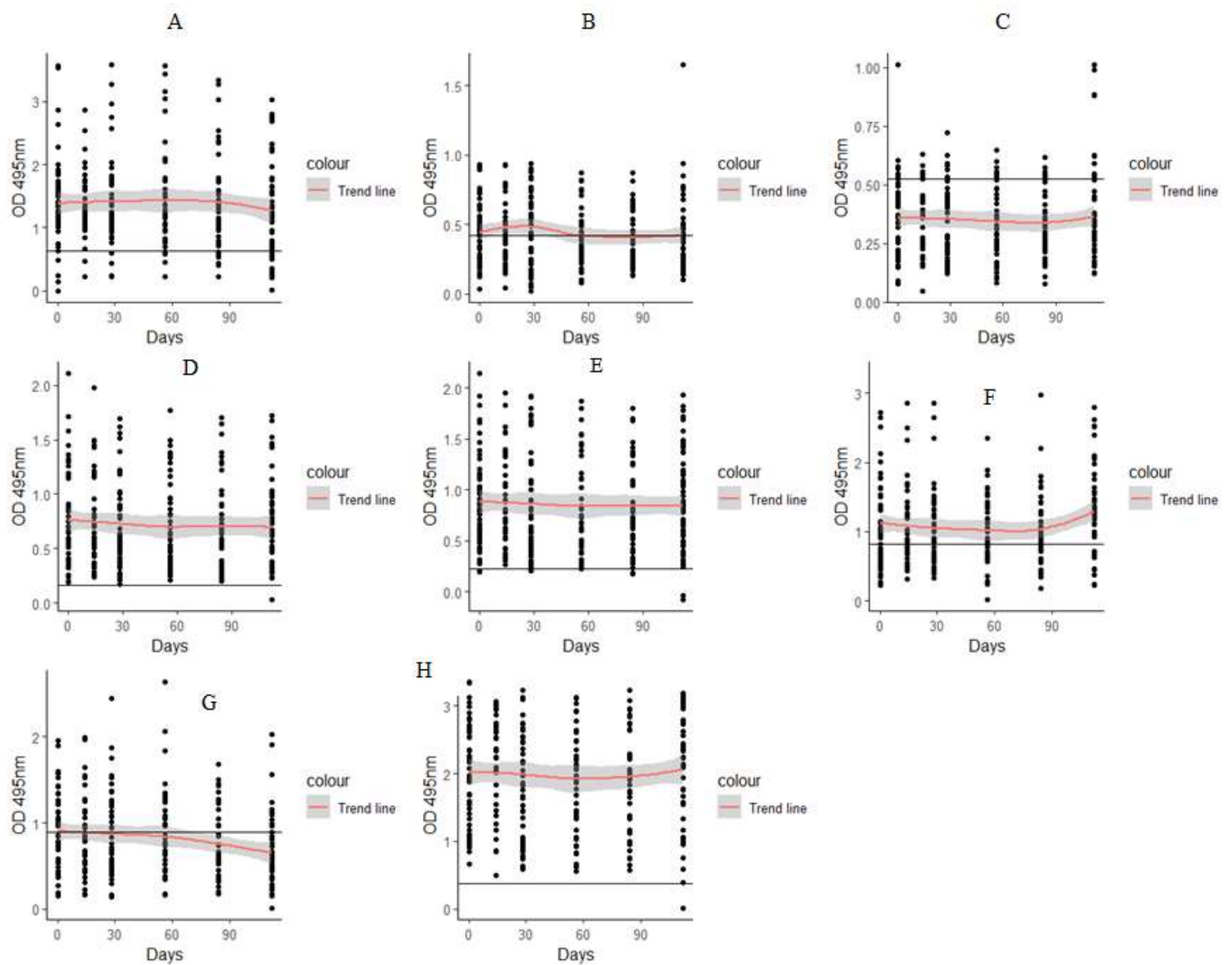



**Figure 4.6(b)** Trend of responses to recombinant gametocyte antigens over time

The x-axis on the plots shows the time points in days and the y-axis shows the optical density (OD) values. Here, the days are represented continuously on the x-axis and the recombinant proteins shown in the graph as: A-CVMPPP, B-PEBP, C-PSOP1, D-PSOP25-3D7, E-PSOP25-10668, F-MDV1, G-CPP4 and H-AMA1. A trend line, indicated by the solid lines on the graphs is included to show the trend of antibody responses over time, with the grey shaded area around the red lines showing the confidence intervals.

A similar analysis to determine a trend with parasitaemia to assess if the density of parasitaemia would influence the responses observed for the different antigens was performed. The density of parasites was measured by qPCR, while antibody responses to the recombinant gametocyte specific proteins were measured over the six time points using ELISA and antibody intensity is given as the ELISA optical density (OD) values read at 495nm. To determine the influence of parasite density on the responses to the recombinant proteins over time, the mean responses to the proteins were plotted for each of the days of follow-up. The days of follow-up are day 0, 14, 28, 56, 84 and 112, with sample sizes of D0=52, D14=37, D28=50, D56=46 D84=47 and D112=52 respectively. Here, the days are represented continuously on the x-axis and the recombinant proteins shown in the graphs are as follows: A-CVMPPP, B-PEBP, C-PSOP1, D-PSOP25_3D7, E-PSOP25_10668, F-MDV1, G-CPP4 and H-AMA1. The graphs also the trend line as indicated by the red solid line. The responses to the recombinant gametocyte-specific proteins were correlated with qPCR over the different time points and are presented in the scatter plots in Figure 4.6(c) and show a steady and consistent upward trend for responses to all the recombinant proteins with parasite density.

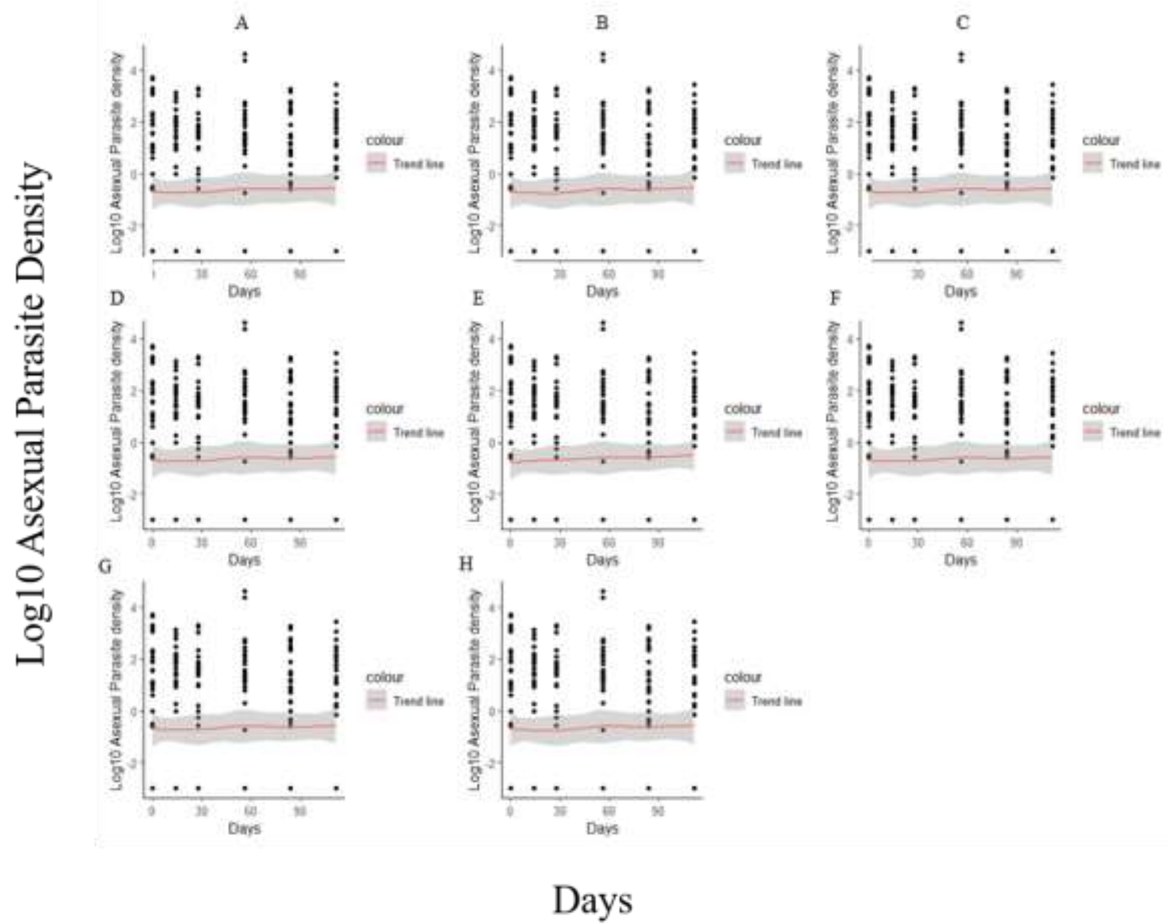



**Figure 4.6(c)** Trend of parasitaemia as measured by qPCR over time

Day of follow-up are represented on the x-axis, against the log transformed density of parasites measured by qPCR, as shown on the y-axis.

### 4.6.1 Correlation of responses between the recombinant proteins

Furthermore, a spearman's rank correlation test to determine the correlation of responses between antigens included in the analysis was performed. Responses to the two versions of PSOP25, i.e. PSOP25_3D7 and PSOP25_10668 had a strong correlation ($\rho=0.83$, $p=<0.05$). Figure 4.6(d) shows a summary of the correlation matrix between responses to the recombinant proteins and includes the significance of the correlation ($p<0.05$). The strength of correlation is represented by the intensity of the colour presented in the scale to the right of the plot. There is was a significant correlation between responses to MDV1 and PSOP25_3D7, MDV1 and PSOP25_10668, PEBP and CPP4, CVMPPP and CPP4, CVMPPP and PSOP25_3D7, CVMPPP and PSOP25_10668, CPP4 and PSOP25_3D7 and between CPP4 and PSOP25_10668. While there is a correlation observed, there is no statistical significance between responses to PEBP and AMA1, PSOP1 and AMA1 and between PSOP1 and CVMPPP.

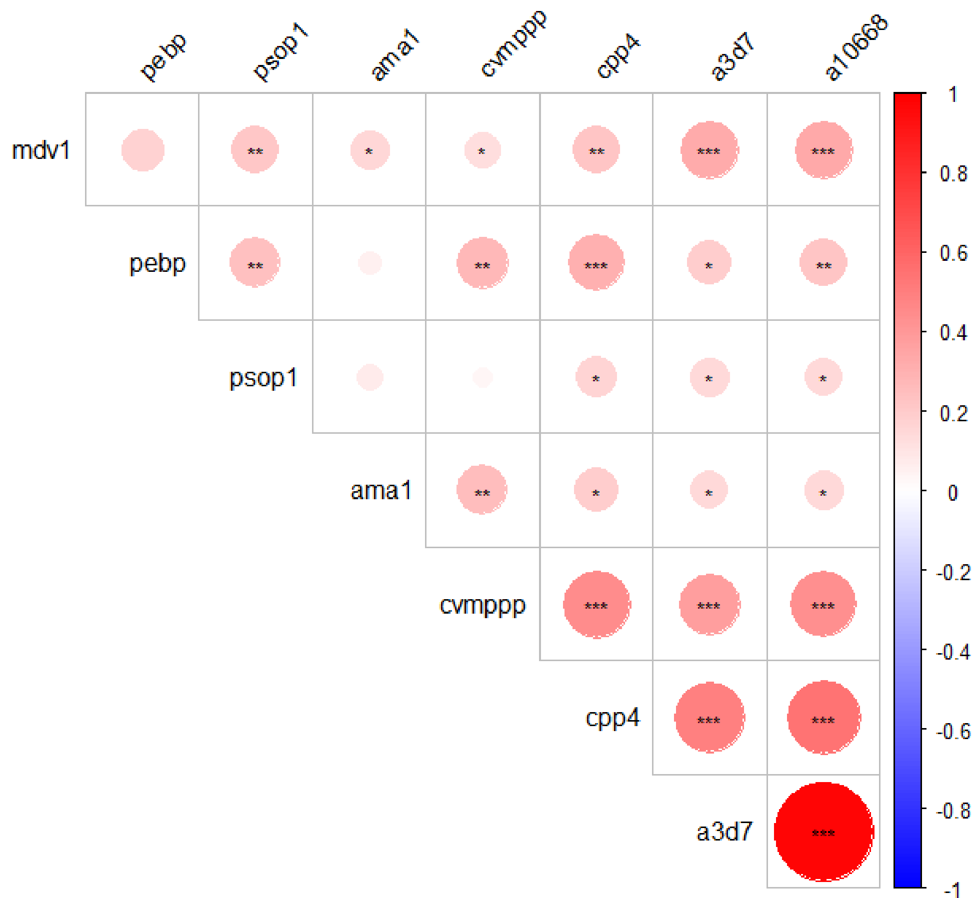



**Figure 4.6(d)** Correlation of responses between the recombinant proteins

The asterisk show the significance of correlation between any two recombinant (*p<0.05* ~ *, *p<0.01* ~ **, *p<0.001* ~ ***). The scale to the right-hand side of the plot shows the colour scale indicating the strength of correlation based on the intensity of the colour shown.

### 4.6.2 Multivariable Regression Analysis: Multivariable analysis for the LAMB cohort

To determine the association between parasitaemia with variables such as the mean responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age, sickle gene and α-thalassemia a multivariable logistic regression model was used. The summary of the regression analysis is presented in the forest plot in Figure 4.6(e). The regression analysis presented in the plot, shows the association between various predictor variables and parasite density as measured by qPCR. The results summarised in Table 4.7 (a) show that for one-unit increase in the mean response to AMA1, the odds of parasitaemia are 56% significantly ($p<0.05$) higher when adjusted for responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age, sickle gene and α-thalassemia.

Age appears to result in a 96% increased odds of being negative for parasites with increase in age, while adjusting for responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age, sickle gene and α-thalassemia. Individuals homozygous for α-Thalassemia had 18% decreased odds of having detectable parasites while heterozygote individuals had 49% increased odds of presenting with detectable parasites in comparison to those with the normal trait while adjusting for responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age and sickle trait. The variance inflation factors associated with adding covariates to the model are summarised in Table 4.7 b.

Participants heterozygote for the sickle gene (AS) had 6% increased odds of presenting with detectable parasite density compared to normal participants, while adjusting for responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age, sickle gene. Responses to the recombinant proteins show that with one-unit increase in responses to the antigens the odds of detectable parasitaemia increase by 5% for CVMPPP, 111% for PEBP, 161% for PSOP1, 45% for PSOP25_3D7, 83% for PSOP25_10668 and 8% for MDV1. Presenting with a fever showed to increase the odds of having detectable parasitaemia by 18%, compared to those who did not present with fever, all while adjusting for responses to the recombinant gametocyte-specific proteins, the day of follow-up, age, sickle gene and α-thalassemia.

**Table 4.7 (a)** Logistic regression for odds of parasitaemia

| | **Multivariable Analysis** | | | |
|---|---|---|---|---|
| | **Co-variate** | **Std error** | **95% C.I** | **P-value** |
| Recombinant protein | AMA1 | 0.21 | 0.04, 0.9 | **<0.05** |
| | CVMPPP | 0.27 | -0.5, 0.6 | 0.85502 |
| | PEBP | 0.73 | -0.7, 2.2 | 0.30240 |
| | PSOP1 | 1.03 | -1.1, 3.01 | 0.35188 |
| | PSOP25_3D7 | 1.51 | -2.6, 3.4 | 0.80700 |
| | PSOP25_10668 | 1.38 | -3, 2.5 | 0.89306 |
| | MDV1 | 0.31 | -0.5, 0.7 | 0.79803 |
| | CPP4 | 0.41 | -0.9, 0.7 | 0.75479 |
| Day of follow-up (Ref Day 0) | Day 14 | 0.49 | -0.5, 1.5 | 0.34318 |
| | Day 28 | 0.45 | -0.8, 0.9 | 0.89814 |
| | Day 56 | 0.45 | -0.4, 1.4 | 0.30741 |
| | Day 84 | 0.47 | -0.8, 1.1 | 0.75007 |
| | Day 112 | 0.60 | -1.1, 1.3 | 0.90366 |
| Age of participants | | 0.014 | -0.1, -0.01 | **<0.01** |
| Alpha-Thalassemia (Ref Hb normal) | Homozygotes | 0.6 | -1.4, 0.97 | 0.74188 |
| | Heterozygotes | 0.34 | -0.3, 1.1 | 0.24245 |

| Sickle Gene (Ref AA normal) | Heterozygotes (AS) | 0.41 | -0.7, 0.9 | 0.87925 |
|---|---|---|---|---|
| Fever | | 0.63 | -1.1, 1.5 | 0.79171 |

**Table 4.7 (b)** Variance Inflation Factors calculated for the covariates included in the model

| Co-variate | VIF (Variance Inflation Factor) | DF (Degrees of Freedom) |
|---|---|---|
| AMA1 | 1.12 | 1 |
| CVMPPP | 1.38 | 1 |
| PEBP | 1.12 | 1 |
| PSOP1 | 1.07 | 1 |
| PSOP25_3D7 | 4.44 | 1 |
| PSOP25_10668 | 4.67 | 1 |
| MDV1 | 1.26 | 1 |
| CPP4 | 1.33 | 1 |
| Day of follow-up | 1.01 | 5 |
| Age | 1.18 | 1 |
| Alpha-Thalassemia | 1.15 | 2 |
| Sickle gene | 1.21 | 1 |
| Fever | 1.04 | 1 |

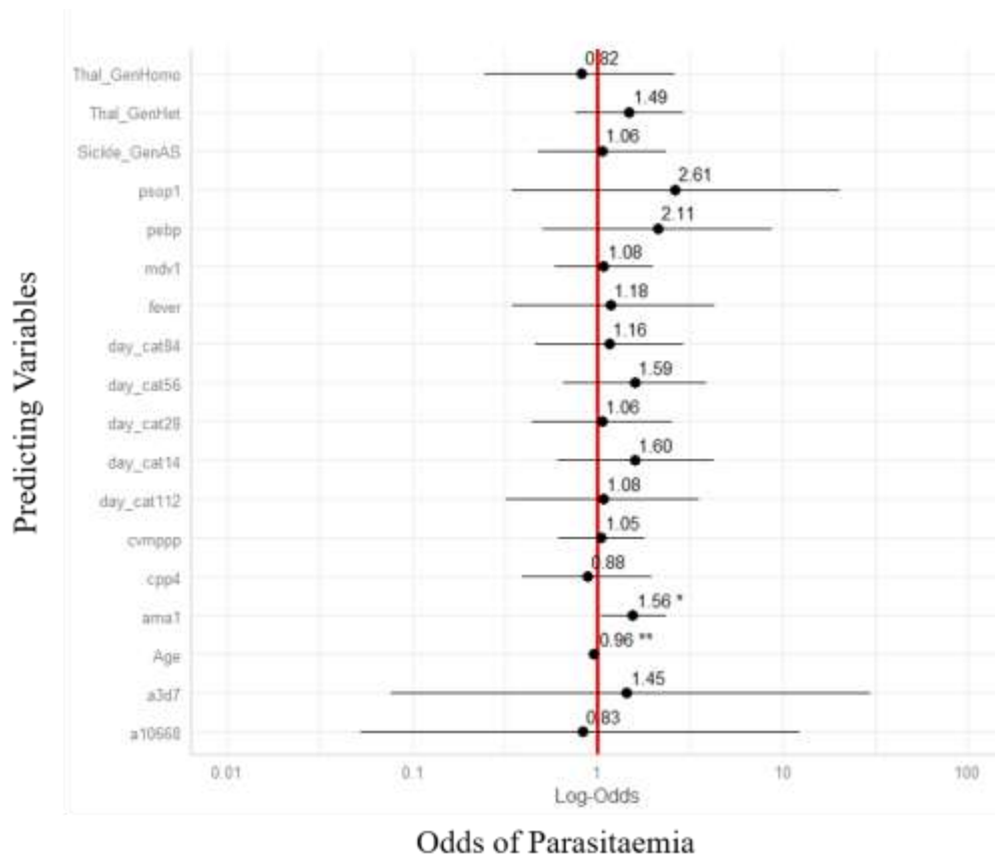
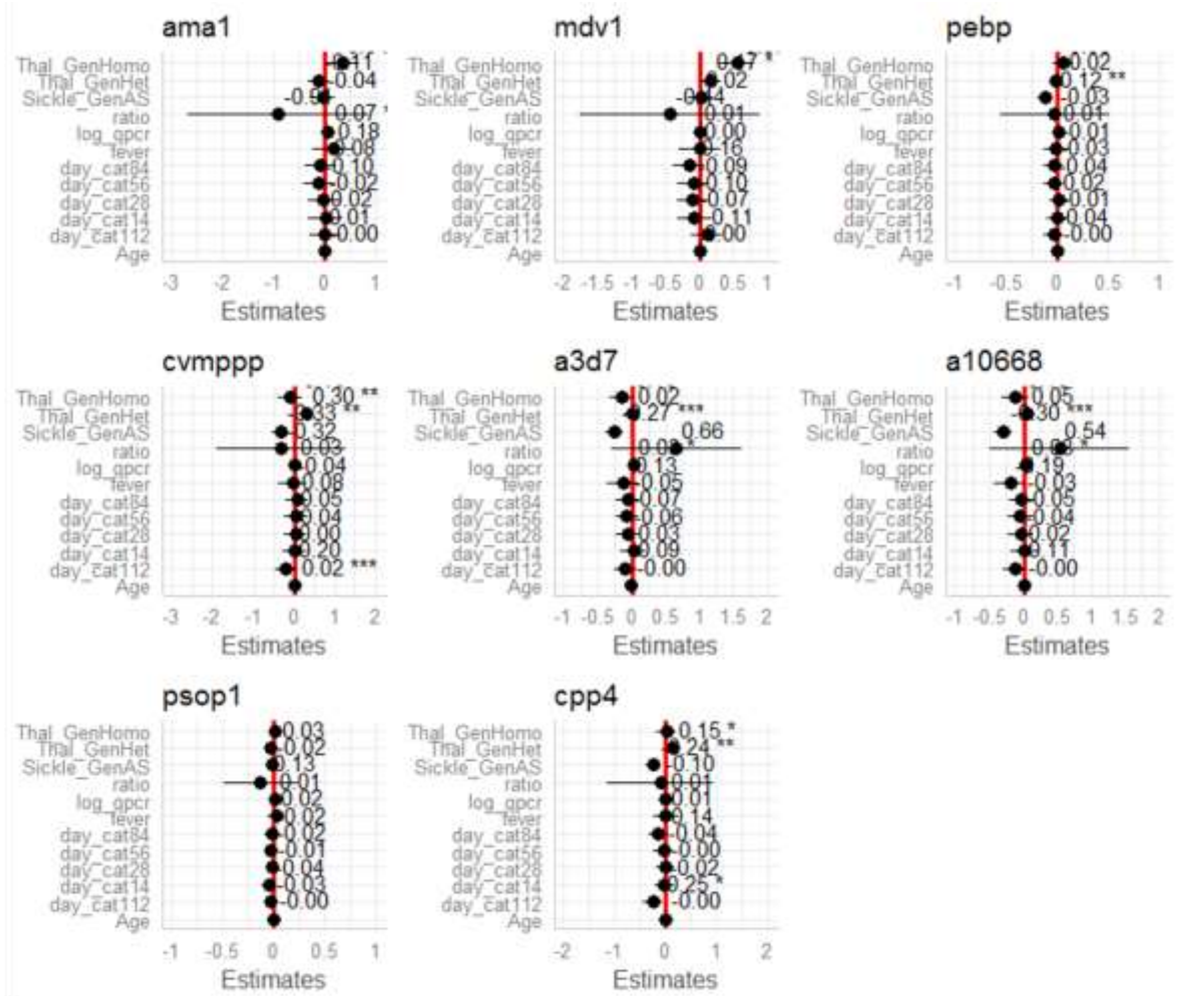



**Figure 4.6(e)** Forest plots showing a summary of the multivariable logistic regression analysis with parasite density as measured by qPCR as the outcome

The forest plots above show a summary of the logistic regression analysis for the odds of parasitaemia as detected by qPCR. The model includes the association between parasitaemia with variables such as the mean responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age, sickle gene and α-thalassemia.

A multivariable linear regression model was used to relate the outcome, responses to the different antigens to a set of predictors of interest. The following predictors were included in the model: age, time-point in days, and density of parasites as measured by qPCR, sickle gene, α-thalassemia, fever (≥37.5°C) and ratio of monocytes to lymphocytes. From the model (Figure 4.6(f)), the influence of age on the responses to the recombinant proteins is only significant for CVMPPP where it results in an increase in the mean responses by 0.02 of 1 OD ($P$ <0.01), while adjusting for all other factors. The day of follow-up, as per the model, did not significantly influence responses to the recombinant proteins. Fever, according to the model, did not significantly influence the responses to the recombinant proteins.




Predicting Variables

Responses to Antigens

**Figure 4.6(f)** Forest plots showing a summary of the multivariable linear regression analysis

The forest plots show the association between the responses to the recombinant gametocyte-specific proteins and variables such as age, time-point in days, and density of parasites as measured by qPCR, sickle gene, α-thalassemia, fever (≥37.5°C) and ratio of monocytes to lymphocytes.

# CHAPTER FIVE

## 5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

### 5.1 DISCUSSION

In the design and development of interventions to block such as vaccines, it is paramount to understand the dynamics of antibody responses *P. falciparum* to gametocytes. In this study, both the cell-free wheat germ expression system and the mammalian HEK293 system were used to produce recombinant gametocyte-specific antigens. Further, antibody responses of malaria-exposed individuals to recombinant proteins were assessed. As reported in numerous other studies, this work found evidence of antibody responses against the produced *P. falciparum* recombinant proteins (Dinko *et al*., 2016) (Drakeley *et al*., 2006) (Bousema *et al*., 2007).

While the precise mechanisms of TBI have yet to be properly elucidated, evidence of antibodies from malaria-exposed persons have shown the ability to block gamete fertilization and any further development in the mosquito midgut (Greenwood *et al*., 2005), (Arévalo-Herrera *et al*., 2011). These immune responses have been found to vary with factors such as exposure to the parasite, parasite density and strain, genetics and age of the host.

The impact of age on responses to the recombinant proteins was assessed by categorising the AFIRM (cross-sectional) study into three age groups. Antibody responses among individuals older than 15-year were consistently higher for all antigens in comparison to responses seen in the other two age groups. Responses among the 5 to 15 year age group were also consistently higher for all antigens as compared to responses by individuals under 5-years of age.

The findings here are consistent with previous studies that sought to understand the dynamics of antibody responses to gametocytes (*Bousema and Drakeley, 2011*) (Ouédraogo *et al*., 2011). This can further be explained given the fact that immunity to the parasite has been reported to require repeated exposure (Paul *et al*., 2007) (Ouédraogo *et al*., 2011). This trend was also observed for responses to AMA1, which serves as a marker for exposure to asexual parasites.

The age of participants was also found to influence the density of parasites as seen from the LAMB study multivariable analysis predicting parasitaemia. Here, a unit increase in age was found to significantly decrease one's odds of having detectable parasites by 96% ($p<0.05$), when adjusting for responses to the recombinant gametocyte-specific proteins, the day of follow-up, fever, age, sickle gene and α-thalassemia. In addition to the impact of age on responses, responses based on the gametocyte status were assessed. To do so, the data set from the cross-sectional AFIRM study, was categorised as gametocyte positive or negative based on the presence or absence of gametocytes as detected by NASBAPfs25.

Significantly higher responses amongst the gametocyte-positive group were found as compared to the gametocyte-negative group, for all the antigens. Upon correlating responses to the produced antigens with gametocyte density (NASBAPfs25), significant correlations with all the gametocyte-specific antigens was found. This infers that the produced recombinant proteins are gametocyte-specific as individuals presenting with gametocytes are able to recognise them due to their exposure to gametocytes therefore having acquired antibodies over time.

The impact of season on responses to the recombinant proteins was also assessed using data from the AFIRM study. Other studies have reported an increase in antibody responses to sexual stage-specific parasites during the transmission season (Gadalla *et al*., 2016). The data herein did not reveal any trend with season, a fact suspected to be as a result of antibodies that are acquired over time with increases exposure, or the maintenance of the parasite during the dry season by undetectable sub-microscopic or low-density gametocytes carried over from the wet season (Zhou *et al*., 2016) (Shekalaghe *et al*., 2007).

In the AFIRM study, gametocyte density and its influence on responses was also assessed by correlating responses to the recombinant proteins and gametocyte density as measured by NASBAPfs25. This study found significant correlations between gametocyte density and the responses to all the recombinant gametocyte antigens. When responses to the recombinant proteins were correlated with asexual parasitaemia as measured by NASBA18s, significant positive correlation was observed for all the recombinant gametocyte-specific proteins.

The longitudinal LAMB study provided a unique opportunity to determine a trend in parasitaemia and in turn a trend in antibody responses over the different time points. From the analysis, there appears to be a rather consistent trend in the responses to the recombinant proteins based on the parasite density over time. A multivariable logistic regression model was fitted for the AFRIM study to determine the influence of various predictor variables on gametocyte status.

Here, the influence of each variable on the outcome was determined as well as determining if there was any interaction between the predictor values and how this interaction influenced the overall outcome variable. The mean responses to the recombinant proteins served as the outcome of the model and the predictors were, age in categories, gametocyte status, season, parasite density (as measured by NASBAPfs25, NASBA18s and qPCR18S), mean responses to AMA1, sickle gene and α-thalassemia.

Similarly, a model was fitted to predict the odds of gametocytaemia among participants of the AFIRM study. Here, the following predictors were included: responses to the produced recombinant gametocyte-specific proteins measured by OD, age group in categories, and season of sampling, parasite density as measured by qPCR, sickle gene and α-Thalassemia. As expected, the presence of asexual parasites as measured by qPCR significantly ($p<0.01$) increased the odds of being gametocyte positive by 1% for every unit increase in asexual parasite density. This observation is consistent with published studies, that asexual parasites are the precursors of sexual stages of the parasite (Guttery, Holder and Tewari, 2012).

When adjusted for parasitaemia, participants who were sampled during the dry season had a 2% increased odds of presenting with gametocytes compared to those sampled during the wet season. This supports what other studies have reported as sub-microscopic levels of gametocytes that are maintained during the dry season and are able to re-establish transmission in the appropriate conditions and vectors (Okell *et al*., 2012), (Lamptey *et al*., 2018).

Haemoglobinopathies have been reported to protect against malaria and are significantly high in individuals at risk of malaria, particularly in Africa (Taylor, Parobek and Fairhurst, 2012 and Kohne, 2011). The influence of haemoglobinopathies was assessed, in this case α-Thalassemia and Sickle trait on both asexual and sexual parasitaemia using a multivariable model. A look at the sickle cell trait (HbAS) and its association with parasitaemia revealed that these individuals have a 6% (confidence interval ~ -0.7, 0.9), increased chance of presenting with asexual parasites (LAMB study) although this increase was not statistically significant.

Further, when parasitaemia is accounted for, then HbAS individuals (AFIRM study) have only 1% (confidence interval ~ 0.004, 0.02) higher odds of having detectable gametocytes compared to their normal (HbAA) counterparts, after adjusting for responses to recombinant gametocyte-specific proteins measured by OD, age group in categories, season of sampling, parasite density as measured by qPCR, sickle gene, α-Thalassemia as well as interaction between the predictors.

This findings are consistent with numerous studies that have shown the protective effect against malaria, of the sickle cell trait (Gong *et al*., 2013). Alpha-thalassemia has been hypothesized to reduce the adhesion of parasitized red blood cells to the walls of capillaries in essential organs (Krause *et al*., 2012). This adhesion serves as the cause of severe conditions such as cerebral malaria (Fedosov *et al*., 2010).

In the case of the AFIRM study the influence of these disorders in predicting the odds of gametocytaemia, individuals homozygous for α-Thalassemia had 68% higher odds of having no detectable gametocytes. A similar observation was seen in the LAMB study where homozygous participants had 82% higher odds of detectable asexual parasites although this was not statistically significant (Confidence interval- 1.4, 0.97).

## 5.1 CONCLUSION

*Plasmodium falciparum* infection can stimulate antibodies that can alter the existence of the parasite in the mosquito, when they are ingested in an infectious blood meal. The body of work presented in this thesis shows the ability to produce recombinant gametocyte-specific proteins using a cell-free expression system, wheat-germ and mammalian expression system, HEK293, to produce recombinant proteins for purposes of immunoprofiling.

Moreover, this study was able to show using ELISA, that the antigens produced were recognised by the sera of malaria-exposed individuals from an area of low malaria transmission such as Kilifi, Kenya. The unique aspect of this study is that it offers a look at responses to recombinant proteins based on both a cross-sectional as well as longitudinal study. The analysis herein shows that host antibody responses to gametocyte antigens are influenced by aspects such as age of participants, the density and presence of parasites and season. This study proves important, as it adds on to providing insight into mechanisms of immunity to malaria. To further improve on the findings presented herein, it would be important to carry out similar immunoprofiling analysis with a different cohort to account for genetic factors that may influence responses, as well as in different endemicity settings. Although this strategy would not directly stop individuals from infection with malaria, it would halt its transmission by allowing for the expansion and prioritisation of antigenic targets that could be prioritised for functional characterisation as more potent candidates for blocking transmission. Introduction of a vaccine to block transmission would greatly boost existing vector control strategies, enabling the elimination and possibly eradication of malaria in the majority of populations exposed to the parasite.

## 5.2 RECOMMENDATIONS

In future, it would be interesting to measure transmission-blocking activity using mosquito feeding assay, which though an expensive and time-consuming venture, would further solidify the reported observations. Moreover, understanding the mechanism underlying the recognition of gametocyte-specific antigens, and further finding a highly immunogenic target(s) will go a long way in advancing the development of an effective vaccine to block malaria transmission.

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