ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM* 3D7 RH5 AND EBA-175 ANTIGENS IN AN ACUTE AND CONVALESCENT COHORT OF CHILDREN FROM JUNJU SUB-LOCATION IN KILIFI, KENYA

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DECLARATION

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

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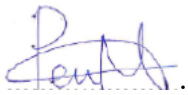
Signature ……  …… Date……25th May, 2020……

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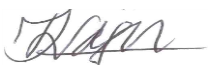
Signature ……  …… Date… 26/05/2020……...

DEDICATION

To my daughter, Teresa. Baby, you are my source of inspiration and strength.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMA 1:	Apical membrane antigen 1
Amp®:	Ampicillin
APS:	Ammonium persulfate
ASCs:	Antibody secreting cells
AT:	Adenine and Thymine
BCA:	Bicinchoninic acid
bp:	base pair
CCD:	Charge coupled device
CO_2:	Carbon dioxide
CR1:	Complement receptor 1
CMV:	Cytomegalovirus
Ct:	Carboxyl terminal
CyRPA:	Cysteine-rich protective antigen
DBL:	Duffy binding-like
DBP:	Duffy binding protein
DF:	Degrees of freedom
dH_2O:	Distilled water
DNA:	Deoxyribonucleic acid
EBA:	Erythrocyte binding antigen
EBL:	Erythrocyte binding-like
ECL:	Enhanced chemiluminescence
E. coli:	*Escherichia coli*
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
Fc:	Fragment of crystallization
Glog:	Generalized logarithm
GP:	Glycophorin
HBsAg:	Hepatitis B surface antigen

HF: High fidelity

His: Histidine

HIV: Human immunodeficiency virus

HRP: Horseradish peroxidase

HRP2: Histidine-rich protein 2

H_2SO_4: Sulfuric acid

HSPGs: Heparan sulfate proteoglycans

IgG: Immunoglobulin G

IPT: Intermittent preventive treatment

IRS: Indoor residual spraying

ITNs: Insecticide-treated nets

kb: Kilobase

KEMRI: Kenya Medical Research Institute

kDa: Kilo Dalton

LB: Luria-Bertani

MIG: Malaria immunoglobulin

Min: Minutes

MSP: Merozoite surface protein

NEB: New England Biolabs

neg: Negative

nlme: Non-linear mixed effect

OD: Optical density

OPD: O-phenylenediamine dihydrochloride

PBMCs: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

P. falciparum: *Plasmodium falciparum*

*Pf*Ripr: *Plasmodium falciparum* Rh5-interacting protein

PHIS: Pooled hyperimmune serum

4-PL: 4-Parameter logistic

Pos: Positive

ppy:	Per person year
PV:	Parasitophorus vacuole
PVDF:	Polyvinylidene fluoride
RBC:	Red blood cell
RDT:	Rapid diagnostic test
Rh:	Reticulocyte-binding protein homolog
RII:	Region II
S.D:	Standard deviation
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE:	Standard error
Sec:	Seconds
SERU:	Scientific and Ethics Review Unit
SMC:	Seasonal malaria chemoprevention
SOC:	Super optimal broth with catabolite repression
SNPs:	Single nucleotide polymorphisms
SP:	Sulfadoxine-Pyrimethamine
SPECT:	Sporozoite protein essential for cell traversal
Std. Error:	Standard error
TE:	Tris-EDTA
TBE:	Tris-Borate-EDTA
TEMED:	N,N,N,N -Tetramethylethylenediamine
TBS/ T:	Tris-buffered saline/Tween
UK:	United Kingdom
UV:	Ultraviolet
WHO:	World Health Organization
6×His:	Hexa-histidine

ABSTRACT

Repeated exposure to malaria parasites broadens the repertoire of antigens recognized by the host immune system leading to acquisition of immunity to the disease. This immunity is inefficient in the absence of exposure as it wanes with time. Antibodies mediate humoral immunity against malaria and are majorly directed towards the extracellular merozoite. The reticulocyte binding-like protein homologue 5 (Rh5) and erythrocyte binding antigen (EBA-175) are merozoite antigens that are immunogenic and they are potential malaria vaccine candidates. The sequence conservation of Rh5 across *Plasmodium falciparum* (*P. falciparum*) strains makes it a desirable vaccine candidate that elicits strain-transcending immune responses. Rh5 also maintains the malaria parasite's viability as shown by futile attempts to delete the gene encoding the antigen. A recent study has shown that EBA-175 shed post merozoite invasion mediates clustering of uninfected red blood cells (RBCs) around infected RBCs promoting parasite growth as progeny merozoites can easily invade other uninfected RBCs. In naturally malaria-exposed populations, Rh5 and EBA-175 may be targets of protective immunity. However, there is limited knowledge on how the magnitude of the antigen-specific antibody titres changes following an acute malaria episode. Thus, the objective of the study was to determine whether the magnitude of antibody responses to Rh5 and EBA-175 from the *P. falciparum* 3D7 strain changes over time following an acute malaria episode. The two recombinant proteins were expressed by mammalian Expi293F cells. Antibody responses to the expressed antigens were measured by enzyme-linked immunosorbent assay (ELISA) using plasma samples collected during a cross-sectional bleed (baseline), acute malaria infection, 4, 6 and 24 weeks following treatment. The differences in antibody titres between all the time-points were determined using the non-parametric Kruskal-Wallis test while the differences between any two time-points were assessed using the non-parametric Wilcoxon signed-rank test. A non-linear mixed effects model was used to model antibody responses as a function of time. The two antigens were immunogenic (OD above 0.05) as they elicited antibody responses in the plasma samples tested. There was also a marked heterogeneity in antibody responses to both antigens in the children aged between 0-15 years from Junju, Kilifi. The data also showed that infection with *P. falciparum* boosted antibody responses to Rh5 ($p = 0.044$). For EBA-175, acute malaria infection did not significantly boost antibody responses to the antigen. However, children older than 5 years of age elicited greater antibody

responses to EBA-175 (Estimate = 0.14, SE = 0.05, p = 0.01), suggesting an acquisition of malarial immunity as the children grew older. Generally, antibody responses to both antigens declined during the follow-up period of 24 weeks (Estimate = -0.04, SE= 0.01, p = <0.01) for Rh5 and (Estimate = -0.07, SE = 0.02, p = <0.001) for EBA-175, showing the short-lived nature of immune responses to the two antigens. In conclusion, the two antigens were immunogenic. Antibody responses to the two antigens were heterogeneous and waned after the acute malaria infection. While infection with *P. falciparum* boosted antibody responses to Rh5, there was an effect of age on antibody responses to EBA-175. These results have the implications for the development of a more efficacious malaria vaccine based on RH5 and EBA-175, and also inform the timing of serological studies. These two antigens are important blood stage antigens that could be included in a multi-component vaccine for a better vaccine efficacy.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

An effective malaria vaccine is yet to be achieved despite the current efforts by scientists in search for one. Consequently, malaria remains a threat in vulnerable populations (Desai *et al.*, 2007; Eckert *et al.*, 2017), with the enormity of the disease burden being borne by sub-Saharan Africa (WHO, 2020). *Plasmodium falciparum* (*P. falciparum*) is the most virulent malaria parasite and it is widely spread in sub-Saharan Africa while in the same area, infection with *P. malariae, P. ovale* and *P. vivax* is less common (Roucher *et al.*, 2014; Howes *et al.*, 2015). In order to successfully complete its multi-stage life cycle, the malaria parasite requires two hosts: human beings and the female *Anopheles* mosquito. In the human host, the cyclic asexual blood stage of infection initiated by the extracellular merozoite leads to an exponential increase in the parasite population and eventual destruction of erythrocytes during merozoite egress causes malaria-associated pathology.

Understanding the merozoite stage of infection and the host immune responses directed towards the merozoite coat and secreted proteins is crucial for malaria vaccine development. This is because these proteins can be recognized by the immune system of the host which mounts a defense mechanism by generating antibodies (Moormann *et al.*, 2013). The development of a vaccine candidate that would successfully interrupt red blood cell (RBC) invasion by the malaria parasites could reduce morbidity and mortality due to the disease. However, obtaining such a viable malaria vaccine has been challenging, due to the diverse nature of merozoite antigens brought about by host immune selection pressure, maintained by balancing selection (Polley *et al.*, 2003). The polymorphisms of the parasite antigens, therefore, lead to allele-specific immune responses where individuals are protected against previously encountered alleles (Polley *et al.*, 2007). When an individual is given a vaccine containing two variants (A and B) of a polymorphic antigen, the individual is protected against both the A and B variants, while the non-vaccine variants increase within the individual and in the population. When the same individual is infected with a parasite containing a different variant, say C, this leads to what are known as breakthrough infections (Ouattara *et al.*, 2015). Therefore, for sufficient clinical protection, individuals have to encounter a large number of alleles. Identifying a vaccine candidate with the ability to induce a

robust immune response and neutralize diverse parasite variants could aid in the design of a more efficacious vaccine (Ord *et al.*, 2015).

The reticulocyte binding-like protein homologue 5 (Rh5) and the erythrocyte binding antigen (EBA-175) are important blood stage antigens that have pivotal roles in erythrocyte invasion and their receptors on RBCs, basigin and glycophorin A (GPA), respectively, are well characterized (Wanaguru *et al.*, 2013; Chiu *et al.*, 2016). Rh5 lacks a transmembrane domain making it the smallest member of the superfamily (~63 kDa) (Wright *et al.*, 2014). To facilitate the invasion process using the sialic acid-independent pathway, Rh5 interacts with cysteine rich protective antigen (CyRPA), P113, and *P. falciparum* Rh5 interacting protein (PfRipr) (Chiu *et al.*, 2014). This interaction helps Rh5 form a complex with its receptor on the RBC membrane. EBA-175 is a ~175 kDa protein that invades erythrocytes via the so called sialic acid dependent pathway and belongs to the erythrocyte binding-like (EBL) protein family including EBA-140 and EBA-181.

Rh5 has limited polymorphisms (Bustamante *et al.*, 2013) while EBA-175 is polymorphic (Soulama *et al.*, 2011) and highly immunogenic (Osier *et al.*, 2008). Full-length Rh5 has previously been targeted by strain-transcending antibodies induced by the antigen-based vaccine (Douglas *et al.*, 2011), which were capable of inhibiting the blood stage of invasion *in vitro* (Patel *et al.*, 2013a; Ord *et al.*, 2014; Tran *et al.*, 2014). Elsewhere, vaccination of *Aotus* monkeys with the Rh5-based vaccine protected them against heterologous *P. falciparum* strains (Douglas *et al.*, 2015). Human vaccination with viral-vectored Rh5 induced an antibody response that inhibited Rh5 invasion complex interactions, again supporting this antigen as an immunogenic protein (Payne *et al.*, 2017). Rh5 gene knockout experiments have proven futile, suggesting that this antigen is required for the viability of the parasite (Baum *et al.*, 2009). EBA-175 region II (RII) was also tested in malaria semi-immune adults from Ghana as a vaccine candidate where they showed that it was safe, immunogenic and well-tolerated by the individuals (Koram *et al.*, 2016). Naturally acquired antibodies to RII of EBA-175 significantly inhibited merozoite invasion suggesting an important role of the antigen-receptor interaction (Badiane *et al.*, 2013; Irani *et al.*, 2015). This further supported EBA-175 as a potential vaccine candidate.

In naturally malaria-exposed populations, Rh5 and EBA-175 may be targets of protective immunity. However, there is limited knowledge on how the magnitude of the antigen-specific antibody titres changes following an acute malaria episode. Therefore, the aim of this study was

to determine whether the magnitude of antibody responses to *P. falciparum* 3D7 Rh5 and EBA-175 antigens changes over time following an acute malaria episode. Specifically, recombinant Rh5 and EBA-175 antigens were generated in a mammalian expression system and antibody responses to the two antigens subsequently examined in acute and convalescent plasma samples that were obtained from a cohort of children from Junju Sub-location in Kilifi, Kenya. Furthermore, changes in antibody titres over time in the acute and convalescent plasma samples were determined.

1.2 Problem statement

Owing to the high burden of malaria that rests in expectant women and children, vaccines have the potential to provide long lasting protection and a massive impact on the global public health. An ideal malarial vaccine candidate should have the ability to induce a robust immune response against the diverse parasite antigens. Designing a vaccine candidate which covers the whole diversity of the malaria parasite antigens is a great hurdle (Conway *et al.*, 1992; Escalante *et al.*, 1998). There is a large body of evidence indicating that the polymorphisms of the malaria parasite antigens are maintained by balancing selection and they come about as a result of immune pressure from the parasite's host (Polley and Conway, 2001; Baum *et al.*, 2003). The licensed malaria vaccine, RTS, S, (Mahmoudi and Keshavarz, 2017) and other malaria vaccine candidates based on polymorphic antigens, including apical membrane antigen 1 (AMA1) (Spring *et al.*, 2009), have a reduced efficacy as they target a limited number of alleles circulating in the population. The antigenic polymorphisms allow the parasite to exhibit variant-specific immune responses and therefore escape the host immune responses (Bustamante *et al.*, 2013). Hence, individuals have to encounter a variety of alleles in order to develop sufficient clinical protection.

1.3 Justification of the study

A clear understanding of how antimalarial immunity is developed and maintained in naturally malaria-exposed populations is essential for improving prospects for successful vaccine development and further to guide the timing of serological studies. Few data are available on how the magnitude of antibody responses to full-length Rh5 and EBA-175 antigens from the *P. falciparum* 3D7 strain changes over time following an acute malaria infection. Thus, the study purpose was to determine if the magnitude of antibody responses to Rh5 and EBA-175 changes over time following an acute malaria infection. The mammalian expression system was used in

the expression of full-length Rh5 and EBA-175. It is a scalable system that has repeatedly been applied in the expression of recombinant functional and full-length *Plasmodium* proteins (Srivastava *et al.*, 2010; Bustamante *et al.*, 2013; Chiou *et al.*, 2014). Rh5 and EBA-175 antigens are rhoptry and microneme proteins, respectively, expressed during the blood stage of infection by the merozoite. These proteins are secreted by the merozoite to facilitate the invasion process by binding to their respective receptors on RBC membrane. Studies have shown that the host can recognize and raise antibodies against the extracellularly secreted proteins which can be measured using enzyme-linked immunosorbent assay, ELISA (Okenu *et al.*, 2000; Ohas *et al.*, 2004; Reddy *et al.*, 2014; Tran *et al.*, 2014). Furthermore, Rh5 is relatively conserved, its presence is vital for the maintenance of the malaria parasite's viability, and it elicits strain-transcending antibody responses (Baum *et al.*, 2009; Bustamante *et al.*, 2013). EBA-175 is a highly immunogenic antigen that has recently been shown to promote parasite growth during the blood stage after being shed from merozoite membrane post-invasion by engaging GPA (Osier *et al.*, 2008; Paing *et al.*, 2018). These form clusters where uninfected RBCs come around infected RBCs providing daughter merozoites a quick access to uninfected cells. These antigens were used in the present study to determine antibody responses in the acute and convalescent plasma samples from malaria-exposed children in Kilifi to inform the changes in antibody responses after an acute malaria infection. This study provides more data regarding the design of a malaria vaccine based on Rh5 and EBA-175 antigens.

1.4 Null hypothesis

Antibody responses to Rh5 and EBA-175 do not change with time following an acute malaria episode.

1.5 Objectives

1.5.1 General objective

To determine antibody responses to recombinant Rh5 and EBA-175 in plasma samples obtained from an acute and convalescent cohort of children from Junju sub-location, Kilifi County, Kenya.

1.5.2 Specific objectives

i. To express recombinant Rh5 and EBA-175 antigens in mammalian Expi293F cells.
ii. To determine antibody responses to recombinant Rh5 and EBA-175 antigens.

iii. To determine the changes in antibody titres to Rh5 and EBA-175 antigens in acute and convalescent plasma samples over time.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Life cycle of *Plasmodium falciparum*

2.1.1 The liver stage

The complex multistage life cycle of *P. falciparum* involves the female *Anopheles* mosquito parasite vector and human beings as its two hosts (**Figure 2.1**) (Cowman *et al.*, 2012). *Anopheles gambiae sensu stricto* is best known for its predominant role in transmitting *P. falciparum* (Mbogo *et al.*, 2003). The parasite's life cycle commences as soon as an infectious *Anopheles* mosquito intradermally transmits infective sporozoites into the human host. Some sporozoites die either at the injection site or when they trickle out of the skin, while others are degraded after entering the lymphatic system (Yamauchi *et al.*, 2007; Amino *et al.*, 2008). A few of them actively make their way to the liver through the blood stream to undergo the exo-erythrocytic cycle leading to a 10,000-fold rise in parasitemia. There are three parasite proteins that have been implicated in aiding sporozoite traversal to the liver and they are sporozoite protein essential for cell traversal (SPECT)-1, SPECT-2 as well as a phospholipase (Ishino *et al.*, 2004; Bhanot *et al.*, 2005). The sporozoite's circumsporozoite protein preferentially interact with glycoproteins on liver cells called heparan sulfate proteoglycans (HSPGs) to invade the hepatocytes (Ying *et al.*, 1997). While in the hepatocytes, the sporozoites mature into schizonts after 7 to 10 days with a varied number of daughter merozoites (Soulard *et al.*, 2015). These schizonts subsequently rupture releasing up to 30,000 merozoite forms into the bloodstream to undergo the erythrocytic cycle in RBCs, their second target. This marks the end of the asymptomatic exo-erythrocytic cycle. *P. vivax* and *P. ovale* are notorious for having a dormant stage in the liver called hypnozoites responsible for latency and relapses of malaria (Cowman and Crabb, 2006).

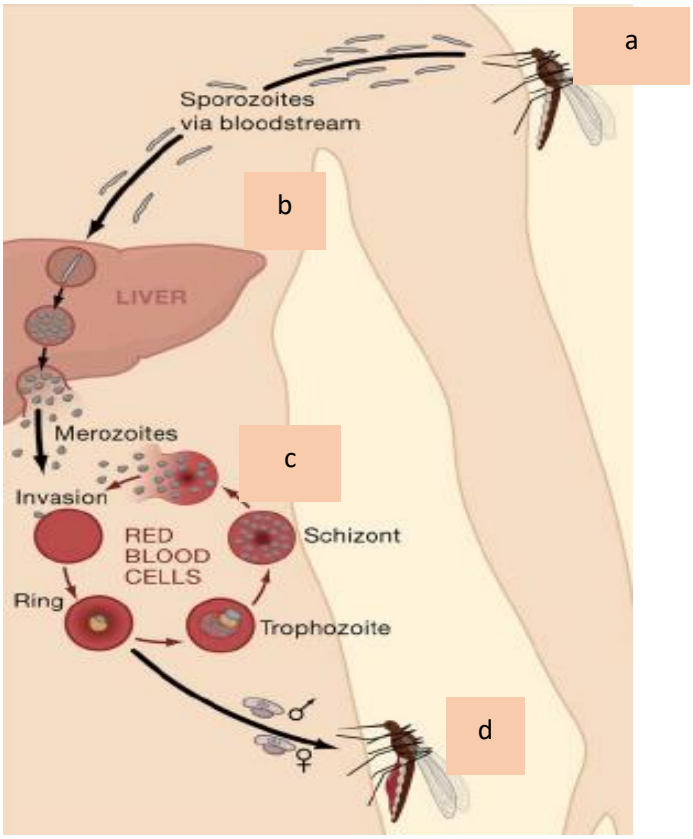

***Figure 2.1*: *Plasmodium falciparum* developmental stages in the human host.** a) An infectious *Anopheles* mosquito feeding from a susceptible human host. Consequently, infective sporozoites are inoculated into the human host. The injected sporozoites traverse the bloodstream to reach their second target, the liver b) Here, they develop into merozoite forms which initiate the blood stage c). Some of the merozoite forms develop into gametes that are taken up by uninfected feeding mosquito d) where the sexual stages are completed. Adopted from Cowman and Crabb (2006)

2.1.2 The blood stage of infection

The merozoite (**Figure 2.2**) is a sporozoan trophozoite released from liver hepatocytes which initiates the blood stage by invading the RBCs and asexually multiplying therein. The invasion process occurs within seconds and involves the merozoite protein coat and proteins secreted by organelles situated in the apical end of the parasite. These proteins bind to erythrocyte receptors to facilitate the invasion process (Cowman *et al.*, 2012). The merozoite forms a contact with the erythrocyte surface. Subsequently, reorientation of the merozoite follows in order that the apical end comes into contact with the RBC. Tight junction formation then follows before pinching of the erythrocyte membrane resulting into a parasitophorous vacuole (PV), a bulb-like compartment

that houses the parasite shielding it from host defense mechanisms (Ward *et al.*, 1993). The merozoites develop through ring stage trophozoites into schizonts. About 1000 merozoites form inside each schizont and are eventually released upon RBC rupture to invade other uninfected RBCs, leading to an exponential increase in parasite population.

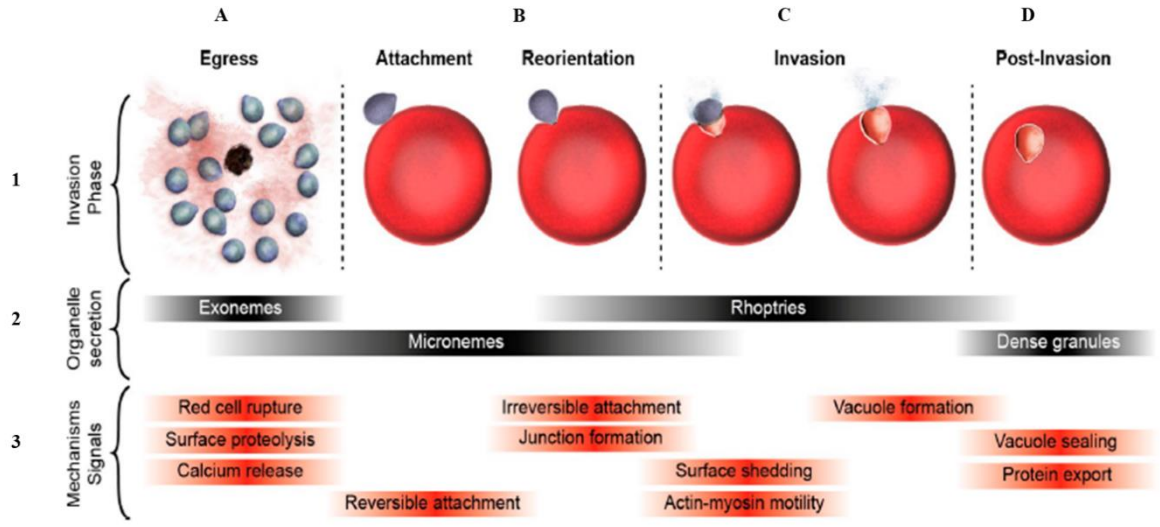

***Figure 2.2*: Time course of RBC invasion by merozoites.** This is a cellular overview of erythrocyte invasion by merozoites. 1) A- Bursting of erythrocytes releasing progeny merozoites into the blood stream. B- The merozoite comes into contact with an RBC before reorienting, bringing the apical end into close proximity with erythrocyte membrane. C and D- The merozoite invades erythrocytes, enclosed in a parasitophorous vacuole. (2) Erythrocyte invasion is associated with timing of secretion from various organelles i.e. rhoptries, micronemes, exonemes as well as dense granules and (3) key signaling steps. Adopted from Cowman *et al.* (2012).

2.1.3 The sexual stage

Inside the RBCs, some merozoites differentiate into gametocytes that remain in circulation until a female *Anopheles* mosquito ingests them as it feeds to undergo the sexual stages. In this host's midgut, the gametocytes develop into microgametes (male) and macrogametes (female) during gametogenesis. The microgamete then fuses with the macrogamete resulting in a zygote that is diploid. The zygotes develop into motile and elongated ookinetes that subsequently burrow into the mosquito's midgut where they develop into oocysts. The ookinete nuclei inside the oocysts undergo sporogony to produce several sporozoite forms. Sporozoites released when the oocysts burst travel through the mosquito cavity into the salivary glands. These sporozoites stay in the

salivary glands awaiting their inoculation into a susceptible human host (Cowman and Crabb, 2006).

2.2 Malaria presentation and control strategies

2.2.1 Presentation of malaria

Malaria manifests during the erythrocyte cycle when infected RBCs continually burst to release merozoites. It can manifest as complicated or uncomplicated disease depending on the severity of the symptoms. Asymptomatic individuals are characterized by the presence of parasites with no fever (Okell *et al.*, 2009; Laishram *et al.*, 2012). Hardly are individuals with clinical or uncomplicated malaria hospitalized if they seek medical attention in time. Any person who presents with severe malaria symptoms like anemia, respiratory distress, or cerebral malaria (Marsh *et al.*, 1995) require admission into a health facility and proper medical care. Severe malaria occurs in a minority of individuals and symptoms vary from person to person. Uncomplicated malaria symptoms including fever, headache, and chills are largely nonspecific and manifest 10 to 15 days after inoculation with sporozoites. Uncomplicated malaria could worsen and become severe malaria, which could lead to death if individuals fail to get treated. Severe malarial anemia majorly afflicts children (<5 years) in high and stable transmission areas. Conversely, cerebral malaria becomes common at older ages in unstable and lower transmission areas (Luxemburger *et al.*, 1997; O'Meara *et al.*, 2008).

2.2.2 Control strategies

Reduction in morbidity and death due to *falciparum* malaria can be attributed to the application of multiple strategies, which include vector control, prophylaxis and improvement of malaria treatment and diagnosis (Nkumama *et al.*, 2017).

As an infected feeding mosquito obtains its blood meal from a susceptible host, infective sporozoites are injected into this host. This interaction that can be prevented by employing vector control strategies that include management of larval breeding grounds, insecticide treated net (ITN) usage as well as indoor residual spraying (IRS) (WHO, 2020). The malaria at risk populations, expectant women and children under 5 years, are recommended by the WHO to sleep under ITNs. Indeed, a decline in death and malaria incidence attributed to ITN usage was observed in endemic areas. According to (Bhatt *et al.*, 2015), from the year 2000 to 2015 in sub-Saharan

Africa, ITN usage accounted for nearly 50% decrease in the prevalence of malarial parasites in children (2 - 10 years). However, the malaria parasites have developed resistance to insecticides such as pyrethroids used in IRS and ITNs threatening the gains in malaria control. Mutations in the *kdr* gene in *Anopheles gambiae* has amounted to resistance to this insecticide. Despite this, ITN remains effective in areas where there is resistance to pyrethroids by acting as a physical barrier against infective mosquitoes. (Ranson *et al.*, 2011).

Chemoprevention strategies have mainly focused on children and pregnant women who are the vulnerable population. They include seasonal malaria chemoprevention (SMC) and intermittent preventive treatment for expectant women (IPTp) and for infants (IPTi). Seasonal malaria chemoprevention is done so that clinical malaria can be prevented in children aged 3-59 months. Use of amodiaquine and SP in regions which exhibit seasonal transmission patterns was also associated with about 80% decline in malaria as well as anemia (Cairns *et al.*, 2012). There was an association between the use of Sulfadoxine-Pyrimethamine (SP) during the second trimester of pregnancy and a decline in neonatal mortality, placental malaria and anemia (ter Kuile *et al.*, 2007; Eisele *et al.*, 2012). A protective efficacy of more than 30% in children living in high malaria transmission areas was achieved after using SP (Aponte *et al.*, 2009). Of concern is the emergence of malarial drug resistance in regions where the disease is endemic which threatens their efficacy (McGready, 2009).

The reduction of malaria-related deaths require that rapid diagnosis of the disease is done in advance before administering an appropriate treatment. To ascertain any speculated case of malaria, a rapid diagnostic test (RDT) and/or microscopy are recommended by the WHO as the diagnostic measures. An RDT, as the name implies, is a rapid way of diagnosing malaria and detects histidine-rich protein (HRP2/3). Nevertheless, its utility is threatened by emerging reports that some parasites in circulation, in Africa as well as South East Asia, lack the *hrp2/3* gene which may potentially impact on the sensitivity of the test (Gamboa *et al.*, 2010; Koita *et al.*, 2012; Kumar *et al.*, 2013; Deme *et al.*, 2014; Bharti *et al.*, 2016). False-negative RDTs can be confirmed with test methods that can detect non-HRP2 antigens such as quantitative real-time Polymerase Chain Reaction (qPCR) (Kreidenweiss *et al.*, 2019) and non-HRP2 alternative RDTs (Berhane *et al.*, 2018).

Vaccines, on the other hand, are considered a cost-effective strategy in the elimination of malaria, and prime the immunity of the host in order that a better response is achieved upon a subsequent infection (Hussein *et al.*, 2015). RTS, S/AS01 is the licensed malaria vaccine that has been engineered making use of genes from the repeat as well as the T-cell epitope of the liver stage circumsporozoite protein of *P. falciparum* and the hepatitis B surface antigen (HBsAg). The AS01 is an added chemical adjuvant meant to improve the immune response (Laurens, 2020). This vaccine is partially protective and the efficacy is short-lived (Rts, 2015). Therefore, there is an urgency for a more effective malaria vaccine because despite of the decline in malaria transmission in endemic areas, the disease is still a burden in these areas.

2.3 Acquisition of antimalarial immunity

People inhabiting endemic regions are incessantly exposed to malaria parasites through infective mosquito bites. With time, they develop tolerance to malaria by acquiring immunity to the disease (Baird *et al.*, 1991; Griffin *et al.*, 2015). Nonetheless, it is unclear how this immunity comes about and how it acts to prevent clinical malaria. The immune system targets remain to be determined (Marsh and Kinyanjui, 2006). According to the study by Rodriguez-Barraquer *et al.* (2018), immunity to malaria infection develops faster with increasing transmission intensity, it is slowly acquired with age (Baird *et al.*, 1991; Griffin *et al.*, 2015) and it is non-sterilizing. This is why some individuals in endemic areas harbor chronic asymptomatic parasitemia and are silent reservoirs of malaria parasites. While asymptomatic parasitemia increases with age and decreases in adulthood, the burden of symptomatic malaria declines as children grow older (Reyburn *et al.*, 2005; Griffin *et al.*, 2015).

Antimalarial immunity is categorized into anti-parasite and anti-disease immunity where the former reduces parasite numbers and the latter prevents clinical disease manifestation. The two types of malarial immunity are acquired in parallel (**Figure 2.3**). As a result, this reduces the chances of developing clinical malaria with subsequent exposure to the parasite antigens (Rodriguez-Barraquer *et al.*, 2018). Studies have also established that immune responses are boosted after an infection as shown by higher concentration of antibodies in parasitized children (Kinyanjui *et al.*, 2004; Partey *et al.*, 2018).

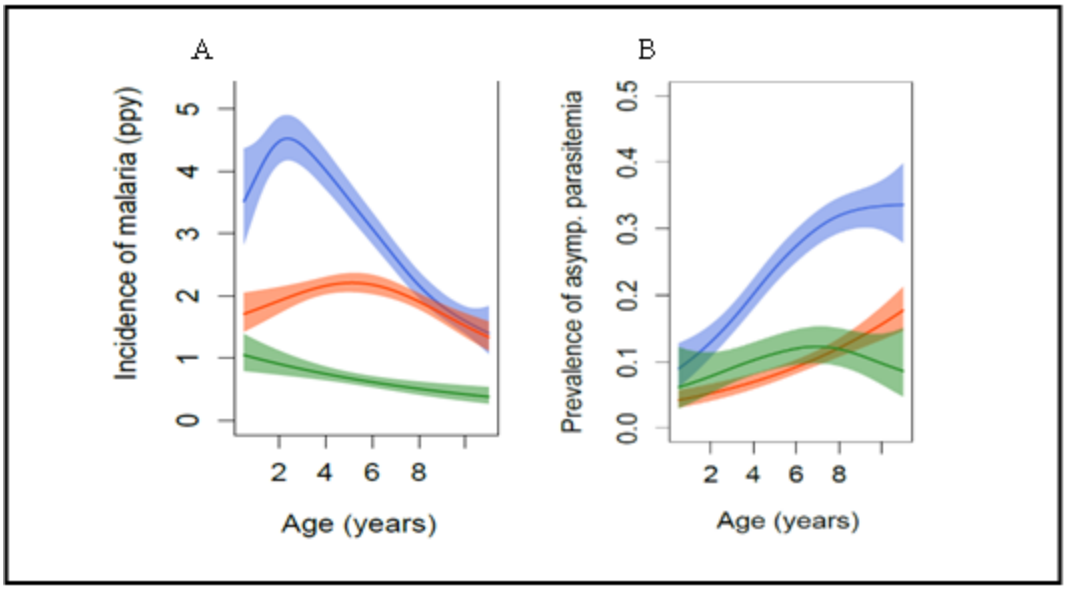

***Figure 2.3*: Gradual increase of antimalarial immunity with age.** This study was carried out in Uganda in three cohorts from an area of varied malaria transmission intensity: low (green), moderate (red) and high (blue). A) A graph of incidence of malaria per person year (ppy) and B) asymptomatic parasitemia prevalence in the three Ugandan cohorts against age (years). The shaded areas represent 95% confidence intervals. In the course of the first 3 years of the children's life, malaria incidence was greatest in the area shaded in blue (the high transmission area) but declined with age in the three cohorts. B) The prevalence of asymptomatic parasitemia increases with age but declines at older ages in the moderate transmission setting. Adopted from Rodriguez-Barraquer *et al.* (2018).

Immunity to malaria can also be achieved through experimental vaccination. However, whether acquired through exposure to the parasites with age or by being vaccinated, it is inefficient as it is partially protective and wanes with time (Frueh *et al.*, 1991; Kinyanjui *et al.*, 2007; Yman *et al.*, 2019). The partial nature of immunity to malaria could be attributed to antigenic diversity brought about by host immune pressure which leads to allele-specific immune responses (Polley *et al.*, 2007). This is a plausible explanation as to why repeated exposure is needed for malarial immunity development. Moreover, immune responses are largely directed towards such polymorphic antigens like AMA1, merozoite surface protein (MSP1) and variant epitopes but less so to less polymorphic antigens such as Rh5.

Pre-erythrocytic immunity is directed to sporozoites while erythrocytic immunity targets the blood stage: the merozoites or the infected RBC. The rapid invasion process results in brief exposure of some essential proteins like Rh5 to the host immunity leading to lower immune responses to such antigens when compared to polymorphic surface antigens. Antibodies raised against the merozoite

antigens are the major mediators of immunity to this stage (Cohen *et al.*, 1961; Osier *et al.*, 2014; Chan *et al.*, 2014).

2.4 The innate and adaptive immunity

There are mechanisms in place before an infection sets in that mediate innate immunity. The innate immune response forms the first line of defense before the adaptive immunity kicks in after exposure to a pathogen. As the name implies, this type of immunity adapts to the particular infection and hence is specific. The innate immunity is mediated by barriers like epithelium, phagocytic cells and the complement system whereas adaptive immunity is mediated by lymphocytes and their associated products (Abbas *et al.*, 2014). The adaptive immunity is exposure-dependent and requires lymphocytes that are activated. This type of immunity can either be cell-mediated or humoral (mediated by antibodies produced by lymphocytes). The latter is crucial for extracellular parasites or their toxins because they can be recognized and neutralized by the antibodies. Cell-mediated immunity, on the other hand and as the name suggests, is mediated by immune cells like T lymphocytes, which destroys intracellular microbes (Abbas *et al.*, 2014).

2.5 Immunity to malaria and other infectious diseases is transferable

Cohen *et al.* (1961) carried out a study where they investigated the role of circulating antibodies on antimalarial immunity in the Gambia. They prepared gamma-globulin (γ-globulin) serum obtained from adults who were immune to malaria and healthy Europeans and tested the same in children who had severe malaria. Surprisingly, the antibodies were effective against *P. falciparum* and *P. malariae* trophozoites but not gametocytes. This was confirmed by another study done by McGregor *et al.* (1963) where they used γ-globulin prepared from sera from West African adults as a therapy for children with severe malaria from East Africa. This step led to recovery and a dramatic decline in parasite density.

Expectant women also passively transfer antibodies to their unborn babies that protect the babies from pathogens once they are born before they start making their own antibodies (I and Ca, 1997). This is not only an active but also a selective process that happens across the placenta. The maternal immunoglobulins (IgG) bind to placental fragment of crystallization (Fc) region and other protein receptors on the placenta (Simister and Story, 1997) enabling transfer of the antibodies into the

fetal capillaries. By the time the fetus is 12 weeks old, small IgG amounts can be detected in the fetus' blood stream (Dancis *et al.*, 1961; Schur *et al.*, 1973). There are rare instances when this transfer can be obstructed like in the case of a preterm birth, placental edema (Bryan, 1977), human immunodeficiency virus (HIV) infection (Moraes-Pinto *et al.*, 1996), hypogammaglobulinemia (Sorensen *et al.*, 1984) and placental malaria (Scott *et al.*, 2005; Cumberland *et al.*, 2007).

Breast feeding mothers can also transfer immunoglobulins (Igs) in breast milk to their babies which protect them against pathogens. These immunoglobulins reduce the risk of acquiring an infectious disease during infancy. It is a recommendation that babies be exclusively breastfed up to six months as there was an association between exclusive breastfeeding and less respiratory and gastrointestinal infections (Goldman *et al.*, 1982; Kilshaw *et al.*, 1984; Harrison *et al.*, 2004; Duijts *et al.*, 2010).

2.6 The genetic diversity of merozoite antigens

Of the approximately 5300 protein coding genes of *P. falciparum* (Gardner *et al.*, 2002), about 2700 of them are expressed during the merozoite stage (Bozdech *et al.*, 2003; Hu *et al.*, 2010). A small number of the widely characterized merozoite proteins whose receptors are known are Rh4, Rh5, EBA-175 and MSP1, which bind to complement receptor (CR)1, basigin, GPA and band 3, respectively (Orlandi *et al.*, 1992; Tham *et al.*, 2010; Crosnier *et al.*, 2011). The merozoite can invade the erythrocytes using either the sialic acid dependent pathway or the sialic acid independent pathway (Duraisingh *et al.*, 2003; Gaur *et al.*, 2007; Awandare *et al.*, 2018). In mutant parasites where EBA-175 was truncated through gene targeted disruption, approximately 85% of erythrocyte invasion was made possible via the sialic acid-independent pathway. This illustrates the redundant nature of the invasion process and also the parasites adaptation to circumvent the erythrocyte receptor diversity as well as the host immune pressure (Reed *et al.*, 2000).

The majority of the proteins on merozoite surface are diverse. This can be attributed to immune-dependent balancing selection (Bustamante *et al*., 2013), a selection force in favor of heterozygotes (Hedrick, 2007). Immune responses to the polymorphic antigens are strain-specific as shown by a study where they examined the *P. falciparum* 3D7 MSP2 variant as a vaccine candidate (Flück *et al.*, 2004). To further show the diversity of the merozoite antigens, AMA1 obtained from *P.*

falciparum that naturally and repeatedly infected children (n = 100) over a 3-year period was sequenced. This revealed that the antigen is highly polymorphic as evidenced by 214 unique combinations of altered nucleotides in about 500 separate infections (Takala *et al.*, 2009). Since antibody responses to the polymorphic antigens target a particular variant to which they are generated against, they do not exhibit strain-transcending immune responses that would ideally protect against all variants of the antigen (Soulama *et al.*, 2011). According to Osier *et al.* (2008), a higher level of protection from clinical malaria is achieved by antibodies with a greater breadth and magnitude.

The malaria parasite's essential genes are relatively conserved and their ability to accept mutations, although limited, would ideally lead to loss of function. Zhang *et al.* (2018) undertook a study that explored the *P. falciparum* genome AT richness enabling them to tell apart non-mutable essential versus mutable dispensable genes. This study identified 2680 antigens which were essential during the blood stage. Among those genes were the drug resistance genes, drug targets and leading vaccine candidates. Rh5 is one of the essential antigens with limited polymorphisms (Bustamante *et al.*, 2013) but less immunogenic when compared to other merozoite surface antigens like MSP1 and AMA1.

2.7 Rh5 and EBA-175 as invasion ligands

2.7.1 Rh5

The Reticulocyte binding-like protein homologue (Rh) superfamily comprises Rh1, Rh2a and Rh2b, Rh4 and Rh5 and are located in merozoite rhoptries. Excluding Rh1, all the Rh superfamily members mediate erythrocyte invasion via the sialic acid independent pathway (Rayner *et al.*, 2001). Apart from Rh4 and Rh5 that bind to CR1 and basigin, respectively, the receptors for the other superfamily members are unknown. Rh5 does not have a transmembrane domain making it the smallest member (63 kDa) in the superfamily (Wright *et al.*, 2014). Rh5 also interacts with P113 (Galaway *et al.*, 2017), *Pf*Ripr (Chen *et al.*, 2011) and CyRPA (Reddy *et al.*, 2015). This helps the antigen form a complex with its basigin receptor on the erythrocyte surface.

Rh5 relocates towards the moving junction during the invasion process (Rodriguez *et al.*, 2008; Baum *et al.*, 2009). This antigen undergoes processing where in culture supernatants, both the 63 kDa full-length antigen and the major processed 45 kDa carboxyl-terminal (Ct) end of the protein,

Rh5Ct, are detected (Baum *et al.*, 2009). It has been established by Bustamante *et al.* (2013) that Rh5 N-terminal domain is also cleaved from the full-length protein.

A study done on Rh5 showed that this antigen was present across all strains *in vitro*, an indication of the importance of the antigen in the strains (Baum *et al.*, 2009). A systematic protein interaction screening approach identified basigin as the Rh5 receptor (Crosnier *et al.*, 2011). Further demonstration that the ligand-receptor interaction was crucial for the invasion process was done using a soluble basigin that competes with the membrane bound basigin, basigin knock-out and anti-basigin antibodies that were serially diluted. Anti-basigin antibodies of ≥10 μg/mL concentration inhibited merozoite invasion in culture, establishing that Rh5 was indeed a crucial erythrocyte invasion ligand (Crosnier *et al.*, 2011).

2.7.2 EBA-175

EBA-175 has a molecular weight of 175 kDa and belongs to the same family as EBA-140, EBA-181, EBA-165 (a pseudo gene) and Erythrocyte Binding Ligand (EBL-1), termed as the EBL protein family. The EBL family members have cysteine-rich regions at both the N- and C-terminus (Adams *et al.*, 1992; Adams *et al.*, 2001). RII of EBA-175 has two duffy binding-like domains (DBL), F1 and F2 (**Figure 2.4**), homologous to *P. vivax* and *P. knowlesi* Duffy binding proteins (DBP). Of all the superfamily and other merozoite antigens, EBA-175 was the first merozoite antigen shown to bind to RBCs and it is well-characterized. EBL-1 binds to GPB, EBA-140 to GPC and EBA-181 to an unknown receptor. EBA-175 facilitates RBC invasion via the sialic acid dependent pathway by engaging GPA, an interaction that anchors the merozoite on RBC (Sim *et al.*, 1994). Specifically, the highly conserved DBL 616 amino acid region II of the antigen binds to the heavily sialylated mucin domain of the GPA receptor (Liang and Sim, 1997; Tolia *et al.*, 2005). GPA is a dimeric 131 amino acid glycoprotein on RBC membrane with each monomer spanning the membrane once and is the major glycoprotein on RBCs (MacKenzie *et al.*, 1997; Tolia *et al.*, 2005).

When the function of EBA-175 is ablated, some strains use the interaction of RH4 and its receptor CR1 on RBC to switch the invasion pathway (Gaur *et al.*, 2006). Removal of the sialic acid residues from RBCs by neuraminidase or mutating the O-linked glycans prevents EBA-175-GPA interaction (Salinas *et al.*, 2014).

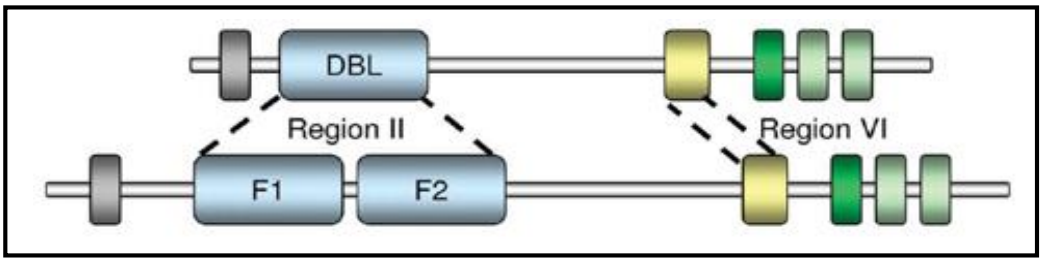

***Figure 2.4*: The structure of the EBL superfamily ligands.** The DBL domain has cysteine-rich regions — F1 and F2. Shown in grey is the signal sequence while the transmembrane and the putative cytoplasmic domains are shown in dark green and light green, respectively. Region VI is shown in yellow. Adopted from Tolia *et al.* (2005)

It has been established that EBA-175 is a micronemal protein and the functional domains of the antigen are structural vaccinology targets (Chen *et al.*, 2013; Batchelor *et al.*, 2014).

EBA-175 shed post-invasion mediate erythrocyte clustering by binding to GPA on other uninfected RBCs bringing the cells close together around infected cells (**Figure 2.5**, circled in blue). This enhances growth of parasites by providing progeny merozoites ready access to uninfected erythrocytes after egress (**Figure 2.5, E**) and additionally shields them from host defense mechanisms (Paing *et al.*, 2018). The RBC clusters have been shown to occur in parasite cultures (**Figure 2.6,** circled in red) (Paing *et al.*, 2018).

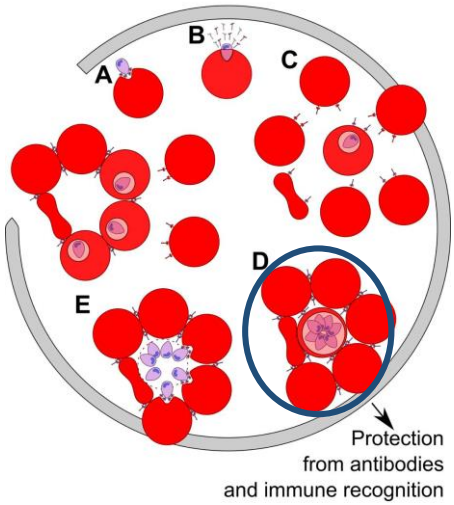

***Figure 2.5*: Immune evasion and parasite survival mediated by EBA-175 shed post-invasion.** A) EBA-175 protein secreted by merozoite (purple) engages its receptor, GPA, on the target RBC (Red). B) Post-invasion, this antigen is then shed from the merozoite and diffuses to nearby RBCs where it binds to GPA on these cells recruiting them around infected RBCs C). This leads to erythrocyte clustering D). Meanwhile, the merozoite is multiplying inside RBCs (in purple, D) and when the RBCs burst, the daughter merozoites readily invade uninfected RBCs E). Adopted from Paing *et al.* (2018).

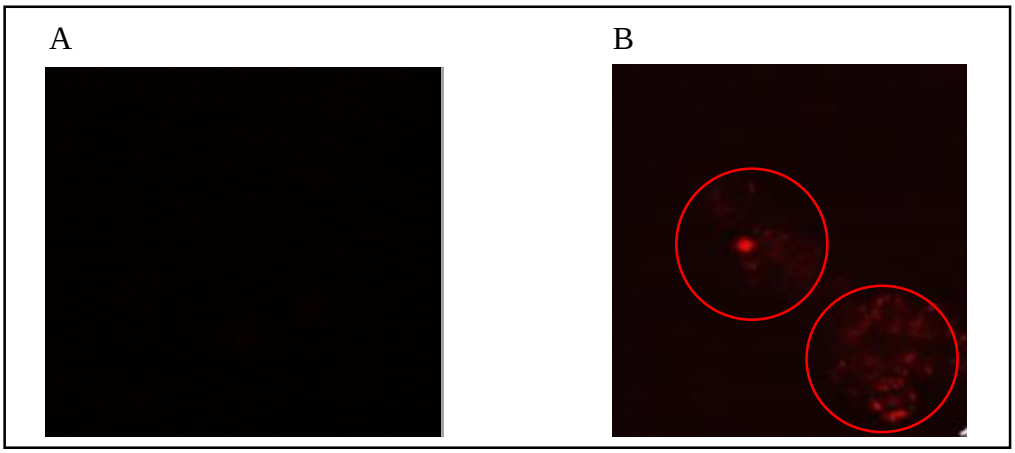

***Figure 2.6*: Erythrocyte clusters in parasite culture.** In parasite culture, RBC clusters were not observed in absence of EBA-175 RII (A) while the presence of the same led to the formation of RBC clusters circled in red (B). Adopted from Paing *et al.* (2018)

2.8 *Plasmodium falciparum* Rh5 and EBA-175 antigens as vaccine candidates

A malaria vaccine of a higher efficacy is a health priority particularly in malaria endemic areas. Decades of malaria vaccine research have yielded about 22 parasite antigens at various stages of the parasite's life cycle that have been tried in clinical trials (Tuju *et al.*, 2017). The partially effective RTS, S/AS01 is the licensed malaria vaccine with a short-lived efficacy (Bojang *et al.*, 2001). This could be a consequence of the parasite antigenic variability and high thresholds

required for protective antibody levels (John *et al.*, 2005; Murungi *et al.*, 2013). The WHO's 2030 strategic goals on licensure of malaria vaccines states that the vaccine should elicit a minimum efficacy of 75%. Therefore, more research needs to be done to identify malaria vaccine candidates of a higher efficacy.

2.8.1 Rh5

Rh5 is a desirable blood stage vaccine candidate whose gene sequence is highly conserved making the antigen to elicit strain-transcending antibody responses. Vaccination of mice and rabbits with the Rh5 antigen inhibited the invasion of erythrocytes by merozoites with a lower EC50 (Williams *et al.*, 2012; Douglas *et al.*, 2014). Antibody responses to Rh5 inhibited all *P. falciparum* lines as well as field isolates tested (Douglas *et al.*, 2011; Bustamante *et al.*, 2013; Reddy *et al.*, 2014). This antigen has been tried as a vaccine candidate in animal models and human beings with promising results. A quantitative study done by Douglas *et al.* (2015) in *Aotus* monkeys assessing the immunogenicity and efficacy of a Rh5-based vaccine to a heterologous *P. falciparum* challenge reported significant protection from *P. falciparum*. Protection was associated with the magnitude of antibody responses and parasite-neutralizing activity.

Recently, Rh5 has been tried in healthy, malaria-naive adult volunteers whereby intramuscular vaccination with Rh5 in a heterologous prime-boost regimen induced cross-strain neutralizing antibodies that inhibited the interaction of Rh5 and the other complex proteins i.e. P113, basigin and CyRPA, *in vitro*. It was also well-tolerated (Payne *et al.*, 2017). This vaccination induced greater antibody responses when compared to those induced naturally after exposure to malaria in African adults (Payne *et al.*, 2017). However, there is limited knowledge on how the magnitude of antibody responses based on full-length *P. falciparum* 3D7 Rh5 antigen expressed by mammalian cells change following vaccination and/or acute malaria episode.

2.8.2 EBA-175

Region II of EBA-175 is a vaccine candidate that has been tried in clinical trials. Naturally acquired antibody responses against this region significantly inhibited invasion of Senegalese parasites (Badiane *et al.*, 2013). Vaccination of rabbits, mice and monkeys with plasmids containing EBA-175 RII DNA elicited high immune responses which blocked the EBA-175-GPA interaction. The immune responses were boosted once the animals were challenged with living parasites (Sim *et*

al., 2001). Region II of EBA-175 was also assessed in Ghanaians who received increasing concentrations of the vaccine at three different time-points, where each dose elicited high antibody responses. The antibody response moderately inhibited the *in vitro* growth of *P. falciparum*. The vaccine was safe, well-tolerated and also immunogenic (Koram *et al.*, 2016).

2.9 The mammalian expression system

One of the important challenges in malaria research is expressing *P. falciparum* antigens in their native form (Birkholtz *et al.*, 2008). The mammalian expression system has repeatedly been applied in the expression of recombinant full-length *Plasmodium* proteins, which are functional and it is scalable (Srivastava *et al.*, 2010; Bustamante *et al.*, 2013; Chiou *et al.*, 2014). Different protein expression systems that include bacteria, yeast, plant, algae and cell-free (Ghosh *et al.*, 2002; Tsai *et al.*, 2006; Tsuboi *et al.*, 2008; Gregory *et al.*, 2012) have also been utilized to express Plasmodial proteins. Among these systems, the bacterial system is the most widely used because of its simplicity and cost-effectiveness although the success rates are as low as 6% (Mehlin *et al.*, 2006). Furthermore, the expressed proteins are hardly soluble and full-length, and have to be re-folded to the functional native protein (Mehlin *et al.*, 2006; Vedadi *et al.*, 2007). This is because the proteins are produced in a reducing environment which inhibits the formation of disulfide bonds that are abundant in secreted and membrane proteins (De Marco, 2009). Furthermore, bacteria are not powered with the appropriate machinery to express the proteins that undergo extensive post-translational modifications (De Marco, 2009). The wheat germ is a eukaryotic system that is cell-free and has successfully expressed soluble proteins although the bigger the size of the protein the less the solubility resulting in the separate expression of protein segments (Doolan *et al.*, 2008; Tsuboi *et al.*, 2008; Crompton *et al.*, 2010).

Rh5 and EBA-175 antigens were utilized in the present study to determine antibody responses against the antigens in acute and convalescent plasma samples obtained from children inhabiting an area where malaria remains endemic in Kilifi, Kenya. This study will inform the longevity of antibody responses to Rh5 and EBA-175 antigens after an acute malaria infection and the timing of serological studies.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study population and ethical approval

This study was carried out in Kilifi, a county along coastal Kenya, approximately 60 km North of Mombasa. The study population involved a cohort of 300 children from Junju sub-location, aged between 0 - 15 years, who are under active long-term surveillance for clinical malaria. Children who were born into the study households during the period of the study were continually recruited into the cohort, while older children were dropped out of the cohort after their 15th birthday. Children whose parent(s) or guardian(s) did not consent to be in the study were excluded from the study. The study population was predominantly from the Chonyi ethnic group, one of the smaller tribes of the Mijikenda, who are residents of Junju and the majority of whom practice subsistence farming. More information concerning the study population has been given elsewhere (Ndungu *et al.*, 2015). Junju sub-location remains a malaria endemic region despite a gradual decrease in the intensity of transmission in Kilifi County as a whole (Okiro *et al.*, 2007). This may be due to scaling up of ITN usage and change from sulfadoxine-pyrimethamine to artemisinin-based combination therapy. In this region, the transmission intensity of malaria peaks for the duration of the rainy season (May to July and November to December) (Mwangi *et al.*, 2005).

The study approval was obtained from the Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) under protocol 3149. Oral or written, thumb-printed or signed and witnessed informed consent was given by the parent(s) or legal guardian(s) of the study participants.

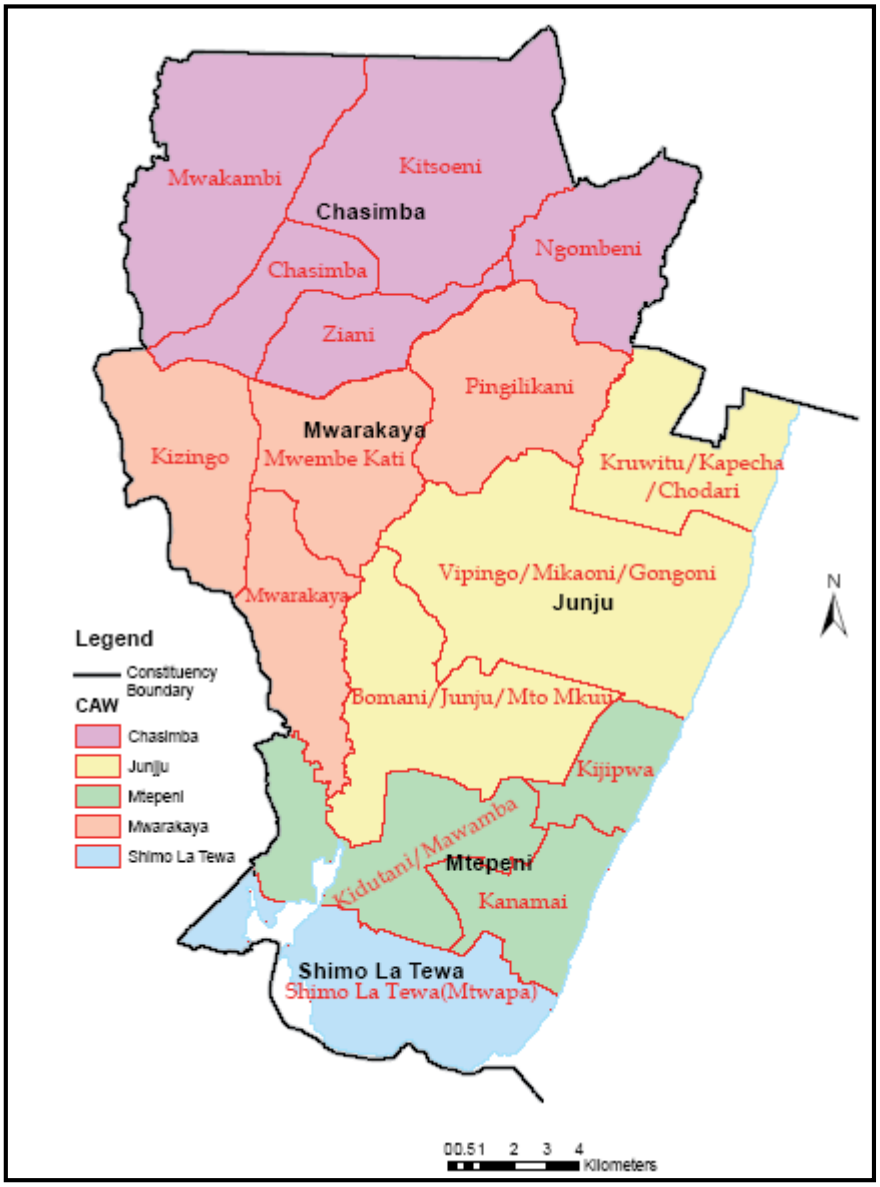

Figure 3.1: **A map of Kilifi South Constituency showing Junju Sub-location**

3.2 Sample size calculation and study design

A sample size of 300 was determined using the *pwr.anova.test* function as applied in the pwr package version 1.3-0 in R (Champely, 2020). An effect size of 0.09 (calculated in the formula below) and a 0.05 level of significance were assumed. This sample size was enough to detect the minimum antibody response (optical density (OD) of 0.05 calculated as mean OD of malaria naïve sera plus 3 standard deviations) to Rh5 and EBA175 at the six time-points with sufficient power (90%), assuming a 10% loss to follow-up.

$$f = \sqrt{\frac{\sum_{i-1}^{k} p_i * (\mu_i - \mu)^2}{\sigma^2}}$$

Where pi = ni/N,

n_i = number of observations in group i

N = total number of observations

μ_i = mean of group i

μ = grand mean

σ^2 = error variance within groups

An annual cross-sectional bleed was done between March and April in 2016 and 2017, before the long rains, which marked the beginning of the first malaria transmission season, where a venous blood sample (5 mL) was drawn from all the children. This constituted the baseline (B) sample. Following the cross-sectional bleed, active and passive surveillance for episodes of clinical malaria was done throughout the year. Briefly, active surveillance was done by field workers who visited the children in their homes on a weekly basis to check if they had malaria for the duration of the study, while passive surveillance was done at the Junju dispensary to capture missed clinical malaria episodes during the weekly visits. If axillary temperature was ≥37.5 °C, a slide and an RDT was done to ascertain if the child had malaria. A 5 mL blood sample was only taken from febrile RDT positive children which constituted the acute (A) sample and antimalarial drugs (Artemether-Lumefantrine tablets, coartem) given. Three subsequent convalescent (C) blood samples, C1, C2 and C3, 5 mL each, were drawn at 4, 6 and 24 weeks, respectively, following treatment. If the child was defined as severely ill, the parent/guardian was advised by the study physician to visit the nearest health facility. Plasma was separated from cells by centrifuging the blood samples at 700 × *g* for a period of 5 min. The plasma part of the blood and peripheral blood mononuclear cells (PBMCs) were stored at -80 °C.

3.3 Expression of recombinant Rh5 and EBA-175 antigens by mammalian cells

3.3.1 Plasmid vector description

The pTT3 vector (**Figure 3.1**) is a high copy number plasmid that was used to express the Rh5 and EBA-175 antigens in a mammalian expression system, Expi293F cells (ThermoFisher Scientific, Waltham, USA). The vector size was 6678 base pairs (bp), with a gene for ampicillin resistance and a rat C-terminal Cd4 d3+4 tag. Codon-optimized full-length Rh5 and EBA-175 antigens with a 6×His tag at the C-terminal end for protein purification and without the N-linked glycans, were

expressed using this vector. Expression was driven by cytomegalovirus (CMV) promoter. Rh5 and EBA-175 gene inserts already cloned in the pTT3 expression vector were kind gifts from the Professor Faith Osier laboratory, KEMRI-Wellcome Trust, Kilifi.

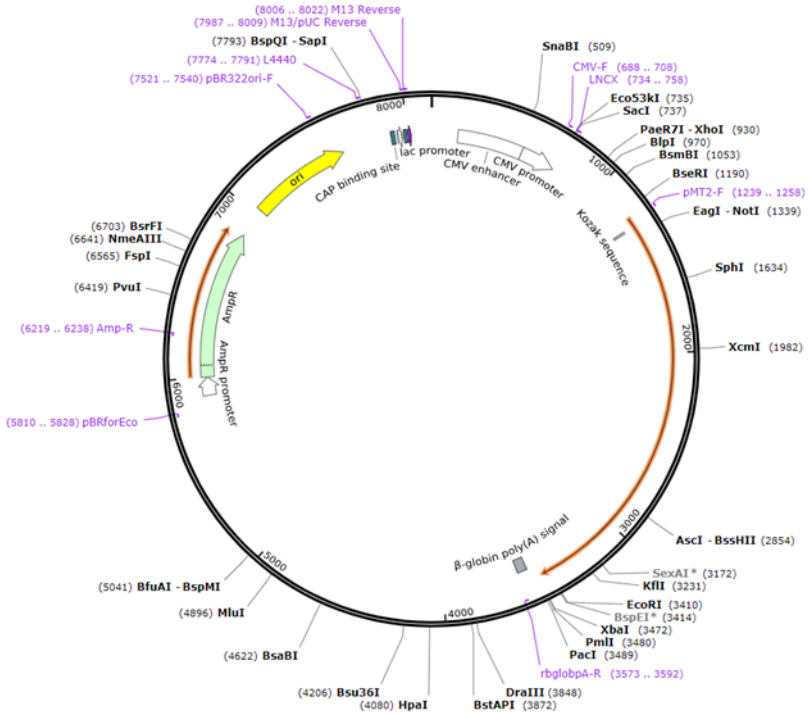

Figure 3.2: The pTT3 vector. This vector was used to express Rh5 and EBA-175 antigens. The features of the plasmid include a Kozak sequence (red), a gene for ampicillin resistance (Amp®, green), origin of replication (yellow), promoter region (White), β-globin poly(A) signal (grey) and the plasmid backbone (black). Restriction sites (text in black) and other features are labelled in purple text and are located on the plasmid backbone. Adopted from https://www.addgene.org

3.3.2 Transformation into competent *Escherichia coli* cells

One Shot® TOP10 chemically competent *Escherichia coli* (*E. coli*) cells (Invitrogen) from -80 °C storage were thawed on ice. Into 25 µL of the thawed cells in a 1.5 mL microcentrifuge tube, 2 µL of plasmid (100 ng/µL) with either Rh5 or EBA-175 as the insert were added separately. The microcentrifuge tube containing the mixture of the competent cells and plasmid was flicked 5 times to mix the contents before being incubated in ice for a 30 min duration. In a 42 °C water bath, the cells were then heat-shocked for 30 sec and quickly returned on ice. Heat-shocking creates pores in the bacterial cell membrane which allows the plasmid to enter the bacterial cell. The cells were

resuspended in 250 µL of super optimal broth with catabolite repression (SOC) medium (Invitrogen) which is a nutrient-rich microbial broth used in the recovery step of the bacterial cells after heat-shocking to maximize transformation efficiency. At 37 °C, the cells were incubated for 1 h in an incubator shaker (New Brunswick™ Innova® 40, Eppendorf, Hamburg, Germany) while shaking at 0.8 × *g*. The culture was spread on petri plates containing LB agar that was supplemented with ampicillin before being incubated overnight at 37 °C. The colonies which grew were picked and cultured in a 1000 mL glass bottles containing 250 mL of Luria-Bertani (LB, ThermoFisher Scientific) medium supplemented with ampicillin (100 µg/mL). The cells were then allowed to grow overnight at 0.8 × *g* at 37 °C in the same shaker incubator.

3.3.3 Isolation and quantification of plasmid DNA

The bacterial cells were harvested by centrifuging for 15 min at 4 °C at 6000 × *g* using Centrifuge 5810R (Eppendorf, Hamburg, Germany). Plasmid isolation was performed as per the Qiafilter plasmid Midi kit protocol (Qiagen, Maryland, USA) and resuspended in 200 µL of 1× Tris-EDTA (TE) buffer. Quantification was done using the NanoDrop 2000 UV-Vis spectrophotometer (ThermoFisher Scientific, Waltham, USA).

3.3.4 Confirmation of the gene insert into the plasmid vector

Double restriction digestion of 10 µL of plasmid (100 ng/µL) was performed overnight at 37 °C in a 20 µL reaction (**Table 3.1**) using 0.2 µL high fidelity (HF) NotI and 0.2 µL AscI (New England Biolabs (NEB)) restriction enzymes. The two restriction enzymes have 100% activity in 1× CutSmart buffer (NEB) and so 2 µL of this buffer and top-up to 20 µL using 7.6 µL of nuclease free water was done. This reaction was incubated overnight at 37 °C.

***Table 3.1*: Double restriction enzyme digestion to confirm gene cloned into the plasmid vector**

Reagents and plasmid constructs	**1 reaction**	**4 reactions**
CutSmart buffer	2 µL	10 µL
High fidelity NotI	0.2 µL	0.8 µL
AscI	0.2 µL	0.8 µL
Nuclease free water	7.6 µL	28.4 µL
Plasmid construct	10 µL	

This is a table showing the amount of the reagents used for double restriction digestion for four reactions.

The products of the restriction digest alongside a 10,000 bp molecular size marker (Hyperladder I, Bioline) were separated using 1% (w/v) agarose gel electrophoresis based on size. The sizes of the ladder fragments ranged from 200 to 10,000 bp with the brightest band at 1000 bp. Into 100 mL of 0.5× Tris-Borate-EDTA (TBE) buffer in a 250 mL flat-bottomed conical flask, 1 g of agarose was added and microwaved for 2 min to completely dissolve the agarose. This was followed by addition of 5 µL of 20,000× RedSafe™ Nucleic Acid Staining Solution (iNtRon Biotechnology) before the agarose solution completely cooled and swirled gently to mix. RedSafe staining solution emits green fluorescence when bound to DNA. While the agarose solution cooled, it was slowly and gently poured into the gel tray before immersing the gel comb. For a duration of 30 min, the agarose gel was allowed to cool further until it polymerized and 10 µL of the samples added. Hyperladder I was loaded to the first well of the agarose gel before performing electrophoresis at 90 V for 90 min. Separation of the Rh5 and EBA-175 gene inserts was visualized under UV illumination using Molecular Imager®, ChemiDoc™ XRS+ Imaging System (Bio-Rad, Hercules, USA).

3.3.5 Recombinant protein expression

Expi293F mammalian cells from liquid nitrogen were quickly thawed for 2 min in water bath at 37 °C while being gently agitated. The thawed cells were then resuspended in 30 mL of pre-warmed Expi293 Expression Medium (ThermoFisher Scientific) in 125 mL vented cap, Corning®

Erlenmeyer cell culture flask. Thereafter, the cells were incubated in a humidified orbital shaker platform (New Brunswick™ S41i, Eppendorf, Hamburg, Germany) at 37 °C and 8% carbon dioxide (CO_2) rotating at 0.4 × *g*. Expi293F cells have a doubling time of 24 h. Total cell count as well as viability was measured making use of an Countess™ automated cell counter, (Invitrogen, Waltham, USA). Then, the cell culture was monitored after every 48 h until the cell density was 1.0×10^6 cells/mL. Dilution of the cell culture to 5×10^5 cells/mL was done and the cell culture monitored after every 48 h until a maximal density of 6×10^6 viable cells/mL. Cell cultures with a viability less than 90% were discarded. Similarly, cells which were in culture for a period of over 3 months were also disposed off before thawing a new batch. Before transfection, cell viability and count was again checked using the same counter. The cells were then diluted to 2.0×10^6 viable cells/mL using pre-warmed Expi293 Expression Medium. Transfection of the diluted Expi293F cells with the expression plasmid containing either Rh5 or EBA-175 antigens was done according to the ExpiFectamine™ 293 Transfection Kit (ThermoFisher Scientific, Waltham, USA), a commercially available kit that is designed for scalable transient transfection of mammalian cells (Chiou *et al.*, 2014). The transfected cells were incubated in a benchtop shaker incubator (New Brunswick™ S41i) set at 37 °C, 8% CO_2 while shaking at 0.4 × *g*.

The medium containing the Expi293F cells and the secreted protein was poured into clean well-labeled 50 mL falcon tubes 4 days post transfection. The contents of the falcon tube were centrifuged at 125 × *g* for 5 min to pellet the cells. The supernatant containing the protein of interest was poured into clean well-labelled 50 mL falcon tubes and thereafter kept at 4 °C prior to purification while the pellet was discarded.

3.3.6 Protein purification and quantification

The expressed proteins were purified using the ProBond™ Purification System (Thermo Fisher Scientific). Into 50 mL supernatant in a 50 mL falcon tube, 1 mM (50 µL) nickel (II) chloride was added to prevent non-specific binding of other proteins. Following that, 300 µL of ProBond™ nickel-chelating resin (Invitrogen), in binding buffer supplemented with 10 mM imidazole to reduce binding of contaminating proteins, was added and rocked at 4 °C for 45 min in order that the resin bound to the 6×His tag of Rh5 and EBA-175 antigens. The resin bound to the protein of interest was allowed to settle for 10 min and gentle aspiration done to remove about 45 mL of the supernatant. The remaining media was poured into 5 mL propylene columns already placed on a

QIArack and let to flow making use of gravity. The column containing the protein of interest was washed with 30 mL 1× native wash buffer supplemented with 20 mM imidazole. Protein elution was done with 500 µL of native elution buffer with 250 mM imidazole into clean well-labeled 1.5 mL Eppendorf tubes.

Protein quantification was done according to the Pierce™ BCA (bicinchoninic acid) Protein Assay Kit (ThermoFisher Scientific, Waltham, USA). Briefly, 10 µL of the purified protein and 10 µL of each protein standard whose concentration ranged from 2000 µg/mL to 125 µg/mL was dispensed in duplicate in Microplate Immulon 4HBX 96 wells. The native elution buffer used to elute the proteins during the purification process was also loaded in duplicate into the wells to act as a blank and 200 µL of reagent A and B that were mixed in the ratio of 50:1, respectively, was then added to the wells. The contents of the wells were mixed by gently pipetting up and down and incubated at 37 ºC in an incubator shaker (New Brunswick™ Innova® 40, Eppendorf, Hamburg, Germany) for 30 min without shaking. Optical density (OD) reading was taken at 592 nm. The unknown concentration of the expressed proteins was interpolated from the standard curve. Aliquots of the quantified Rh5 and EBA-175 were kept at -20 ºC and were thawed at 4 ºC before being used.

3.3.7 The size and integrity of the expressed proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to www.nationaldiagnostics.com to determine the integrity and confirm the sizes of the purified proteins. An 8% (w/v) separating gel was made by adding 6.7 mL of distilled water (dH_2O) to 13.3 mL of ProtoGel Quick-Cast (12%) (National Diagnostic, Leicestershire, UK) in a 50 mL falcon tube. This was followed by addition of 200 µL of 10% ammonium persulfate (APS) and 20 µL of N, N, N, N -tetramethylethylenediamine (TEMED) and mixed briefly by inverting the falcon tube thrice. The separating gel was quickly transferred into the gel cassette before polymerizing and 50% isopropanol added to give a level gel. The gel was allowed to set for 20 min, the isopropanol discarded and the gel washed with a gentle stream of tap water. The stacking gel (4.8%) was prepared by adding 3.6 mL of dH_2O to 2.4 mL of ProtoGel Quick-Cast (12%) in a 15 mL falcon tube. Following this was addition of 30 µL of 10% APS and 5 µL of TEMED and brief mixing done by inverting the falcon tube thrice. The stacking gel was then cast over the separating gel and a comb quickly inserted before allowing the stacking gel polymerize at ambient

temperature. The protein samples were mixed with 2× Protein loading buffer Blue (National Diagnostics) in the ratio of 1:1 and the proteins denatured at 100 ºC for 5 min. After this, 10 µL of the denatured protein samples was loaded into the wells after removing the comb and 5 µL of Amersham™ ECL™ Rainbow™ Marker (full range) loaded into one of the wells to determine band sizes. The gel was then allowed to run for 90 min at 115 V and stained using InstantBlue® Coomassie Protein Stain (Expedeon) to visualize the separated bands of Rh5 and EBA-175 antigens.

3.3.8 Western blot detection of Rh5 and EBA-175

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as per the protocol in section 3.3.7 except that the gel was not stained with InstantBlue® Coomassie Protein Stain. Western blotting was performed according to https://www.abcam.com/protocols/general-western-blot-protocol. The gel was placed in 1× transfer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol) buffer for 15 min. Similarly, 2 sponges and 2 fibre pads were soaked in 1× transfer buffer for 5 min. The Polyvinylidene fluoride (PVDF, Invitrogen) membrane was soaked in methanol for 1 min to activate it, then in water for 2 min and lastly in 1× transfer buffer. The transfer sandwich was assembled cautiously to ensure air bubbles were not trapped before placing the cassette in the transfer tank, to transfer the separated protein bands from the gel to the PVDF membrane for a period of 1 h at 100 V. After rinsing the membrane with water, 1 h blocking at ambient temperature with 3% (w/v) blocking buffer (Blotto, non-fat dry milk) was done. The membrane was then incubated with an appropriate dilution (1:5000) of primary antibody conjugated with horseradish peroxidase (HRP) (Anti-His, Sigma) in 3% (w/v) blocking buffer, which bound to the hexa-histidine tag of the target protein. This was followed by washing of the membrane 3 times, 5 min each wash, using 1× Tris-buffered saline/0.05% (v/v) Tween 20 (TBS/T) at ambient temperature. Novel enhanced chemiluminescence (ECL, Invitrogen) substrate for horseradish peroxidase conjugated to the primary antibody was applied to the blot for signal development, and incubated in the dark for 5 min. The signals were captured by a charge-coupled device (CCD) camera-based imager (Molecular Imager®, ChemiDoc ™ XRS+ Imaging System (Bio-Rad) and image analysis software employed to determine the band intensity of Rh5 and EBA-175 recombinant proteins.

3.4 Measurement of antibody responses to recombinant antigens by standard ELISA

3.4.1 Optimization for the coating concentration of the antigens

The expressed and quantified Rh5 and EBA-175 antigens were serially diluted in carbonate-bicarbonate coating buffer (Sigma-Aldrich) starting at a concentration of 70 µg/mL and 53µg/mL, respectively. Microplate Immulon 4HBX 96 well (Dynatech, ThermoFisher Scientific, Loughborough, UK) plates were coated with the serially diluted antigens in duplicate and an overnight incubation at 4 ºC done. The plates were washed 4 times using 1× Phosphate buffered saline/Tween 20 (PBS/T) (washing buffer) using an ELISA plate washer before blocking with 200 µL of 1% (w/v) Marvel skimmed milk powder dissolved in 1× PBS/T (blocking buffer) to prevent non-specific binding. The plates were incubated at ambient temperature for 3 h and washed 3 times with washing buffer. The concentration of primary and secondary antibody was held constant at 1:1000 and 1:5000 (v/v) dilution in blocking buffer, respectively. Pooled hyperimmune serum (PHIS) from Kenyan adults who were immune to malaria and malaria naïve serum were used as the primary antibodies. The plates were incubated with 100 µL of the primary antibodies and an overnight incubation at 4 °C done. The plates were washed 5 times and incubated with Rabbit Anti-Human IgG HRP Gamma-Chain Specific (Dako Ltd, Cambridgeshire, UK) for 3 h at ambient temperature and thereafter washed 5 times. The enzyme substrate (100 µL of SIGMAFAST™ O-phenylenediamine (OPD) dihydrochloride, Sigma-Aldrich) was then added after which 25 µL of the substrate stop solution (2 M H_2SO_4) was added. Optical density reading was done at 492 nm.

3.4.2 ELISA on actual plasma samples

Out of the 300 children that were included in this study, the ELISA assay was done for samples from 83 children who developed an acute malaria infection and had plasma samples from at least five time-points i.e. baseline (B), acute (A), convalescent 1 (C1), convalescent 2 (C2), and convalescent 3 (C3). Children whose plasma samples were not taken at any of the mentioned time-points were excluded from the study. Out of the 83 children who were assayed, 2 of them reported to hospital with another acute malaria infection between A and C1 time-points and were termed as AC1, while 33 reported to hospital with an acute malaria infection between C2 and C3 and were termed as AC3. None of the 83 children reported to hospital with an acute malaria infection between C1 and C2, that would have been termed as AC2.

Plasma levels of immunoglobulin G (IgG) specific for the expressed Rh5 and EBA-175 antigens were determined by ELISA in the acute and convalescent plasma samples. Briefly, in Microplate Immulon 4HBX 96 well plates, 100 µL of the mentioned antigens in coating buffer, was added. The plates were incubated at 4 °C overnight and washed 4 times using washing buffer before blocking with 200 µL of blocking buffer to prevent non-specific binding. An incubation of the plates at ambient temperature was done for 3 h after which 3 times washing with wash buffer was repeated as above. The plates were incubated with test plasma (100 µL) in duplicate at 1:1000 dilution. An overnight incubation of the plates at 4 °C was done. The plates were washed 5 times before being incubated with the secondary antibody, Rabbit Anti-Human IgG HRP Gamma-Chain Specific at 1: 5,000 dilution in blocking buffer at room temperature for 3 h. The plates were washed 5 times and the enzyme substrate, OPD dihydrochloride substrate added. After this was the addition of 25 µL of substrate stop solution (2 M H_2SO_4) and OD reading done at 492 nm. A total of 5 negative controls obtained from non-exposed individuals from United Kingdom (UK) were used in the establishment of the negative cut-off, defined as mean OD of naive sera plus three standard deviations, while pooled hyperimmune sera (PHIS) from adults who were immune to malaria was used in each plate as a positive control. A serially diluted malaria immunoglobulin (MIG) of known concentration (50 mg/mL) from Malawian adults was included in each plate to normalize variation from one plate to another.

3.4.3 Evaluation of changes in antibody titres over time

Data were analyzed in R version 3.6.1. While antibody levels were given as antibody titres, the seropositivity cutoff was defined as mean OD of naive sera plus three standard deviations. Malaria Immunoglobulin (MIG) standard of known concentration (50 mg/mL) was serially diluted and run in each plate and was later used to determine the antibody titres by interpolating the OD values of the acute and convalescent plasma samples. A four-parameter logistic curve (4-PL) was derived from the MIG standard concentrations and measurements in OD at the different time-points of the test plasma samples interpolated from the curve to obtain antibody titres. Differences in antibody titres across all the time-points were compared using the non-parametric Kruskal-Wallis test. The Wilcoxon signed-rank test was applied to compare the antibody titres between B and A, and between A and either C1, C2 and C3 time-points.

A non-linear mixed effect model (*nlme*) analysis for repeated measure designs was used to model antibody titres as a function of time to determine the changes in antibody titres over time. The *nlme* package was used to perform the non-linear mixed effect analysis of antibody titres (the dependent variable) as a function of time-point (the independent variable). As fixed effects, gender, age of the children and parasite density at baseline were entered into the model. A random effect for the unique identifier for each child was included to account for variation inherent due to individual differences.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of the study population

Table 4.1 is a summary of the characteristics of the study population. Out of the 83 children who were assayed, 47 (56.6%) were male while 36 (43.4%) were female. The age of the study participants ranged from 0 to 14 years, inclusive, with a median of 9 years. Children over 5 years represented 72.3% of the 83 children. The median age for the children above 5 years was 9 years and ranged from 6 to 14 years, while the median age of those ≤5 was 3 years ranging from 0 to 5 years. Children with baseline parasitemia represented 15.7% of the 83 children. Baseline parasitemia ranged from 0.18 to 95234 parasites by Polymerase Chain Reaction (PCR).

***Table 4.1*: Characteristics of the study population**

Number of study participants	83
Gender	
Male [n(%)]	47 (56.6)
Female [n(%)]	36 (43.4)
Median Age (Range)	9 (0-14)
≤5 [n, (%) (median, range)]	23, 27.7 (3, 0-5)
>5 [n, (%) (median, range)]	60, 72.3 (9, 6-14)
Percentage of participants with baseline parasitemia [n (%) (Range)]	13 (15.7) (0.18-95234)

4.2 Plasmid vector validation

Double restriction digest of the pTT3 plasmid using HF NotI and AscI restriction enzymes, confirmed the insertion of Rh5 and EBA-175 into the pTT3 vector. In **Figure 4.1A**, the two bands visualized were of the 1515 bp Rh5 insert and the 6678 bp pTT3 vector backbone, while those visualized in **Figure 4.1B**, were of the same vector backbone and the EBA-175 insert of 4218 bp. The double restriction digest was complete as only two bands of the vector backbone and the gene of interest were visualized.

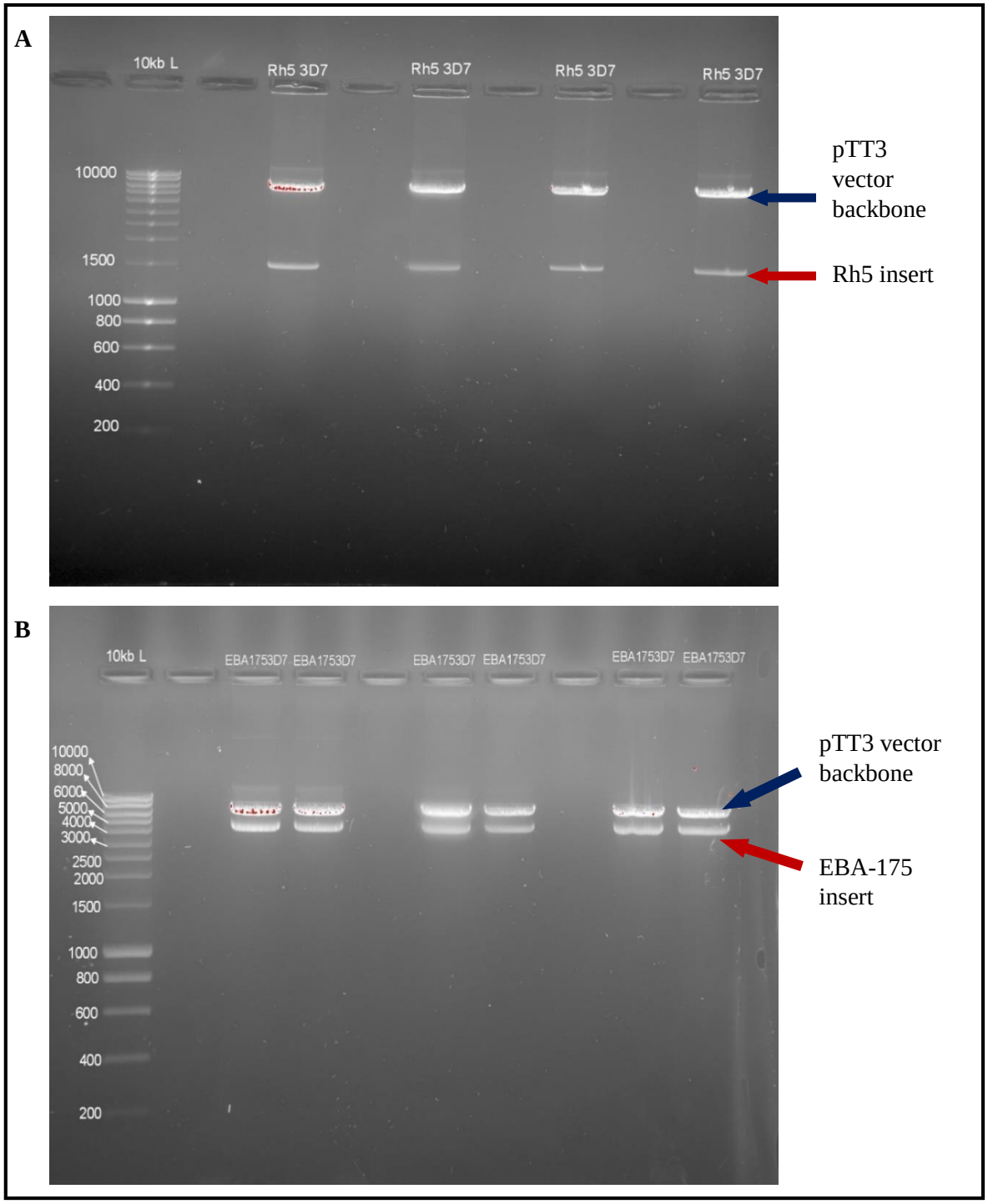

***Figure 4.1*: Screening of recombinant plasmids by restriction enzyme analysis using NotI/AscI.** (A) This figure shows a 1% agarose gel used to resolve the 1515 bp Rh5 (red arrow) and 6678 bp pTT3 vector backbone (blue arrow) products of the restriction digestion. A 10 kb ladder (L) was used to determine the band sizes. Four reactions for Rh5 were done. (B) This agarose gel was for EBA-175. The antigen size is 4218 bp (red arrow) and the vector backbone is of 6678 bp (blue arrow). Three reactions for EBA-175 were performed.

4.3 The size of the expressed proteins

4.3.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

A full-length Rh5 (~63 kDa) and EBA-175 (~175 kDa) were successfully expressed by Expi293F cells. **Figure 4.2 A** represents the SDS-PAGE for Rh5 while **Figure 4.2 B** represents that of EBA-175. The Amersham™ ECL™ Rainbow™ Marker, a full range ladder ranging in size from 12 kDa to 225 kDa, was used to confirm the band sizes of Rh5 and EBA-175. Three protein elutions each are shown for Rh5 and EBA-175. The expressed proteins had no contaminating proteins as only one band for each elution was observed.

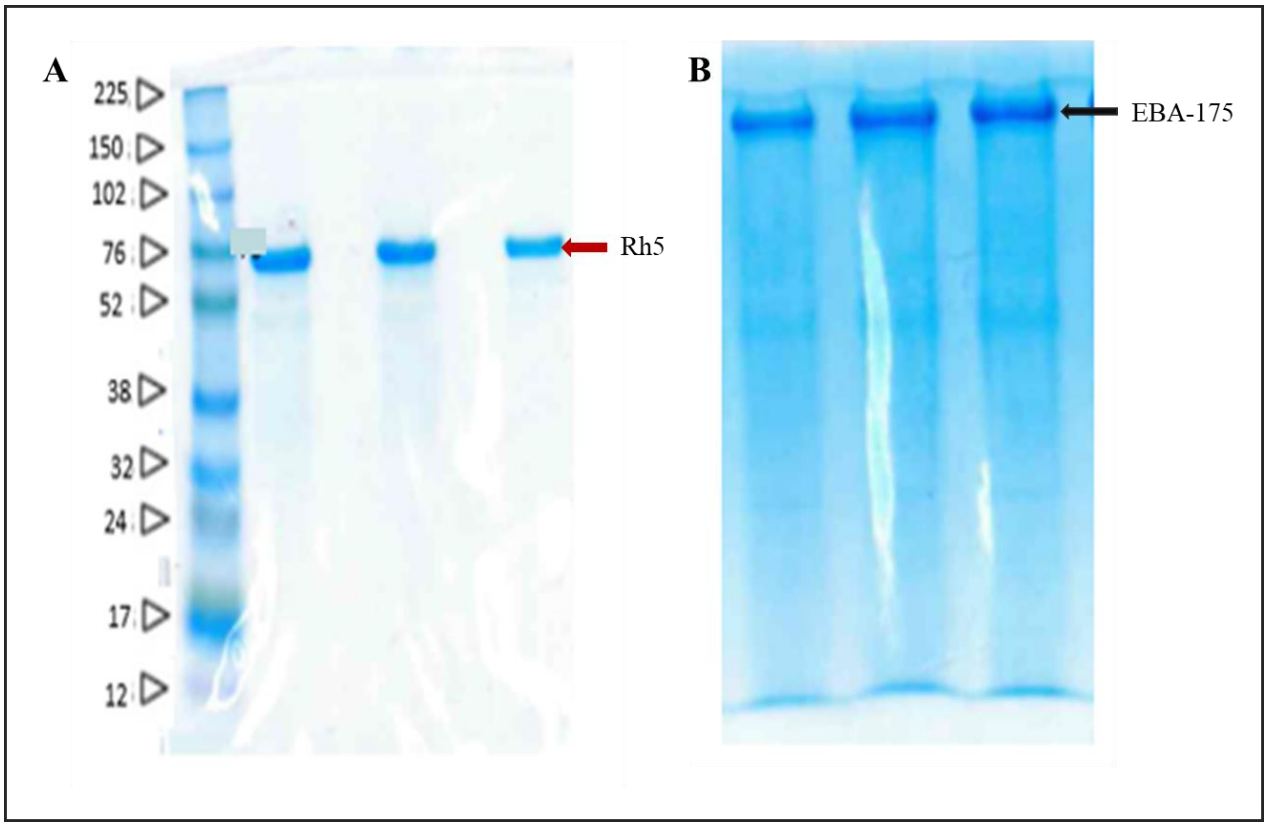

***Figure 4.2*: The size and integrity of Rh5 and EBA-175 antigens.** A and B are SDS-PAGEs for Rh5 and EBA-175, respectively. L represent Amersham™ ECL™ Rainbow™ Marker – full range ladder. The size of the ladder ranges from 12 kDa to 225 kDa. Three protein elutions for Rh5 and EBA-175 are shown.

4.3.2 Western blot analysis

The hexa-histidine tagged Rh5 and EBA-175 were successfully expressed using Expi293F cells (**Figure 4.3**). The HRP-conjugated antibody reacts with native and denatured histidine-tagged

proteins independent of the location of the histidine-tag in the protein sequence. AMA1 and Rh5 variants 1-10 were used as controls for the western blot to ensure the anti-his and enhanced chemiluminescence (ECL) substrate for horseradish peroxidase conjugated in the primary antibody were working. However, the bands of focus are for the ~63 kDa Rh5 and ~175 kDa EBA-175 antigens.

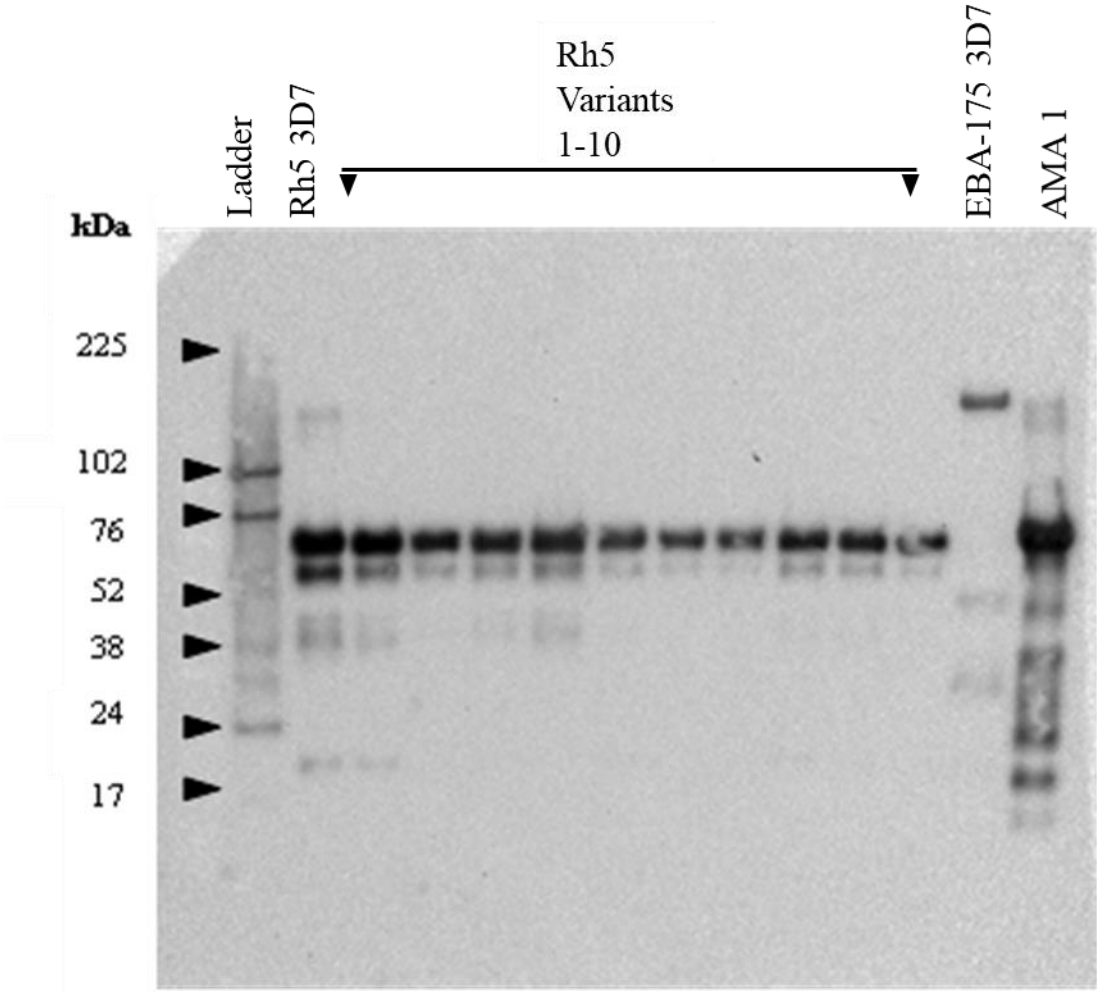

***Figure 4.3*: A Western blot for Rh5 and EBA-175 antigens.** This is a Western blot for Rh5 3D7, Rh5 variants 1-10, EBA-175 3D7 and AMA1. Rh5 3D7 and Rh5 variants 1-10 are approximately 63 kDa while EBA-175 is a ~175 kDa protein. AMA1 is ~62 kDa. kDa: KiloDalton.

4.4 Optimization of the Rh5 and EBA-175 protein coating concentration

Optimization of the Rh5 and EBA-175 protein coating concentration was done to ensure that the optimal amount of antigen that produced detectable antibody response was used for ELISA.

4.4.1 Rh5

The OD of the positive control (PHIS) and the negative control (Naïve serum) was plotted against the coating concentration of Rh5 (**Figure 4.4)**. The OD increased with increasing concentration of the antigen, in the case of the positive control, before saturation. For the negative control, OD increased slightly with increasing concentration of the antigen before reaching a plateau phase. This shows that the naïve serum had antibodies against Rh5 antigen but less compared to the positive control. Therefore, Rh5 antigen was coated at a concentration of 35 µg/mL (**Figure 4.4**).

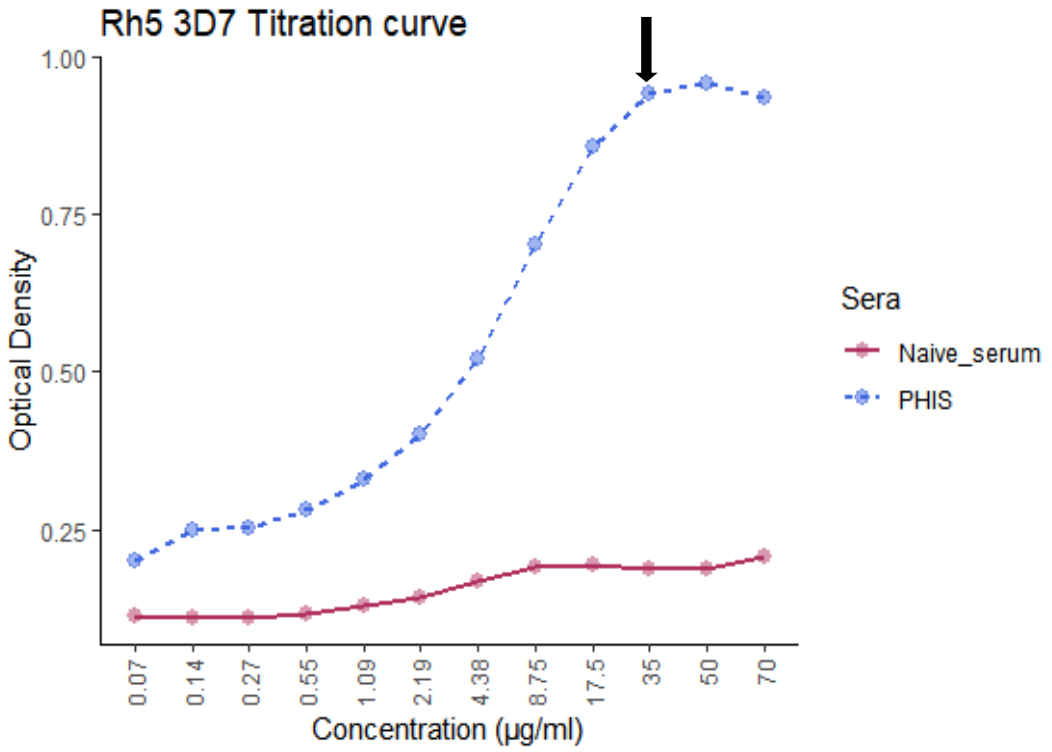

***Figure 4.4*: Rh5 3D7 titration curve.** This is a graph of optical density (OD) of pooled hyperimmune sera (PHIS, blue) and malaria naïve serum (maroon) against increasing concentration of Rh5 antigen in µg/mL. The black arrow points at the concentration of Rh5 that was used to coat the ELISA plates. To the right of the graph is a legend showing the two types of sera that were used as the primary antibodies. PHIS was used as a positive control while naïve serum was used as a negative control. Each dot represents mean OD of two experimental replicates. The dots are joined with either a dashed and blue line or continuous and maroon line.

4.4.2 EBA-175

Figure 4.5 represents a graph of OD of both PHIS and naïve serum against increasing EBA-175 concentration. The OD of the positive control increased with increasing concentration of EBA-

175 until a point where further increase in the concentration of the antigen led to no increase in OD. The negative control showed no response to EBA-175 because there was no increase in OD with increasing concentration of the antigen. EBA-175 was coated at a concentration of 6.62 µg/mL (**Figure 4.5**).

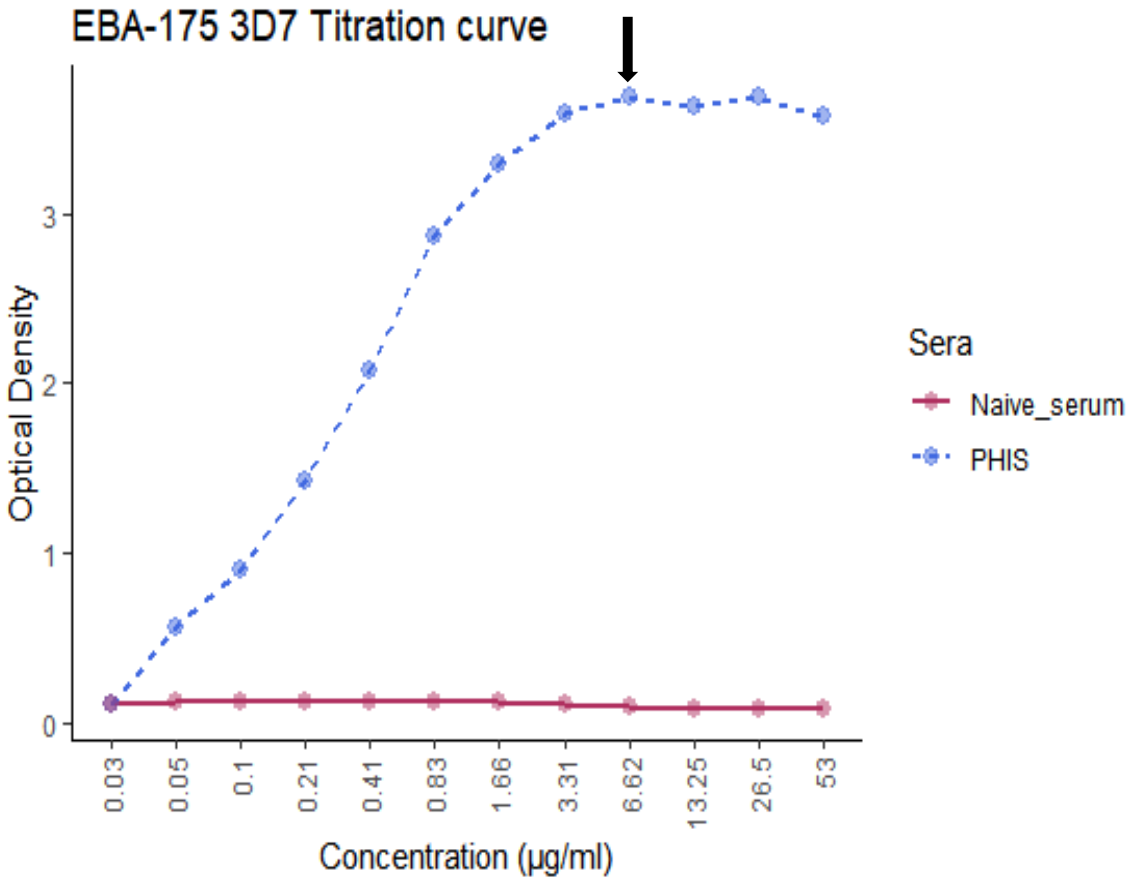

***Figure 4.5*: EBA-175 3D7 titration curve.** This is a graph of optical density (OD) of pooled hyperimmune sera (PHIS, blue) and malaria naïve serum (maroon) against increasing concentration of EBA-175 antigen in µg/mL. The black arrow points at the concentration of EBA-175 that was used to coat the ELISA plates. The legend to the right of the graph represent the two types of sera that were used as primary antibodies. Naïve serum was the negative control while PHIS was the positive control. Each dot represents mean OD of two experimental replicates The dots are joined with either a dashed and blue line or a continuous and maroon line.

4.5 Heterogeneity in antibody responses to Rh5 and EBA-175

4.5.1 Rh5 general profiles

The number of children assayed were 83. Data from 82 children was considered for analysis because at one time-point, one child had a low OD value that could not be interpolated from the MIG standard curve to get antibody titres. Rh5 was immunogenic as it elicited antibody responses in the plasma samples tested. **Figure 4.6** represents a graph of log-transformed antibody titres against time-point (B, A, C1, C2 and C3). Antibody responses to Rh5 were heterogeneous. Some children had high antibody titres (~104648.95) throughout the follow-up period while in others, antibody titres remained low (~942), with the majority of the children having average antibody titres (~17278.29). The median antibody titre was 13835.48. The antibody titres ranged from 942 to 104649. There was variability in antibody responses at baseline which could have resulted from the differences in the degree of previous exposure to malaria among the children. In addition, some children were asymptomatic and others uninfected at baseline which could also have contributed to the variability.

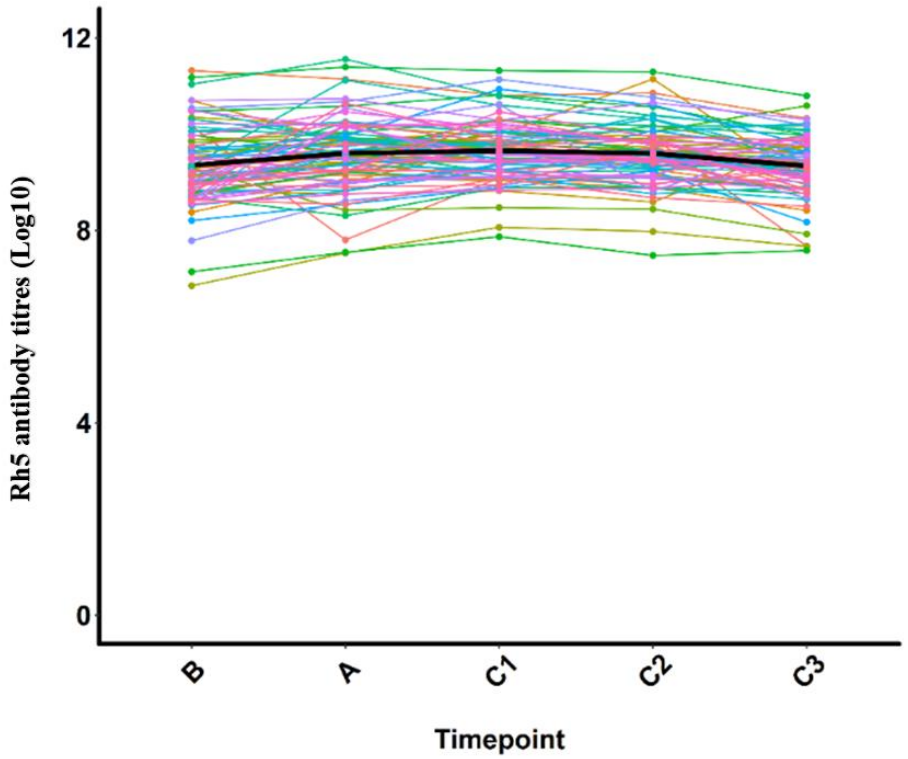

***Figure 4.6*: General antibody profiles for Rh5.** Plasma levels of log-transformed antibodies titres specific to Rh5 in 82 malaria-exposed children at baseline (B), acute malaria infection (A, Week 0), and after 4 weeks (C1), 6 weeks (C2) and 24 weeks (C3), following treatment are shown. Each dot represents a time-point. Temporal changes in antibody titre levels in each child and in the cohort mean is represented by the distinctly colored lines and the heavy black line, respectively. The distinctly coloured lines (except the heavy black line) connect data from individual children.

4.5.2 Individual profiles for Rh5

Figure 4.7 represents the individual profiles for 82 children for the Rh5 antigen. In a majority of the children, 28/82 (34.1%), antibody titres peaked during the acute malaria infection and in some children (28.6% of the 28 children whose antibody titres peaked at acute malaria infection), antibody titres kept rising between A and C1 before declining to baseline. In 26/82 children (31.7%), there was no change in antibody titres with time. In 13/82 children (15.9%), antibody responses peaked at C1 while in 7/82 children (8.5%) antibody responses peaked at C2. Whether the antibody responses peaked at C1 or C2, they generally declined to baseline after 24 weeks (C3). In 7/82 children (8.5%), antibody responses were highest at baseline but waned throughout

follow-up and in 1 child, antibody responses were lowest at baseline but increased throughout follow-up.

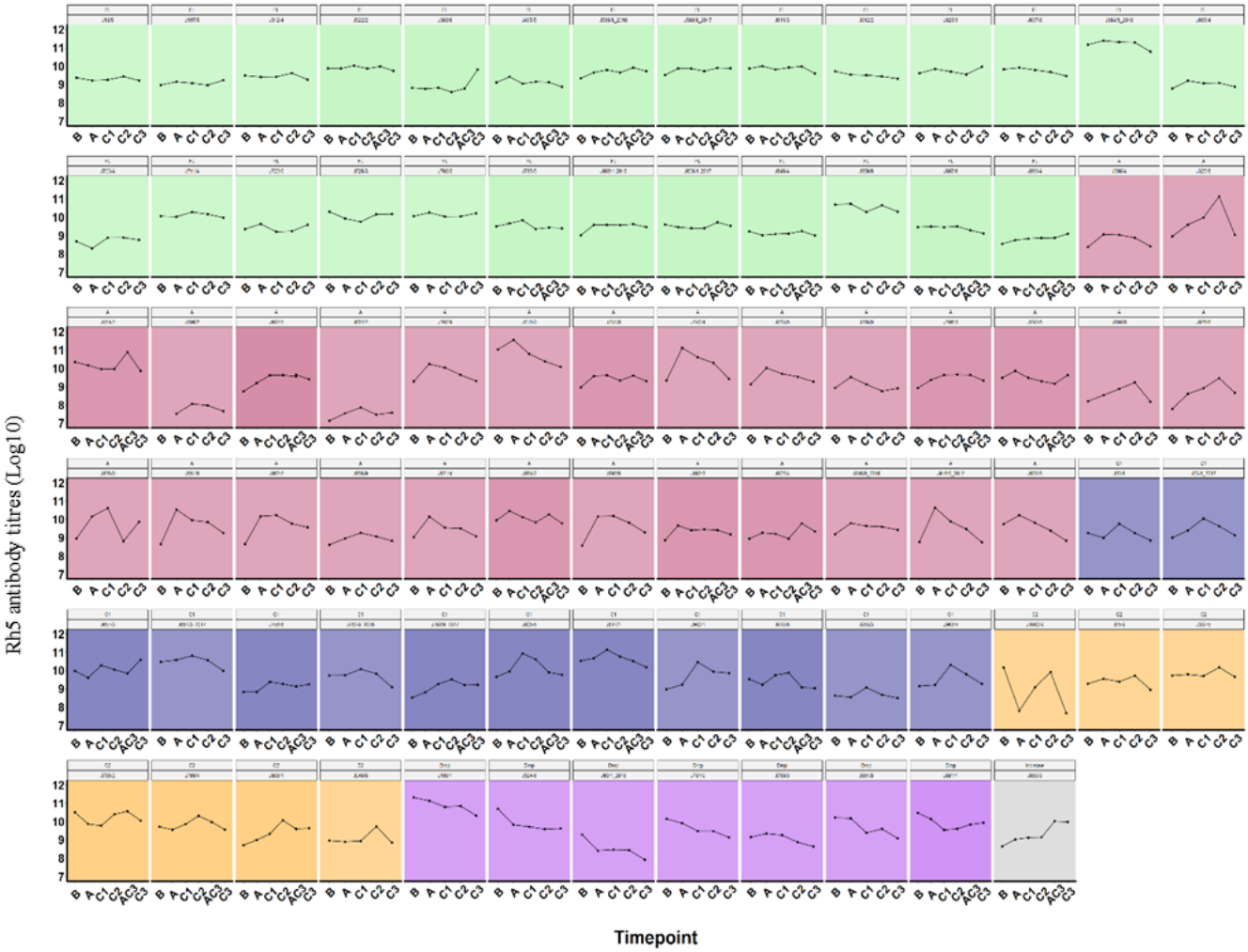

***Figure 4.7*: Individual profiles for Rh5 antigen.** This is a graph of log-transformed antibody titres for Rh5 against time-points (B, A, C1, C2, AC3 and C3). The different profiles are highlighted in distinct colours with similar profiles highlighted with the same colour. The individual profiles are ordered from the most common to the least common. Green: Represent profiles where there was no change in antibody titres with time. Maroon: Profiles where antibody responses peaked at acute malaria infection followed by a decline during the convalescent period. Blue: Antibody responses peaked at C1 and declined during the recovery phase. Orange: Antibody responses peaked at C2 before declining to baseline by the 24th week (C3). Purple: Represent the profiles of children where antibody responses were highest at baseline and decreased throughout follow-up. Grey: Antibody responses were lowest at baseline and increased throughout follow-up. Above each individual profile is a unique identifier for each child and profile type. Each dot represents a time-point and the scale on the y-axis runs from 7 to 12.

4.5.3 General antibody profiles for EBA-175

The number of children assayed were also 83. One child had high antibody responses at all time-points that could not be interpolated into the standard curve to obtain antibody titres reducing the number of children whose antibody titres was obtained to 82. EBA-175 was also immunogenic as it elicited antibody responses in the plasma samples tested. **Figure 4.8** represents a graph of log-transformed antibody titres against time-point (B, A, C1, C2, and C3). For some time-points, antibody responses to EBA-175 were either too high or low to be interpolated from the MIG standard curve and hence the varied number (n) of children assayed at each time-point. Antibody responses to EBA-175 were more heterogeneous than those of Rh5. Two children had high antibody titres throughout follow up (~79160.9), while a majority of them had average antibody titres (3389.8). The antibody titres ranged from 26.2 to 79160.9 with a median antibody titre of 866.1.

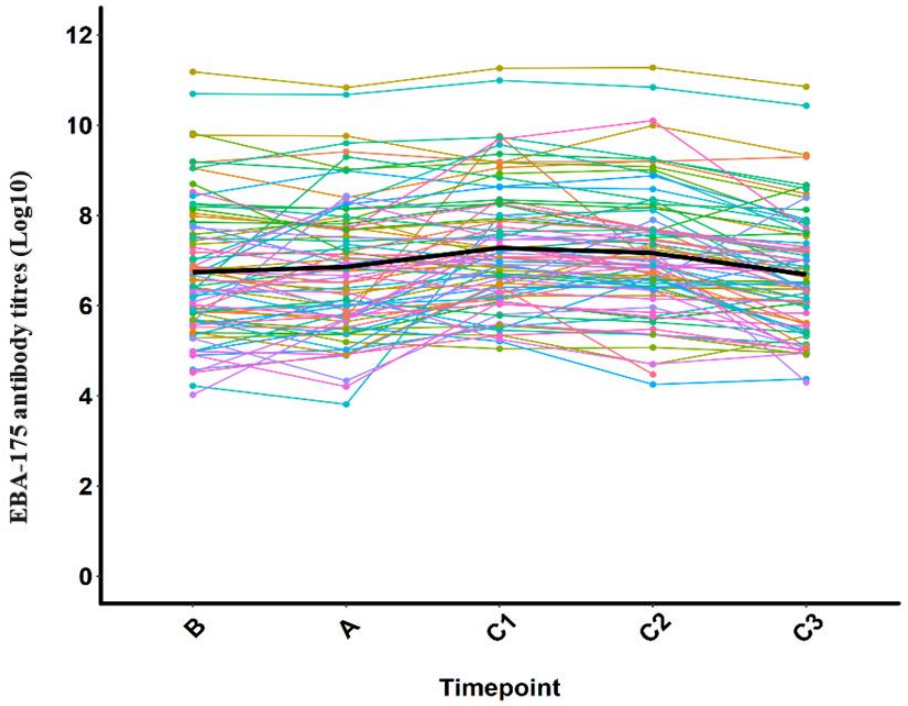

***Figure 4.8*: General antibody profiles for EBA-175.** This is a graph of log-transformed antibody titres against time-points for EBA-175 antigen. Plasma levels of antibodies specific to EBA-175 in malaria-exposed children at baseline (B), acute malaria infection (A, Week 0), and after 4 weeks (C1), 6 weeks (C2) and 24 weeks (C3) following treatment. Each dot represents a time-point. Temporal changes in antibody titre levels in individual children is represented by the distinctly coloured lines. The heavy black line represents the cohort mean. The lines connect data from individual children.

4.5.4 Individual profiles for EBA-175

The individual profiles for EBA-175 are represented in **Figure 4.9.** As observed, in 32 out of 82 children (36.6%), there was no change in antibody titres with time. In 18/82 children (22.0%), antibody titres peaked at C1 and decreased to baseline during follow-up while in 16/82 children (19.5%), antibody titres peaked during the acute malaria infection but declined to baseline by the 24^{th} week (C3). In 5/82 children (6.1%), antibody responses increased at C2 and declined soon after to baseline. Antibody responses were highest at baseline but declined throughout follow-up in 7/82 children (9.8%), while 4/82 children (4.9%) had the lowest antibody responses at baseline which increased throughout follow-up.

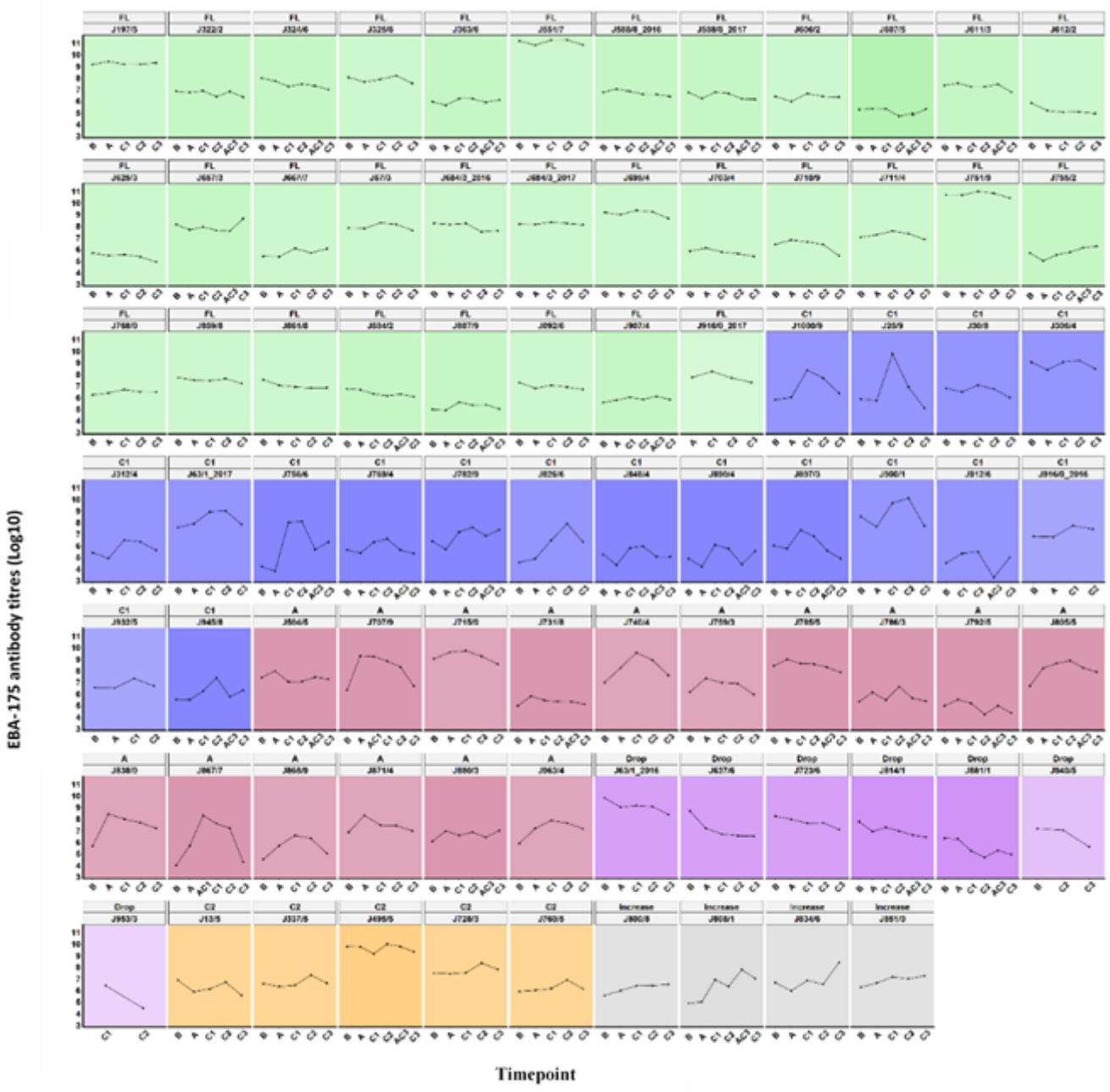

***Figure 4.9*: Individual profiles for EBA-175**. This is a graph of log-transformed antibody titres against time-points. The different profiles are highlighted in distinct colours. Green: Represent profiles where there was no change in antibody titres with time. Blue: Antibody responses peaked at C1 and declined during the recovery phase. Maroon: These are profiles where antibody responses peaked at acute malaria infection followed by a decline during the convalescent period. Purple: Represent the profiles of children where antibody responses were highest at baseline and decreased throughout follow-up. Orange: Antibody responses peaked at C2 before declining to baseline by the 24th week (C3). Grey: Antibody responses were lowest at baseline and increased throughout follow-up. Above each individual profile is a unique identifier for each child and profile type. Each dot represents a time-point and the scale on the y-axis runs from 3 to 11.

4.6 Acute malaria infection boosts antibody responses to RH5

The presence of malaria parasites during an acute malaria infection boosted antibody responses to Rh5 that declined during the convalescent period (**Figure 4.10A**). The antibody responses to Rh5 antigen were higher across all time-points as compared to those of EBA-175 because of the higher coating concentration for Rh5. Generally, there were differences in antibody titres for Rh5 irrespective of time-point ($p = 0.0021$, **Figure 4. 10A).** Antibody responses to Rh5 were significantly boosted ($p = 0.044$) during an acute malaria infection (**Figure 4.10A**) and declined during follow-up such that after 24 weeks (C3), antibody titres were significantly lower ($p = 0.017$) than at acute malaria infection. For EBA-175 (**Figure 4.10B**), there was a significant difference ($p = 0.016$) in antibody titres irregardless of time-point. Antibody titres for EBA-175 were not significantly boosted ($p = 0.62$) during acute malaria infection. Four weeks after the acute malaria episode, antibody titres slightly increased although the increase was not significant ($p = 0.063$), followed by a gradual decline towards baseline.

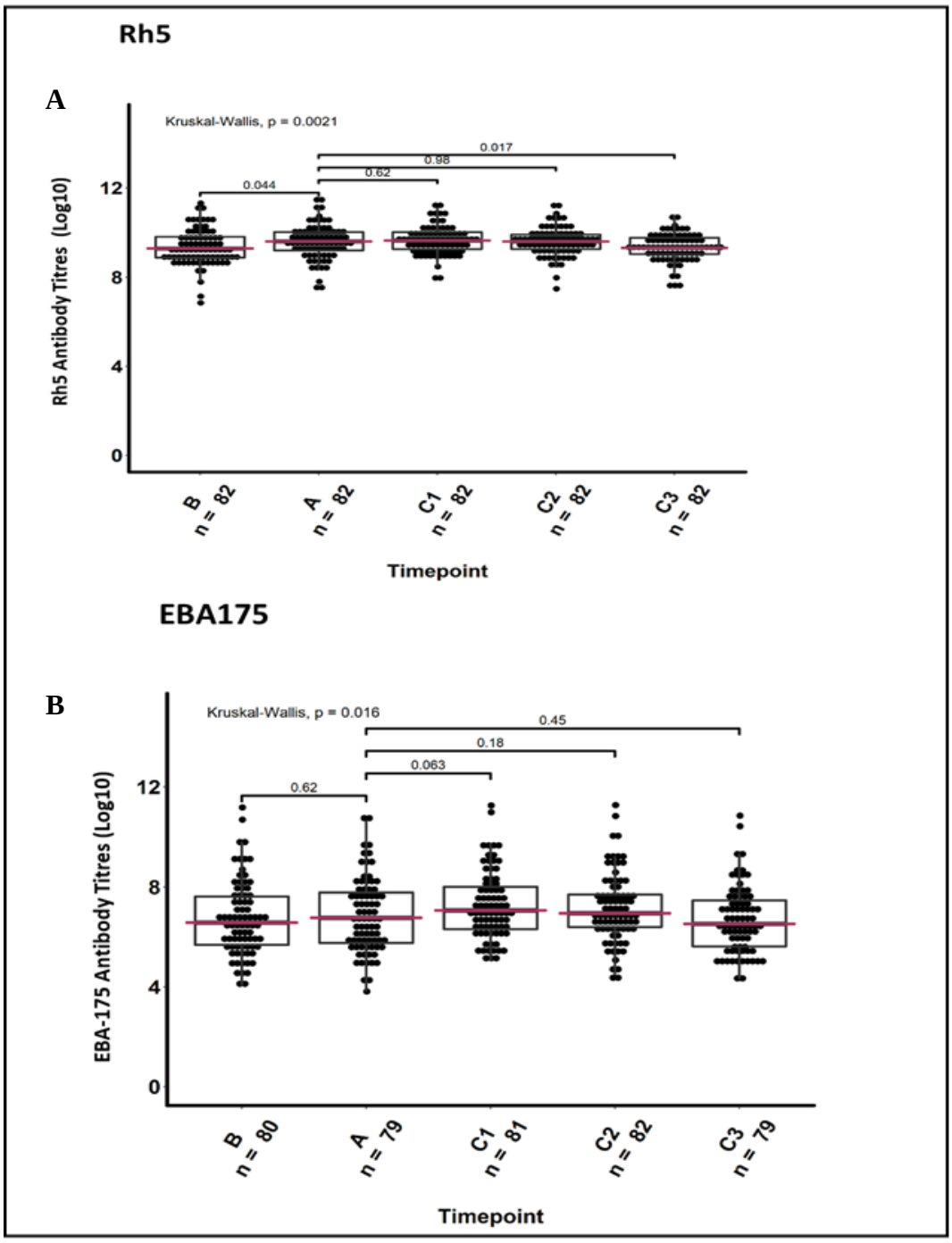

***Figure 4.10*: Acute malaria infection boosts antibody responses to Rh5.** These are box plots of ELISA results of antibody titres for Rh5 (A) and EBA-175 (B) antigens. n stands for the number of participants per time-point. Log-transformed antibody titres were plotted against time-points (B, A, C1, C2 and C3). The boxes indicate the 25th to 75th percentile with the maroon line showing the median antibody titres. Each dot represents a child and the dots outside the boxes represent the outliers. For EBA-175, n is variable because some of the OD values from some children were either too high or low to be interpolated from the standard curve. To the left top corner are p-values obtained after running a Kruskal-Wallis test. A p-value <0.05 shows that the difference is statistically significant.

4.7 Higher antibody responses to Rh5 in children with baseline parasitemia

The children were grouped into two groups: those with baseline parasitemia (pos) and those without baseline parasitemia detected (neg) (see **Figure 4.11**). The children with asymptomatic parasitemia at baseline were few (13/83, 15.66%) possibly because the cross-sectional baseline bleed was taken during the dry season when infection with malaria parasites was lowest. Generally, antibody titres between children with baseline parasitemia and those who were aparasitemic at baseline were statistically significant irrespective of time-point for both Rh5 ($p = 0.000023$, **Figure 4.11A**) and EBA-175 ($p = 0.016$, **Figure 4.11B**). For Rh5, children with baseline parasitemia had significantly higher antibody titres ($p = 0.015$) when compared to those without at the baseline time-point (**Figure 4.11A**). This was not the case for EBA-175 where the presence of malaria parasites at baseline did not boost antibody responses for the antigen (**Figure 4.11B**).

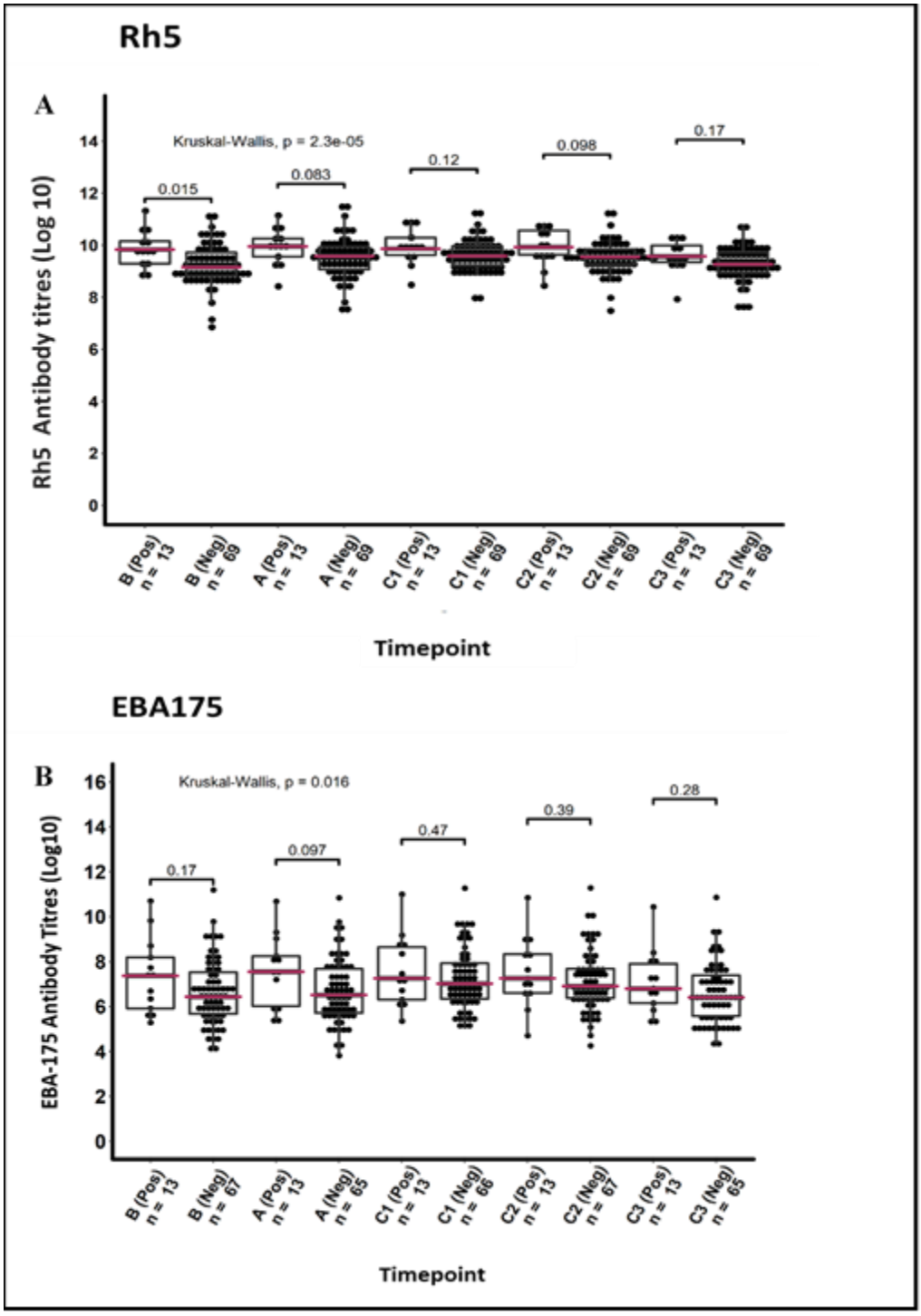

***Figure 4.11*: Higher antibody responses to Rh5 in children with baseline parasitemia**. These are box plots of ELISA results of antibody titres for Rh5 (A) and EBA-175 (B) antigens after grouping the children by being parasite positive at baseline or not. The boxes indicate the 25th to 75th percentile with the maroon line showing the median antibody titres. Each dot represents a child. The dots outside the boxes represent the outliers. A p-value <0.05 shows that the difference is statistically significant. n: number of participants per time-point.

4.8 Children over 5 years elicit greater antibody responses to EBA-175

Generally, antibody titres between children over 5 years and those under 5 years was significantly different ($p = 0.014$) irrespective of time-point for Rh5 (**Figure 4.12A**) and EBA-175 ($p = 0.00061$, **Figure 4.12B**). Antibody responses to EBA-175 were significantly higher in older children (>5 years) ($p = 0.01, 0.033, 0.049, 0.0034$ for B, A, C2 and C3 time-points respectively, than in younger children (≤5), except for the C1 time-point (**Figure 4.12B**). For Rh5, antibody responses between the older and younger children were not significantly different (**Figure 4.12A**).

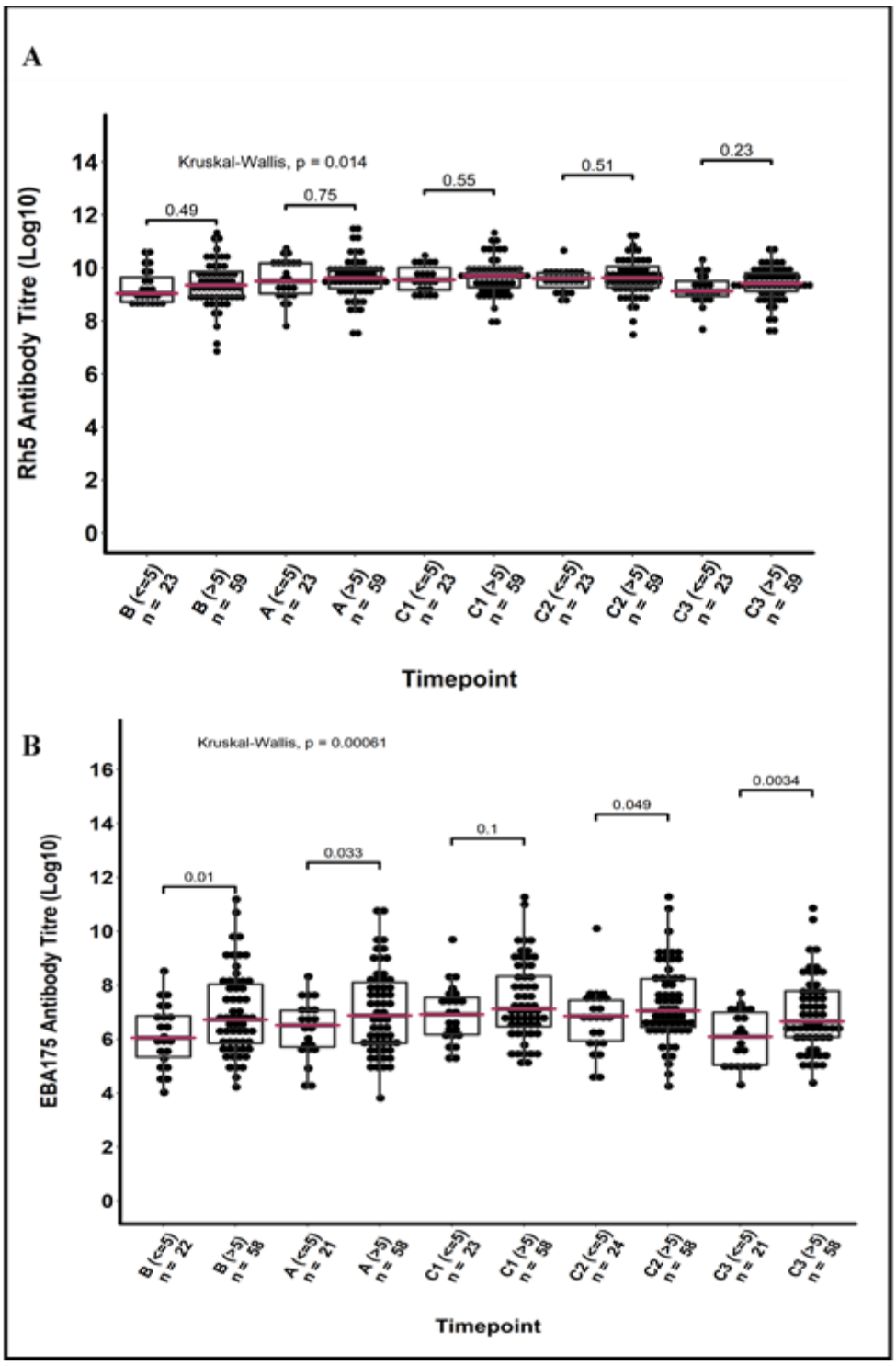

***Figure 4.12*: Higher antibody responses to EBA-175 in older children**. A graph of antibody titres against time-point after grouping the children by age (≤ 5 and >5 years) for Rh5 (A) and EBA-175 (B). The boxes indicate the 25th to 75th with the maroon line showing the median antibody titres. Each dot represents a child. The dots outside the boxes represent the outliers. A p-value <0.05 shows that the difference is statistically significant. n: number of participants per time-point.

4.9 Decline in antibody responses to Rh5 and EBA-175 with time

4.9.1 Decrease in antibody titres to Rh5 with time

For Rh5, the estimates of the fixed effects (**Table 4.2**) were obtained after running the *nlme* model where Rh5 antibody titres were modelled as a function of time in months. Antibody titres were the dependent variable and time the independent variable. The "Intercept" was the reference which is an estimate of the baseline (B) antibody responses for the children. Antibody titres declined with time (Estimate = -0.04, SE= 0.01, p = <0.001). The effect of age, gender and parasite density at baseline on antibody responses to Rh5 antigen was not significant.

***Table 4.2*: Decline of Rh5 antibody titres with time**

	Value	Std.Error	DF	t.value	p.value
(Intercept)	13.72	0.26	359	53.64	**0**
time_months	-0.04	0.01	359	-3.80	**<0.001**
glog_parasite_density	0.05	0.03	78	1.46	0.14
Age	0.01	0.03	78	0.49	0.63
GenderM	0.33	0.20	78	1.69	0.09

This is a table of estimates for the Rh5 antigen. DF: Degrees of freedom, Std. Error: Standard Error, Glog: Generalized logarithm, M: male. P-value <0.05 is statistically significant (in bold).

After controlling for the effects of parasite density, gender and age at baseline, the fitted log-transformed antibody titres was plotted against time in months (**Figure 4.13**). The baseline was treated as time-point 0. The average time between the baseline and acute malaria infection was 153.61 days, ranging from 45 to 306 days. From the acute malaria infection time-point, C1, C2, and C3 plasma samples were taken after approximately 28, 42 and 168 days, respectively. The average time between the C2 and the second acute malaria infection (AC3) was 244.12 days with a range of 158 to 416 days. Antibody responses were boosted at acute malaria infection but declined during follow-up. The baseline antibody responses were highly variable.

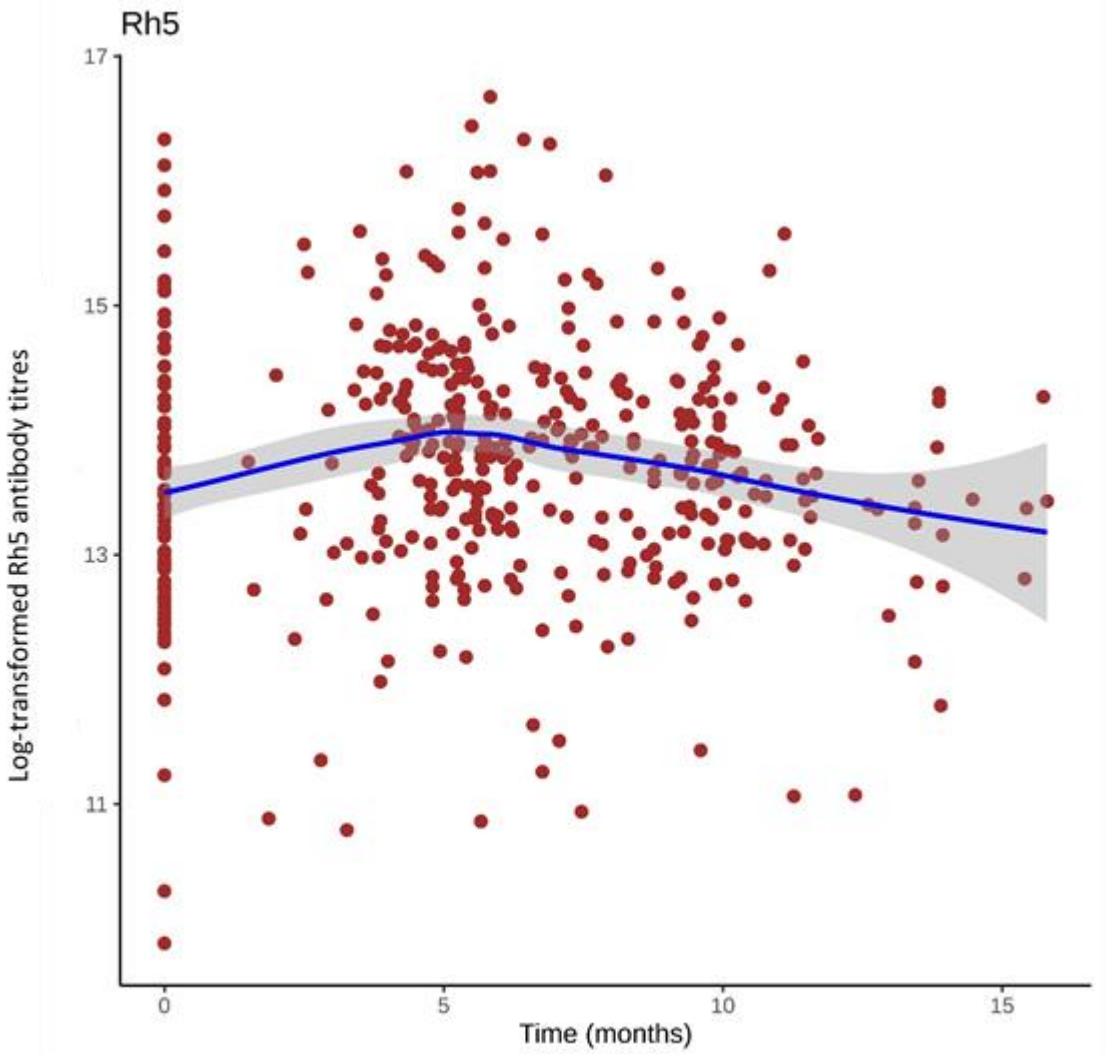

***Figure 4.13*: Decline of Rh5 antibody titres with time.** Each red dot represents a time-point. The blue line represents the fitted log transformed antibody titres while the grey shaded area represent 95% confidence interval.

4.9.2 Effect of age and decline of antibody responses to EBA-175 with time

Antibody responses to EBA-175 declined with time (Estimate = -0.07, SE = 0.02, p = <0.001) (**Table 4.3**). There was a significant effect of age on antibody responses to EBA-175, older children appeared to have greater antibody responses (Estimate = 0.14, SE = 0.05, p = 0.01). The effect of parasite density at baseline and gender on antibody responses to EBA-175 was not significant.

***Table 4.3*: Effect of age and decline of antibody responses to EBA-175 antigen**

	Value	Std.Error	DF	t.value	p.value
(Intercept)	9.42	0.53	351	17.63	**0**
time_months	-0.07	0.02	351	-3.47	**<0.001**
glog_parasite_density	0.06	0.07	351	0.91	0.36
Age	0.14	0.05	79	2.48	**0.01**
GenderM	-0.13	0.41	79	-0.32	0.75

This is a table of estimates for EBA-175 antigen. DF: Degrees of freedom, Std. Error: Standard error, Glog: Generalized logarithm, M: males. P-value <0.05 is statistically significant (in bold).

Figure 4.14 represent a graph of fitted log-transformed antibody titres against time in months, after controlling for parasite density, gender as well as age effects at baseline. Antibody responses were slightly boosted after acute malaria infection but declined during follow-up. The baseline antibody responses were highly variable.

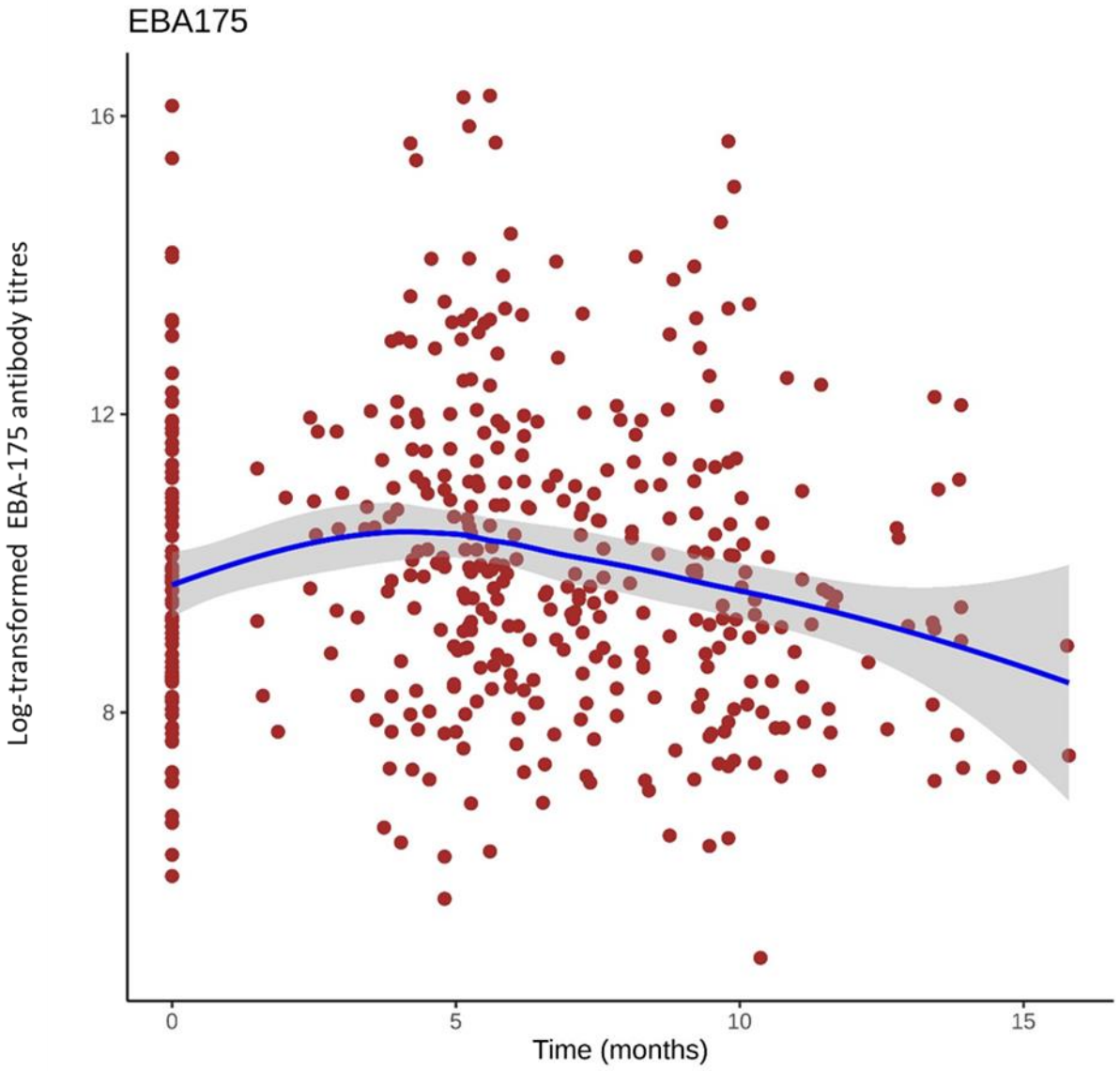

***Figure 4.14*: Decline of EBA-175 antibody titres with time.** Each red dot represents a time-point. The blue line represents the fitted antibody titres and the grey shaded area represent 95% confidence interval.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Expression of Rh5 and EBA-175 antigens

The full-length Rh5 and EBA-175 proteins were expressed by mammalian cells. This system of expressing proteins has repeatedly been applied in the expression of recombinant full-length *Plasmodium* proteins that are functional and it is scalable (Srivastava *et al.,* 2010; Bustamante *et al.,* 2013; Chiou *et al.,* 2014). Different protein expression systems that include bacteria, yeast, plant, algae and cell-free (Ghosh *et al.,* 2002; Tsai *et al.,* 2006; Tsuboi *et al.,* 2008; Gregory *et al.,* 2012) have also been applied to express *Plasmodium* proteins. The bacterial system of protein expression is the most commonly used owing to its simplicity and cost-effectiveness although the success rates are as low as 6% (Mehlin *et al.,* 2006). Furthermore, the expressed proteins are hardly soluble and full-length, and have to be re-folded to the functional native protein (Mehlin *et al.,* 2006; Vedadi *et al.,* 2007). The eukaryotic cell-free wheat germ system has also successfully expressed soluble proteins although the bigger the size of the protein the less the solubility resulting in the separate expression of protein segments (Doolan *et al.,* 2008; Tsuboi *et al.,* 2008; Crompton *et al.,* 2010).

5.2 Evaluation of Rh5 and EBA-175 antibody responses

In this study, Rh5 and EBA-175 antibody responses were assessed in 83 malaria-exposed children under 15 years of age, followed prospectively for 24 weeks after an acute malaria episode. To the best of my knowledge, it is the first longitudinal study determining the change in the magnitude of antibody responses to full-length *P. falciparum* 3D7 Rh5 and EBA-175 antigens expressed by mammalian cells in the same cohort (Junju), following an acute malaria episode. The two antigens were immunogenic as they elicited antibody responses in the baseline, acute and convalescent plasma samples tested. The individual and general antibody profiles for both antigens exhibited heterogeneity which could have resulted from differences in genetics, age and the degree of previous exposure which impacts on pre-existing immunity.

Studies have shown an association between Rh5 antibody responses with protection from clinical malaria episodes and decreased risk of developing malaria in malaria-exposed children in Papua New Guinea (Chiu *et al.,* 2014; Weaver *et al.,* 2016). The presence of antibodies specific to Rh5,

at enrolment, were associated with a longer period of time between the first blood stage infection and the first malaria episode in Malian children and adults (Tran *et al.*, 2014). Rh5 is a relatively conserved antigen that has been reported to have low immunogenicity in naturally malaria-exposed populations (Douglas *et al.*, 2011; Patel *et al.*, 2013a; Douglas *et al.*, 2014; Partey *et al.*, 2018) and therefore it was coated at a concentration higher than that of the more immunogenic EBA-175 antigen (Osier *et al.*, 2008). Rh5 is secreted by merozoite rhoptries prior to the invasion process. Brief exposure to the host immune system as a result of the rapidity of the invasion process could lead to the low immunogenicity (Patel *et al.*, 2013b; Douglas *et al.*, 2014).

In the present study, children with baseline parasitemia elicited greater antibody responses to Rh5 after stratifying the children by being parasite positive at baseline or not. During an acute malaria episode, antibody responses to Rh5 were boosted, suggesting a robust immune memory. Studies have shown that in the presence of malaria parasitemia, antibody responses to Rh5 are boosted (Tran *et al.*, 2014; Partey *et al.*, 2018) which was in line with what was observed in our study. A longitudinal study done in Kenyan children where antibodies specific to Rh5 were assessed prior and after the rainy season showed that exposure to malaria boosted the level of Rh5 antibodies although follow-up samples were not included in this study to examine the changes in antibody titres after the infection (Tran *et al.*, 2014). There was no impact of age on antibody responses to Rh5, an indication that Rh5 could be a marker of recent exposure only.

Some studies have associated EBA-175 antibodies with protecting individuals from clinical malaria (Richards *et al.*, 2010; McCarra *et al.*, 2011; Chiu *et al.*, 2016) while this is not the case in others (Okenu *et al.*, 2000; Ohas *et al.*, 2004; Osier *et al.*, 2008). While the presence of malaria parasites at baseline did not seem to boost antibody responses to EBA-175, there was an effect of age in that antibody responses tended to be higher in older children (>5 years). A study done elsewhere established that antibody responses to two polymorphic *P. falciparum* antigens, EBA-175 and MSP2, were elevated in the older age groups but lower for the Rh5 homologue (Rh2a), further supporting our results (Sennang *et al.*, 2014). Other studies have established an effect of age on antibody responses to EBA-175 antigen, an implication that EBA-175 could be a potential marker of the development of immunity with age (Okenu *et al.*, 2000; Mlambo *et al.*, 2006). In our study, antibody responses to EBA-175 were not significantly boosted during the acute malaria episode. Consistent with the study done by Akpogheneta *et al.* (2010), there was limited boosting

of antibody responses to EBA-175 during acute malaria infection and re-infection did not boost antibody responses to EBA-175 (Morell *et al.*, 1970).

5.3 Decline in antibody responses to Rh5 and EBA-175 antigens

The antibody responses to Rh5 and EBA-175 generally declined to baseline during the convalescent period showing the transient nature of antibody responses to these antigens. These results are in line with what was observed in other studies, that immunity to malaria is short-lived (Frueh *et al.*, 1991; Kinyanjui *et al.*, 2007; Crompton *et al.*, 2010; Yman *et al.*, 2019) and require constant boosting by exposure to the parasite antigens (Tran *et al.*, 2014; Partey *et al.*, 2018). Studies have established that during an acute malaria infection, there is a bias towards the production of short-lived IgG1 and IgG3 which, in part, could explain the brevity of the antibody responses (Rzepczyk *et al.*, 1997; Taylor *et al.*, 1998; Kinyanjui *et al.*, 2007). Furthermore, poor development of antigen-specific memory as well as long-lived plasma cells have been implicated (Anders, 1986; Achtman *et al.*, 2003). Repeated exposure to the malaria parasite antigens has been associated with the greater longevity of antibody responses shown by the maintenance of larger numbers of long-lived antibody secreting cells (ASCs) (Yman *et al.*, 2019). Moreover, the antibodies produced after exposure to the malaria parasites rapidly decay (White *et al.*, 2014).

5.4 Conclusion

- The full-length *P. falciparum* 3D7 Rh5 and EBA-175 antigens were successfully expressed by mammalian cells.
- The two antigens were immunogenic as they elicited antibody responses in the plasma samples tested. There was heterogeneity in antibody responses to both antigens which could have resulted from differences in age, parasite density at baseline and degree of previous exposure which impacts on pre-existing immunity.
- A malaria vaccine with long-lasting immunity is urgently needed. In this study, antibody responses to both antigens waned with time, showing the short-lived nature of antibody responses against the two antigens. This challenges the development of an efficacious malaria vaccine based on Rh5 and EBA-175.
- These results provide data on the changes in the magnitude of antibody responses to Rh5 and EBA-175 antigens following an acute malaria infection and further inform the timing of serological studies.

5.5 Recommendations

The two antigens studied are essential blood stage antigens that could be included in a multicomponent malaria vaccine for a better vaccine efficacy. Repeating the study in a bigger cohort with more closely spaced out time-points within the first few weeks of an episode and more antigens could provide additional information on the changes in the magnitude of antibody responses based on the two antigens following an acute malaria episode. I would also recommend the evaluation of the synergistic action of the two or more combinations of the merozoite antigen antibodies.

CHAPTER SIX

6.0 REFERENCES

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6.1 APPENDICES

APPENDIX 1: ETHICAL APPROVAL

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **January 15, 2016**

TO: **FRANCIS NDUNGU,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. BENJAMIN TSOFA,**
THE DIRECTOR, CGMR-C,
KILIFI

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE RESEARCH, COAST

Dear Sir,

RE: **PROTOCOL NO. KEMRI/SERU/CGMR-C/017/3149 (*RESUBMISSION 2-INITIAL*): SYSTEMS IMMUNOLOGY STUDIES OF *PLASMODIUM FACIPARUM* MALARIA SUSCEPTABILITY IN KILIFI COUNTY.**

Reference is made to your letter dated 5th January, 2016. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on 6 January, 2016.

This is to inform you that the Committee notes that the issues raised during the 243rd meeting of the KEMRI/Ethics Review Committee (ERC) held on September 16, 2015, have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day **15th day of January 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **14th January, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **3rd December 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

RECEIVED
22 JAN 2016
DIRECTOR'S OFFICE

APPENDIX 2: CONSENT FORM

INFORMED CONSENT AGREEMENT FORM

Study title:…….

I, [being a parent/guardian of _____ (name of child)], have had the research explained to me. I have understood all that has been read/explained and had my questions answered satisfactorily.

If you agree, please insert the boxes below where relevant:
- **I agree to samples being stored and used for future research**
- **I agree to samples being exported**

I understand that I can change my mind at any stage and it will not affect to me/my child in any way.

Subject/Parent/guardian's signature: _____ **Date** _____

Subject/Parent/guardian's name: _____**Time** _____
(Please print name)

Where parent/guardian cannot read, ensure a witness* observes consent process and signs below:

I attest that the information concerning this research was accurately explained to and apparently understood by the subject/parent/guardian and that informed consent was freely given by the subject/parent/guardian.

Witness' signature: _____ **Date** _____

Witness' name: _____ **Time** _____

**A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.*

Thumbprint of the parent as named above if they cannot write: _____

I have followed the study SOP to obtain consent from the [participant/guardian]. S/he apparently understood the nature and the purpose of the study and consents to the participation [of the child] in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Designee/investigator's signature: _____ **Date** _____

Designee/investigator's name: _____**Time** _____