THE ROLE OF LEUKOCYTE-ASSOCIATED IMMUNOGLOBULIN-LIKE RECEPTOR 1 (LAIR1)-BEARING ANTIBODIES IN IMMUNITY AGAINST MALARIA

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A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science in Immunology of Pwani University

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DECLARATION

This thesis is my original work and has not been presented in any other University or any other Award.

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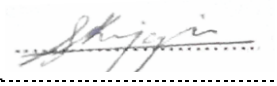
Signature:  Date: 19/04/2022

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DEDICATION

This work is dedicated to my brother, Nguti Kisiangani, in appreciation of his leadership skills and resilience.

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ABSTRACT

Malaria, caused by parasites of the *Plasmodium* genus, was responsible for 241 million cases and over 600,000 deaths globally in 2020. This was an increase from 217 million cases in 2014 despite measures such as vector control using insecticide-treated nets, and insecticide residual spraying being applied. The deaths are high despite the availability of anti-malaria medication, partly due to the emergence of resistance to antimalarial drugs. To augment the control of malaria, several vaccines are in the development pathway. The challenge with vaccine development is the complex biology of the parasite such that in the blood stage, the parasite express variant surface antigens which are highly polymorphic and variant. Therefore, the development of vaccines to this stage requires either targeting conserved epitopes or generation of broadly reactive antibodies. Four studies recently characterised antibodies that contain leukocyte-associated immunoglobulin-like receptor 1 (LAIR1) insert and are broadly reactive to the *P. falciparum* variant surface antigens (VSA).

This study, nested in the Controlled Human Malaria Infection (CHMI) conducted at KWTRP, sought to establish the association between LAIR1-bearing antibodies (LBA) and immunity against malaria. 108 of 142 CHMI study participants came from a region with high malaria transmission and 34 from an area with low malaria transmission. The participants were classified as “treated” or “untreated” depending on whether they became symptomatic or not during follow-up. Pre-challenge (C-1) plasma samples was assayed using ELISA to screen for LBA. The aim was to establish a high throughput assay for LBA screening, to compare the seroprevalence of LBA at C-1 and C+14, to assess the association between LBA and anti-VSA antibody breadth and to evaluate the correlation between prior malaria exposure and LBA levels. Plasma samples from malaria un-exposed UK individuals were used as negative control and 3 standard deviations above their mean absorbance was defined as the positivity cut off. The median

level of anti-VSA to six parasite isolates was used as proxy for anti-VSA breadth. Anti-schizont antibody response was used as proxy for prior malaria exposure.

The seroprevalence of LBA in CHMI samples was 27% (39/142). There was no association between LBA status and malaria endemicity of participants' residence (Chi-squared test, *P*= 0.6150). Additionally, although 64% of LBA positive samples were in the untreated group, there was no significant difference in the median LBA across CHMI outcomes (*P*=0.9610). There was a positive correlation between LBA levels and the number of parasite isolates recognised (Spearman correlation, rho = 0.1875. p-value = 0.02547). Additionally, there was a weak positive correlation between LBA levels and anti-schizont antibody levels (spearman correlation, rho = 0.260821, *P*-value = 0.001718).

In conclusion, nearly a third of the CHMI study participants had LBA and prior malaria parasite exposure seems to drive LBA development. There was a positive correlation between LBA levels and the breadth of anti-VSA antibodies as well as prior malaria exposure. However, there was no association between LBA positivity with CHMI outcomes or the malaria endemicity of participants' residence.

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ABBREVIATIONS

A647	Alexa Fluor 647
ACTs	Artemisinin-based drug combinations
AL	Artemether and Lumefantrine
AMA1	Apical membrane antigen
BSA	Bovine Serum albumin
C +5	5 days post challenge
C +7	7 days post challenge
C+21	21 days after challenge
C+35	35 days post challenge
C-1	A day before challenge
CDRs	complementarity determining regions
CHMI	Controlled Human Malaria Infections
CSA	chondroitin sulfate A
CSP	circumsporozoite protein
CV	coefficient of variation
DAMPs	damage-associated molecular patterns
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
FN	false negative
FP	false positive
FSC-A	forward scatter area
FSC-H	forward scatter height
GEST	Gamete egress and sporozoite traversal protein

GLURP	Glutamate-rich protein
GPI	glycophosphatidylinositol
H_2O_2	hydrogen peroxide
IgG	immunoglobulin G
iRBC	infected red blood cells
ITIMs	immunoreceptor tyrosine-based inhibitory motifs
ITNs	insecticide treated nets
IRS	indoor residual spraying
KWTRP	KEMRI Wellcome Trust Research Programme
LAIR1	leucocyte-associated immunoglobulin-like receptor 1
LBA	LAIR1 bearing antibodies
Mab	Monoclonal antibody
MFI	median fluorescent intensity
MSPs	Merozoites surface proteins
OD	optical density
OPD	o-Phenylenediamine dihydrochloride
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PE	Phycoerythrin
*Pf*NF54	*Plasmodium falciparum* laboratory isolate NF4
*Pf*Rh	Reticulocyte-binding-homologue proteins
RBC	red blood cell
RPM	rotations per minute
SSC-A	Side-scatter area
stevor	Subtelomeric, variable open reading frames

surf	surface-associated interspersed
TN	true negative
TNF-α	tumour necrosis factor alpha
TP	true positive
TRAP	Thrombospondin-related anonymous protein
uRBC	uninfected red blood cells
VSAs	variant surface antigens

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is a disease caused by parasites of the *Plasmodium* genus and results in a substantial health burden to humans especially in Africa (WHO, 2021b). In 2020, there were 241 million cases and 627,000 estimated deaths attributed to malaria. Children are the most affected population – 80% of the reported deaths 602,000 deaths from WHO Africa region were from children under 5 years of age (WHO, 2021b).

The current control strategies of malaria target either the mosquito vector or the malaria parasite. Insecticide-treated nets (ITNs), indoor residual spraying (IRS), and larvicides help in controlling mosquitoes. These vector-control measures as well as the use of effective drugs resulted in a 37% decline in malaria incidence and a 60% reduction in malaria related mortality from 2000 to 2015 (Cibulskis et al., 2016). The challenges with vector control measures, however, include the development of resistance to insecticides as well as the change in mosquitoes' feeding pattern (Cator, Lynch, Thomas, & Read, 2014). For the infected individuals, prompt diagnosis and administration of Artemisinin-based combination therapy (ACT) is vital (FDA, 2020). Nonetheless, the parasites are also developing resistance to Artemisinin derivatives (Rosenthal, 2018).

To augment the existing malaria-control measures and management tools, several vaccine candidates are under development (Draper et al., 2018). These vaccine candidates are focused on the different developmental stages of the parasite with RTS,S/ASO1 (RTSS) targeting the sporozoite stage (Nussenzweig & Nussenzweig, 1989; PATH, 2016). RTS,S/ASO1 gets its name from the substituent components. R represents the tetatrapeptide repeats of circumsporozoite protein (CSP). T represents the T-cell epitope found on CSP. S represents the hepatitis B surface antigen (HBsAg). R, T and S form a single fusion protein that are co-expressed with an additional free HBsAg

hence RTS,S. ASO1 is an adjuvant comprising liposomes, alum + 3-O-desacyl-4′-monophosphoryl lipid A (MPL), and saponin QS-21 from *Quillaja saponaria* extract (Laurens, 2020). RTSS is the only vaccine that has been rolled out for pilot implementation but it has low efficacy, and the immunity it induces wanes over time (Bejon et al., 2008; Olotu et al., 2013; RTSS Clinical Trials Partnership, 2015; WHO, 2019b). It has been established that RTSS when combined with chemo preventive antimalarial drugs has a protective efficacy of above 60% (Chandramohan et al., 2021). None the less, the World Health Organisation recommends its use in 4 doses among children from moderate to high malaria transmission regions in the prevention of malaria (WHO, 2021a). The other vaccine, R21, is made of central repeat and C-terminal of CSP fused to the N-terminal of HBsAg. R21 demonstrated slightly over 70% efficacy at phase IIb of clinical trial (Datoo et al., 2021).

The development of a vaccine against *P. falciparum* is not only complicated by its complex life cycle but also by the multiple immunological targets they expresses across that life cycle. Some of the vaccines in the development pathway target sporozoites, merozoites, and even gametocytes (Clyde, McCarthy, Miller, & Woodward, 1975; Laurens et al., 2013; Lyke et al., 2017; Thera et al., 2010). During the mature asexual stages, the parasite secretes and transports proteins to the surface of infected red blood cells (iRBC). These proteins are implicated in the disease process of malaria (Fried & Duffy, 1996; Marín-Menéndez et al., 2013; Uyoga et al., 2012) are targets of protective immunity against malaria (Kinyanjui, Mwangi, Bull, Newbold, & Marsh, 2004; Warimwe et al., 2013) and are therefore putative vaccine candidates (Draper et al., 2018). However, these proteins, jointly designated variant surface antigens (VSAs), are highly polymorphic and variant (Brown, Brown, & Hills, 1968; Bull et al., 1998; Marsh & Howard, 1986; McGregor, 1987; Mendis, David, & Carter, 1991). To circumvent this, it

is proposed that broadly reactive antibodies targeting VSAs can be used as vaccine candidates.

Recently, Tan *et al.* (2016) established that some individuals could generate antibodies that broadly react to VSAs. These antibodies contain an ectopic insertion of a mutated leukocyte associated immunoglobulin-like receptor-1 (LIAR1) in the variable regions (Hsieh & Higgins, 2017; Pieper et al., 2017; Tan et al., 2016). LAIR1 is an inhibitory receptor found on some cell surfaces and it binds collagen (Meyaard et al., 1997; Meyaard, Hurenkamp, Clevers, Lewis, & Phillips, 1999). However, the presence of the LAIR1 as an insert in the antigen-binding site of antibodies, result in LAIR1-bearing antibodies (LBA) which can cross-react with multiple parasite isolates (Tan et al., 2016). Tan et al established that the LAIR1 insert was dispensable for binding infected red blood cells. Moreover, the LBA mediated opsonic phagocytosis of the infected red blood cells (Tan et al., 2016). Thus, antibodies with the LAIR1 insert are potential tools in the course of vaccine development.

Tan and colleagues performed a mixed agglutination assay on 557 plasma samples using culture adapted Kenyan parasite isoaltes. 3 of the plasma samples formed mixed agglutinates with at least 6 parasite isolates. Samples from 2 donors, C and D, formed mixed agglutnates with 8 parasite isoalates. Most of the monoclonal antbodies generated from these two donaors were broadly reactive and molecular analysis illustrated that the broad;y reactive antibodies had an ectopic insert that was about 90% homologu to LAIR1 in the V and DJ segements of the heavy chain. The LAIR1 insert was mutated with reduced capacity to bind collagen and enhanced binding to infected red blood cells. Using liquid chromatography-mass spectrometry, it was established that the LBA recognised type A RIFIN. Apart from agglutination, LBA demonstrated a strong capacity for opsonic phagocytosis of infected red blood cells.

1.2 Problem statement

In the global technical strategy for malaria report of 2015, WHO advocates for the development of a vaccine whose protective efficacy is at least 75% by 2030 (WHO, 2015). The vaccine will be a complementary tool in the control of malaria. While many vaccine candidates have been developed, an efficacious vaccine remains elusive. RTS,S/ASO1, which was recently rolled out in three African countries, remains the most advanced malaria vaccine despite its low efficacy in phase III trials (Adepoju, 2019; Dunachie, Hill, & Fletcher, 2015; 2015; Olotu et al., 2011; Partnership, 2017; White et al., 2015; WHO, 2019b). R21 in phase IIb of clinical trial has suboptimal efficacy relative to the WHO recommendations (Datoo et al., 2021). Therefore, there is a need to explore other options. Being associated with clinical symptoms, VSAs are a possible target in vaccine development. The challenge, however, is their high degree of polymorphism that would otherwise impede vaccine development. The polymorphism makes it essential to develop a vaccine that would either target conserved epitopes or induce broadly reactive antibodies.

1.3 Justification

Antibodies containing the LAIR1 insert have been linked to broad reactivity to the mature blood stage of *P. falciparum* (Tan et al., 2016). A separate study identified a repetitive interspersed families of polypeptides (RIFINs) as the target antigen for the broadly reactive antibody (Saito et al., 2017). These studies raise the possibility of using the targets of LAIR1-bearing antibodies in malaria vaccine development. However, further evidence on the protective efficacy of these LBA antibodies is required. A major confounder in malaria immunological studies is the failure to account for the exposure among study participants. This study, therefore, utilised samples from controlled human malaria infection (CHMI) studies to circumvent this challenge. The CHMI samples are especially important for this study because the participants got standardised exposure and

uniform measurement of outcome (Kapulu, Njuguna, & Hamaluba, 2019; Tuju, Kamuyu, Murungi, & Osier, 2017).

1.4 Objectives

The main objective of this study is to explore the role of LBA in protection against malaria. The specific objectives are as follows:

1) To establish a high-throughput assay for the screening of plasma samples for LBA
2) To compare the prevalence of LBA among CHMI participants at C-1 and C+14
3) To assess the association between LBA and the breadth of anti-VSA antibodies across CHMI samples.
4) To evaluate the correlation between prior malaria exposure and LBA levels mong CHMI participants

1.5 Hypothesis

Null hypotheses:

1. There is no associations between having LAIR1-bearing anti-VSA antibodies and CHMI outcomes,
2. There is no associations between the levels of LAIR1-bearing and the breadth of anti-VSA antibodies the time of challenge.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lifecycle of *P. falciparum*

Members of the *Plasmodium* genus that cause malaria in humans include *P. vivax, P. ovale, P. malariae* and *P. knolewsi*. In some instances, *P. inui, P. cynomolgi,* and *P. coatneyi* result in zoonotic infections (Maeno et al., 2015). *P. falciparum* is the predominant parasite in Africa and accounts for the majority of the severe cases and deaths from malaria (Maeno et al., 2015; WHO, 2019c).

Plasmodium falciparum has a complex lifecycle. During a blood meal, a female Anopheles mosquito picks up gametocytes from an infected human. Once they mature, the gametocytes differentiate into male and female gametes. Fertilisation of these gametes takes place within the gut of the mosquito with consequent formation of zygotes that further develop to be invasive ookinetes. The ookinetes burrow into the gut wall and within the haemolymph develop into oocysts that contain sporozoites (Crompton et al., 2014). Sporozoites, the infective stage to humans, move into the salivary glands of mosquitoes and are deposited into a different host in the subsequent blood-meal.

When the infected mosquito bites human beings during a blood-meal, it deposits sporozoites from its salivary glands. Some of the sporozoites remain localized in the skin because anti-sporozoite antibodies block them from trickling into blood vessels (Vanderberg & Frevert, 2004), the rest move to join either blood or lymphatic circulations (Vanderberg & Frevert, 2004; Yamauchi, Coppi, Snounou, & Sinnis, 2007). From a mice model, it was established that some of the sporozoites develop into merozoites within parasitophorous vacuoles within the skin (Gueirard et al., 2010). Those in lymphatic circulation can get cleared by immune cells at the draining lymph node while those in blood circulation migrate into the liver (Amino et al., 2006) where they navigate several cells to escape immune clearance (Amino et al., 2008; Mota Maria M.

et al., 2001). Parasites that infect hepatocytes undergo asexual replication to form thousands of merozoites. The infected hepatocytes burst and release merozoites into the peripheral circulation. These merozoites bind to and enter red blood cells (RBCs) where they continue developing from the ring to schizont stages (Gaur, Storry, Reid, Barnwell, & Miller, 2003; Healer et al., 2019; Jaskiewicz, Jodłowska, Kaczmarek, & Zerka, 2019). Majority of merozoites released develop into schizonts and subsequently infect new RBCs leading to the clinical symptoms of malaria. A small fraction of the blood-stage parasites gets committed and become gametocytes which after maturation can then infect another mosquito during a blood meal (Bruce, Alano, Duthie, & Carter, 1990; Kafsack et al., 2014; Kaushal, Carter, Miller, & Krishna, 1980; Poran et al., 2017; Wirth et al., 2014).

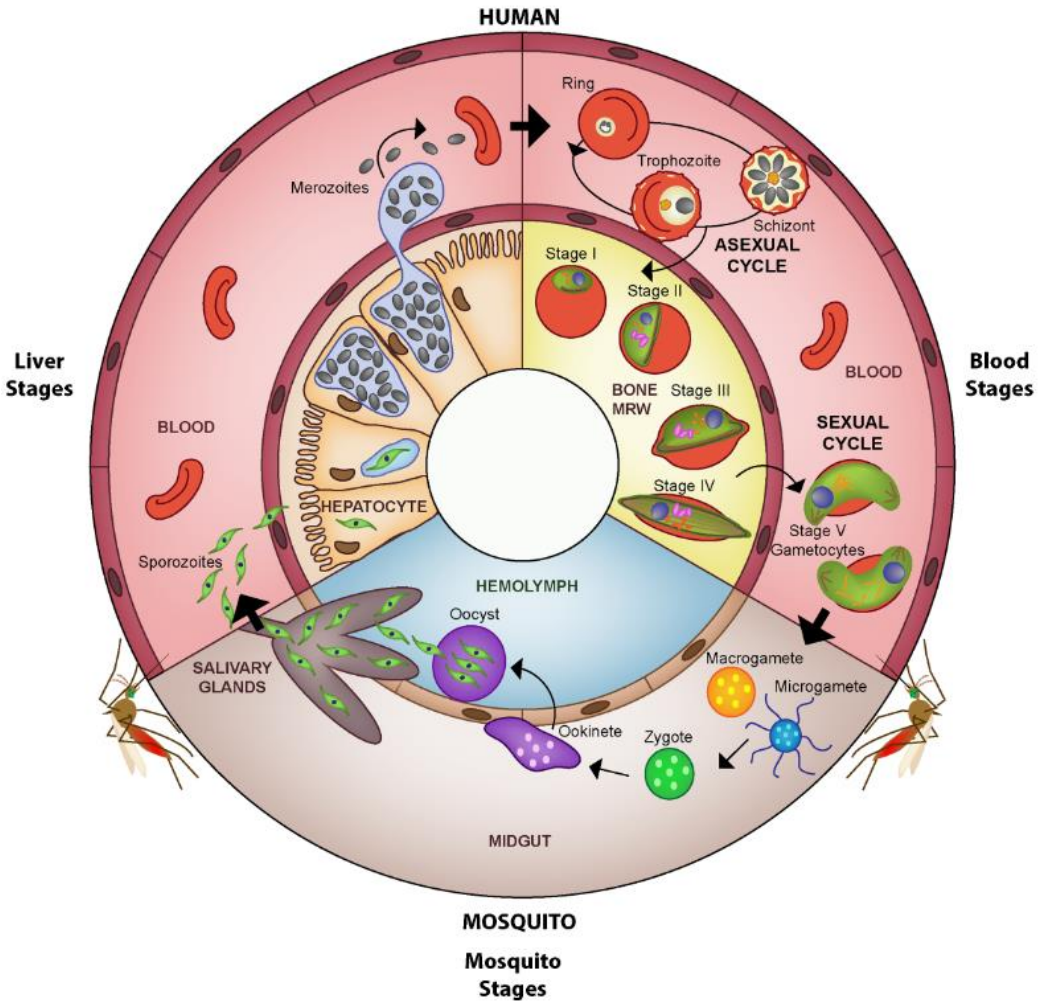

Figure 2.1.1: The life cycle of P. falciparum (Nilsson et al., 2015).

2.2 Malaria control strategies

Numerous measures are available for the control of malaria. Among the measures, insecticide treated nets (ITNs) and indoor residual spraying (IRS) are the recommended primary interventions against mosquitoes (WHO, 2019a). Drug administration for prophylaxis or treatment of malaria cases is an alternative that controls the establishment of an infection by the parasite in a human host. In malaria-endemic regions, Sulfadoxine-pyrimethamine administration is recommended as an intermittent preventive treatment during the second trimester of pregnancy (WHO, 2014). Amodiaquine and Sulfadoxine-pyrimethamine is a preventive measure already in use in the Africa Sahel sub-region as seasonal malaria chemotherapy to children under 5 years of age (WHO, 2012). Artemisinin-based drug combinations (ACTs) are the first line of treatment for

individuals with the diagnosis of malaria. Additionally, ACTs can be administered to help reduce gametocyte density in humans hence resulting in a reduction in malaria transmission by mosquitoes (Okell, Drakeley, Ghani, Bousema, & Sutherland, 2008).

The interventions, as mentioned above, have helped avert millions of cases of malaria since their introduction (WHO, 2018). Nonetheless, there are challenges to these measures. First, mosquitoes are developing resistance to the available insecticides (Awolola et al., 2009; Kanzaa et al., 2013; Liu, 2015; Yahouédo et al., 2017). The resistance could result from a single point mutation that either enhances metabolic detoxification of the insecticides or decreased sensitivity by the mosquitoes (Awolola et al., 2009; Liu, 2015). Other times, there is delayed uptake of an insecticide by mosquitos whose gene mutations result in thicker cuticles (Yahouédo et al., 2017). Second, the ITNs, though generally well utilised are sometimes reused for other purposes when old or are potentially misused in other forms such as fishing (Kibe, Kamau, Gachigi, Habluetzel, & Mbogo, 2019; Millennium & Goals, 2016; Minakawa, Dida, Sonye, Futami, & Kaneko, 2008). According to Minakawa et al. (2008), up to 83% of the total surface area used to dry fish was covered by mosquito nets. In addition, mosquitoes are adapting to control measures and have altered their feeding pattern to enhance infection of the human host (Cator et al., 2014). Rather than feeding during bed time, most mosquito bites occur either early in the morning or in early hours of the evening (Bamou et al., 2021; Sherrard-Smith et al., 2019). On the other hand, *Plasmodium* parasites are developing resistance to the available anti-malaria drugs such as artemisinin (Rosenthal, 2018). Balikagala et al. in 2021 established that there has been an increase in the prevalence of resistance from about 4% in 2015 to approximately 20% in 2019. The established prevalence was significantly associated with a mutation in the *kelch13* gene of the *Plasmodium* parasite (Balikagala, Fukuda, & Ikeda, 2021)

Despite the challenges, there is a possibility of successfully utilising vaccines in the elimination and eradication of malaria, as was the case of smallpox (Elwood, 1989). According to the Global Technical Strategy, a vaccine that has an efficacy of at least 75% is necessary for the eradication of malaria (WHO, 2015). There are several malaria vaccine candidates at different stages of exploration and development summarised in **Figure *2.2.1***. R21/MM is a pre-erythrocytic vaccine candidate that illustrated more than 70% efficacy in phase 2b clinical trial (Datoo et al., 2021). RTS,S-ASO1 is the most advanced of these vaccines with an efficacy of about 35.9% a year post vaccination (Olotu et al., 2016). However, this efficacy wanes off over time (Mugo et al., 2021; Olotu et al., 2011; RTSS Clinical Trials Partnership, 2017; White et al., 2015). Thus, there is the need to continue with the search for other vaccine candidates and interventions.

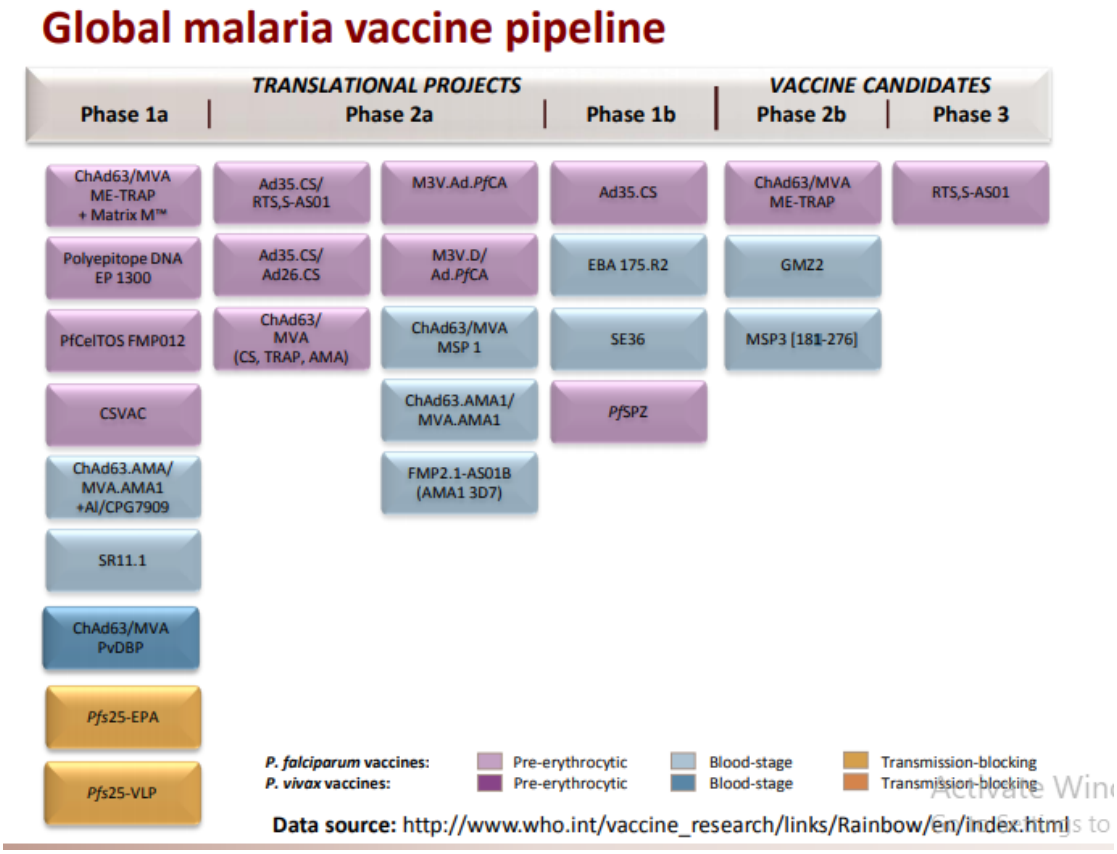

Figure 2.2.1: Malaria vaccine candidates in the development pathway.

2.3 Malaria pathogenesis

Malaria infected individuals may present with fever alone or with a few other mild symptoms when the infection is uncomplicated. On the other hand, complicated malaria may clinically present with symptoms such as coma, convulsion, prostration, anaemia, jaundice, acidosis, pulmonary oedema, significant bleeding, shock, and hyperparasitemia (WHO, 2015). Pregnant women with complicated malaria tend to have underweight babies at birth, preterm births, or even intrauterine death of the foetus (Thompson et al., 2019).

During the blood-stage of *P. falciparum* infection, fever occurs when hemozoin directly disrupts thermoregulation or induces tumour necrosis factor alpha (TNF-α) production (Sherry et al., 1995). Anaemia ensues when haematin, a product of haem degradation, disrupts the cytoskeleton of infected red blood cells (iRBC), making them susceptible to lysis (Omodeo-Salè et al., 2005). Additionally, the phagocytosis of both the infected and uninfected red blood cells by the monocytes could contribute to anaemia (Rogerson et al., 2003). Hypoglycemia and hypertriglyceridemia transpire when glycophosphatidylinositol (GPI), an endotoxin of *Plasmodium*, interferes with the signalling for insulin and disrupts glucose oxidation by the adipocytes (Louis Schofield & Hackett, 1993).

Cerebral malaria has been allied to inflammatory cytokines some of which upregulate the expression of adhesins of the vascular endothelium (Raulf et al., 2019; Velagapudi, Kosoko and Olajide, 2019). The cytokines and reactive oxygen species that induce neuroinflammation and neurotoxicity contribute to the development of cerebral malaria (Mahanta, Kar, Kakati, & Baruah, 2015; Velagapudi, Kosoko, & Olajide, 2019). Enhanced expression of adhensins results in cytoadherence which culminates in vasoconstriction and vaso-oclussion (Cabrales, Zanini, Meays, Frangos, & Carvalho,

2010; Storm et al., 2019). Sequestration of iRBC with granzyme B effector CD3+CD8+ T cell engagement may also occur and contribute to symptoms (Lewallen et al., 2000; Riggle et al., 2020). The sequestration of iRBC along retinal blood vessels results in retinopathy whose severity is positively associated with the degree of iRBC sequestration (Barrera et al., 2015; Lewallen et al., 2000).

Poor outcomes in pregnancy, such as low birth weight, can be attributed to the sequestration of the parasites at the placenta (Rogerson et al., 2003). The sequestration results in localised inflammation, macrophage infiltration and necrosis of the placenta (Beeson, Amin, Kanjala, & Rogerson, 2002; Walter, Garin, & Blot, 1982). Generally, the symptoms tend to be milder among individuals that have a natural immunity to this *Plasmodium* pathogen (Tebo, Kremsner, Piper, & Luty, 2002).

2.4 Immunity

2.4.1 Evidence for immunity to malaria

Immune responses to malaria is species and stage-specific. Both experimental and epidemiological studies have provided evidence of immunity to malaria as detailed below.

Studies have established the role of antibodies in the development of immunity to malaria. In a study that involved the passive transfer of antibodies to children with severe malaria, Cohen and colleagues in 1961 established the role of antibodies in immunity to malaria. Children that received gamma-globulin (γ-globulin) from hyper-immune serum had a rapid reduction in both parasite load and fever. On the contrary, children that antibodies derived from the serum malaria-naive individuals or gamma free globulin fraction had a minimal reduction in parasitemia (Cohen, McGregor, & Carrington, 1961). This study established that antibodies partly mediate the clearance of the *P. falciparum* and alleviate symptoms.

Epidemiological studies demonstrate that immune responses to malaria depends on age, exposure and malaria transmission intensities.

A cross-sectional study in Tanganyika established that children develop severe disease while with an increase in age, the symptoms get milder to a point the older population develop asymptomatic infections (Wilson, 1936). Moreover, the risk for developing clinical malaria decreases with an increase in age (Tran et al., 2013). Another study established that there was a 6% decline in the probability for developing clinical malaria for each additional year lived in a malaria-endemic region (Rodriguez-Barraquer et al., 2016a). The **Figure 2.4.1** below illustrates how in the malaria-endemic areas, over time, the severity of symptoms changes. The burden of malaria shifts to the younger population (Carneiro et al., 2010; Wilson, 1936) such that from about the age of 15 years, individuals in malaria-endemic regions, though infected, are free of symptoms. These asymptomatic carriers tend to have high parasitemia with lower odds of developing clinical symptoms than the non-carriers (Males, Gaye, & Garcia, 2008). Further, a fitted model established that this phenomenon is established gradually (Griffin et al., 2015).

Repeated exposure and high malaria transmission intensities are linked to improved immunity against malaria. repeated exposure to the parasite protects against severe disease (Langhorne, Ndungu, Sponaas, & Marsh, 2008). Upon repeat exposure to the parasite, the symptoms get milder and there is a 2% decline in the risk for developing clinical symptoms for every additional infection (Rodriguez-Barraquer et al., 2016a; Wilson, 1936). A systematic review on 86 articles - analysed for clinical malaria (N=29), hospital admission (N=36) and malaria-diagnosed mortality (N=21) (Carneiro et al., 2010). Additional 50 studies reporting all cause admission (N=22) and all-cause mortality (N=28) were included in the review. Carneiro et al. (2010) established that most of the clinical cases, hospitalisation and admission rates attributed to malaria are

concentrated in the younger population in areas of high malaria transmission intensity with no marked seasonality. Specifically, the median ages for peak malaria cases, hospital admission and mortality were 32, 17 and 12 months respectively in the high transmission regions with low marked seasonality (Carneiro et al., 2010). The findings from Carneiro et al. (2010) illustrated that children in low transmission regions with marked seasonality took at least twice longer before they registered the peak prevalences.

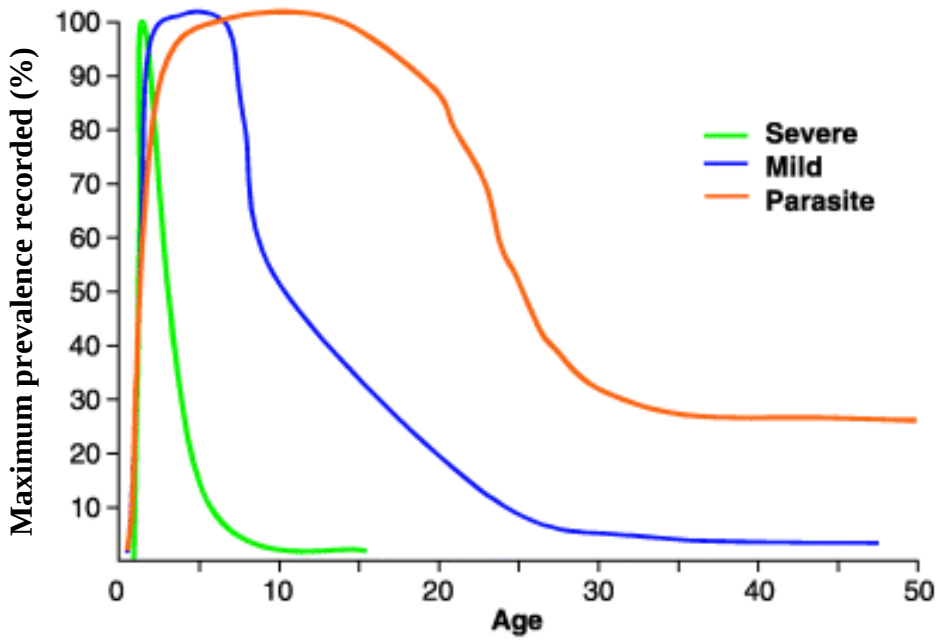

Figure 2.4.1: Epidemiological evidence of immunity to malaria (K. Marsh & Kinyanjui, 2006)

The graph was developed from representative data of studies conducted in Kilifi. It illustrates the age variation in years of asymptomatic parasitemia and the period prevalence of both mild and severe malaria relative to the maximum prevalence recorded. The period prevalence of severe malaria occurs before the 5th year of life while mild malaria has its peak between the age of 5 and 10 years. By about the 15th year of life within the population, the period peak prevalence of asymptomatic parasitemia is recorded.

2.4.2 Targets and mechanisms of immunity to malaria

2.4.2.1 Innate immunity

The innate immunity to malaria involves inflammation, the production of interferon-gamma, which activates T lymphocytes, and antibody production. Utilizing pattern recognition receptors, innate cells such as the monocytes and dendritic cells recognise various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), get activated, and produce cytokines (Artavanis-Tsakonas & Riley, 2002; Knackstedt et al., 2019; Rogerson et al., 2003). One immune target to malaria infection is hemozoin. Hemozoin is a DAMP produced when *P. falciparum* degrades the haem component of haemoglobin when red blood cells are infected. It is released into the extracellular matrix when the iRBCs burst. Haemozoin is recognised by the C-type lectin receptor or the NOD-like receptor that activates the NLRP3 inflammasome and initiates secretion of TNF-α, interleukin (IL)-6, IL-1β, and IL-8 (Griffith, Sun, McIntosh, & Bucala, 2009; Raulf et al., 2019; Velagapudi et al., 2019). Glycosylphosphatidylinositol (GPI) on the other hand, through protein kinase C, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκβ), TNF-α, interleukin 1 (IL-1), and inducible nitric oxide (L Schofield et al., 1996; Tachado et al., 1997). *Plasmodium*'s deoxyribonucleic acid (DNA) is a PAMP that activates innate cells to secrete type 1 interferons (Kalantari, 2018; Kalantari et al., 2014; Sharma et al., 2011; Sisquella et al., 2017).

2.4.2.2 Adaptive immune response

The pre-erythrocytic stage immunity is majorly cellular mediated while the erythrocytic stage is humoral as discussed below.

2.4.2.2.1 Cell-mediated immunity

Cell-mediated immune response against malaria majorly occurs when sporozoites invade hepatocytes. CD8+ T cells are tissue-resident and require priming by innate cells such as dendritic cells and monocytes (Fernandez-Ruiz et al., 2016; Silvie, Amino, & Hafalla, 2017). Once monocytes recognise *Plasmodium* liver-stage-restricted antigens, they process these antigens and present them to CD8+ T cells, which then get activated (Kurup et al., 2019). Activated CD8+ T cells kill the infected hepatocyte within 24 hours of infection (Spencer et al., 2017). A murine model revealed that CD8+ T cells that produce interferon gamma and perforin are contribute to protection against the blood-stage infection of malaria (Imai et al., 2010). The CD8 alpha dendritic cells activate cytotoxic T cells which then carry out lysis of infected red blood cells (Lundie et al., 2008). Moreover, the presences of CD4+ T cells against *Pf*EMP1 that are positive for interleukin 4 in children was associated with protection from clinical malaria (Gitau et al., 2014). Infected red blood cells express major histocompatibility factor class 1 before and after infection hence are recognised by CD8+ T cells in an antigen specific manner (Imai et al., 2013). The cytotoxic T cells through a Fas ligand dependent manner induce the extracellular expression of phosphatidylserine on iRBC hence contributing to the phagocytosis of these iRBCs (Imai et al., 2015).

2.4.2.2.2 Antibody-mediated immunity

Before the blood stage, the main targets of immunity are free sporozoites and liver cells that are infected. Anti-sporozoite antibodies inhibit the invasion of blood vessels by sporozoites (Flores-Garcia et al., 2018). The immunological targets in the erythrocytic stage are the merozoites and red blood cells that are infected. Antibodies are formed against merozoites and result in complement fixation with subsequent activation of the classical pathway as well as opsonisation (Boyle et al., 2015). The antibodies are also

linked to the inhibition of both invasion of erythrocytes by parasites and growth of the parasite, prevention of clinical malaria as well as prevention of high-density parasitemia (Boyle et al., 2015; Hill et al., 2013; Mensah-Brown et al., 2019). In the sexual stage of the parasite, antibodies have been shown to mainly target surface antigens of gametocytes (Stone et al., 2016). Though minimal, antibody-mediated immunity to the immature gametocytes can be naturally acquired (Chan et al., 2019; Dantzler et al., 2019).

2.5 Surface antigens of *P. falciparum*

2.5.1 Stage-specific surface antigen expression of *P. falciparum*

Different surface antigens characterise the *P. falciparum's* life cycle at the different stages of its development. The gametocytes express P230, P48/45, which affect the fertility of male gametes and are essential in the formation of ookinetes (Marin-Mogollon et al., 2018; Van Dijk et al., 2001). Sporozoites, on the other hand, express the circumsporozoite protein (CSP), gamete egress and sporozoite traversal protein (GEST) and thrombospondin-related anonymous protein (TRAP) (Pratt-Riccio et al., 2017; Swearingen et al., 2017, 2016). The CSP is the basis for the subunit RTS,S/ASO1 vaccine (RTSS Clinical Trials Partnership, 2017). Merozoites express apical membrane antigen (AMA1), merozoites surface proteins (MSPs), glutamate-rich protein (GLURP) and reticulocyte-binding-homologue proteins (*Pf*Rh4 and *Pf*Rh5) as some of the surface proteins that mediate binding to red blood cells (Adamou et al., 2019; Healer et al., 2019; Stanisic et al., 2009; Tham et al., 2010). Later blood stages – trophozoite and schizont – are associated with the expression of proteins on the surface of iRBCs, which are collectively called variant surface antigens (VSAs).

2.5.2 Variant surface antigens

There are several VSAs encoded by different genes most of which are situated in the subtelomeric region of *P. falciparum* chromosomes and are transcribed at diverse stages

of the parasite (Gardner et al., 2002; Rubio, Thompson, & Cowman, 1996). These VSAs include the *Pf*EMP1, RIFIN, STEVOR, and SURFIN (Autino et al., 2012). *Pf*EMP1, which is transcribed in the ring stage and expressed in the blood stage of *Pf* development, is encoded by 60 *var* gene (gardener 2002). The expression of *Pf*EMP1 diminishes with parasite growth through the trophozoite stage, while *rif* gene transcription picks up (Kyes, Rowe, Kriek, & Newbold, 1999). The *rif* gene encodes RIFIN which has at least 200 members all of which are diverse with the highest expression at mid trophozoite stage (Kyes et al., 1999). Subtelomeric, variable open reading frames *(stevor)* gene encodes STEVOR protein which has its peak transcription between the late trophozoite and early schizont stages (Kaviratne, Khan, Jarra, & Preiser, 2002). At least 10 surface-associated interspersed *(surf)* genes encode SURFIN, a protein found on the surface of iRBC and merozoites (Winter et al., 2005).

2.5.2.1 VSA mediated pathogenesis

VSAs are involved in the disease process of severe malaria through cytoadherence and rosetting. Cytoadherence is a process where iRBCs adhere to other cells, such as those on the blood vessel walls leading to iRBC sequestration. On the other hand, rosetting is characterised by iRBCs binding to uninfected red blood cells (David, Handunnetti, Leech, Gamage, & Mendis, 1988). Cytoadherence and rosetting contribute to pathogenesis by blocking small blood vessels referred to as vascular occlusion. RIFIN-mediated rosetting is associated with blood group A and is implicated in severe malaria anaemia (Barragan, Kremsner, Wahlgren, & Carlson, 2000; Marín-Menéndez et al., 2013). Additionally, rosetting enhances the transfer of 4-hydroxynonenal from the infected to the uninfected cells leading to enhanced phagocytosis of the uninfected red blood cells culminating in severe malaria anaemia (Uyoga et al., 2012).

*Pf*EMP1 binds to chondroitin sulfate A (CSA) receptors found on the placenta, resulting in sequestration of the parasite at this site during pregnancy-associated malaria (Fried & Duffy, 1996). Consequently, this sequestration leads to poor pregnancy outcomes such as low birth weight, preterm births, and even deaths (Thompson et al., 2019).

Cytoadherence of iRBC to cerebral blood vessels is associated with cerebral malaria. It has been established that relative to non-severe cases, children with cerebral malaria demonstrated adherence of iRBC to endothelial protein C receptor (EPCR) and ICAM1 (Tuikue et al., 2017). Moreover, sequestration in the brain results in a positive feedback loop whereby sequestration results in inflammation while inflammation enhances cytoadherence and further sequestration. *Pf*EMP1 binds to ICAM-1 found on endothelial surfaces in the brain causing localised production of inflammatory mediators (Autino, Noris, Russo, & Castelli, 2012; Ochola et al., 2010; Travassos et al., 2018). These results in cerebral malaria and increases the expression of ICAM-1 with consequent enhancement of adherence (Mota, Jarra, Hirst, Patnaik, & Holder, 2000).

Thus, VSAs contribute to the severity of malaria with symptoms corresponding to the site of parasite adherence or sequestration. However, a wide repertoire of ant-VSA IgG are more likely to prevent clinical symptoms (Kinyanjui, Mwangi, et al., 2004).

2.5.2.2 Immunity to VSAs

Antibodies to *P. falciparum* VSAs are largely variant-specific (Marsh & Howard, 1986). Bull et al demonstrated that children from a malaria endemic region are protected only against variants to which they had pre-existing antibodies (Bull et al., 1998). This observation is reinforced by the finding that symptomatic infections are linked to *Var* gene expression homogeneity and broader host antibody response (Warimwe et al., 2013). Antigenic specificity enables *P. falciparum* to escape immune surveillance by either shifting between different variants of a VSA or to periodically alter the levels of

expression of a given VSA (Abdi et al., 2016). On the other hand, there are reports of cross-reactive antibodies with broader antigen specificity that are associated with milder clinical malaria (Marsh & Howard, 1986; Tuju et al., 2019). While the cross-reactive antibodies may be recognising conserved epitopes, some of those epitopes could be homologous to an immunogen that triggered the development of the antibody (Avril et al., 2008; Marsh & Howard, 1986). For instance, the MSP1 from *P. falciparum* and P. *knowlesi* were >70% similar in their sequence alignment hence recognised by a cross-reactive antibody (Muh et al., 2020). The cross-reactivity may result from unique properties of the antibodies themselves. Recently, Tan and colleagues identified broadly reactive antibodies which contain leucocyte-associated immunoglobulin-like receptor 1 (LAIR1) insert (Tan et al., 2016).

VSA expression occurs under immune selection pressure (Abdi et al., 2017; Bull et al., 2005; Kivisi et al., 2019). When *var* genes from 12 clinical parasite isolates from Kenyan children were sequenced, it was observed that there was a negative correlation between the expression of relatively conserved VSA and the repertoire of anti-VSA antibodies (Bull et al., 2005). Abdi et al. in 2017 established that low antibody titers were positively associated with the expression of the conserved *var* gene subclasses. Similarly, maternal antibodies demonstrate immune selection pressure on the *P. falciparum* parasites isolates that infect infants (Kivisi et al., 2019). A Controlled Human Malaria Infections (CHMI) study where semi-immune adults were recruited, observed that participants with low antibody levels to *Pf*EMP1 had a rapid increase in parasitemia with a broad activation of *var* genes (Bachmann et al., 2019). From the same study, participants with intermediate antibodies developed asymptomatic infections with few *var* gene variants expressed. Additionally, *var* genes coding *Pf*EMP1 that binds to EPRC-binding were rarely detected in this set of volunteers (Bachmann et al., 2019). Similarly, a study observed an increase

in expression of relatively conserved group A *var* gene with age and waning of maternally-acquired anti-VSA antibodies in infants (Kivisi et al., 2019).

Seroreactivity to VSA was shown to be affected by age and exposure to the parasite (Gupta, Snow, Donnelly, Marsh, & Newbold, 1999; Zhou et al., 2019). Individuals establish immunity to severe malaria gradually so that from birth to about the age of three children are at high risk for infection (Dent et al., 2016; Griffin et al., 2015). From a cohort study of 93 Ugandan children, it was established that the risk for clinical malaria decreased by 6% for each additional year lived and a 2% decline for each additional prior infection (Rodriguez-Barraquer et al., 2016b). Similarly, repeat exposure among children was associated with higher parasitemia without clinical symptoms (Ndungu et al., 2015). A separate study established that the prevalence of cases, parasite density as well as clinical severity decreased with an increase in age (Bloland et al., 1999). Zhou et al. (2019) established that adults mounted a better humoral response to STEVOR and RIFIN compared to children. On the contrary, children failed to raise antibodies to a subset of RIFIN, which is associated with severe malarial (Zhou et al., 2019).

Individuals with more recent exposure to malaria parasites had a better IgG titre against RIFIN than those whose exposure had occurred a long time before the study (Zhou et al., 2019). The loss of these antibodies could be caused by antibody decay evidenced by the reported short half-lives of the IgG isotypes. A study on anti-merozoite antibody established that the IgG subclasses had short half-lives that ranged from 6.1 days for IgG3 and 9.8 days for IgG1 (Kinyanjui, Conway, Lanar, & Marsh, 2007).

2.5.2.3 LAIR1 containing antibodies in the control of malaria

2.5.2.3.1 The constitutively expressed LAIR1

LAIR1 is an inhibitory receptor that is encoded on chromosome 19q13.4 (Meyaard et al., 1997). It belongs to the immunoglobulin superfamily and is constitutively expressed on

most of the mononuclear lymphocytes (Meyaard et al., 1997). LAIR1 is found on the surfaces of the CD4+ T cell, CD8+ T cell, natural killer cells, B lymphocyte, monocytes, and thymocytes but not on granulocytes, platelets and red blood cells. The extracellular domain of LAIR1 has only one immunoglobulin-like domain. Via the extracellular domain, LAIR1 binds to collagen or the collagen-like region of complement component 1q (C1q)(Fouët et al., 2021). Its cytoplasmic region has two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Meyaard et al., 1997, 1999). When SH2-containing tyrosine phosphatases 1 and/or 2 (SHP 1 and/or 2) are recruited, they cross-link the ITIMs resulting in the generation of inhibitory signals (Meyaard et al., 1997).

A study was conducted to investigate the inhibitory process of LAIR-1 in the presence of C1q. It was established that C1q binds both LAIR-1 and LAIR-2 (the soluble derivative of LAIR-1) with an affinity similar to the interaction between LAIR-1 and collagen (Son, Santiago-Schwarz, Al-Abed, & Diamond, 2012). The binding resulted in phosphorylation of ITIM. Consequently, there was an inhibition in differentiation of monocytes to dendritic cells. Moreover, the expression of proinflammatory cytokines was inhibited (Son et al., 2012).

LAIR1 alters innate immune responses and affects cytokine production. LAIR1 inhibits the maturation of monocyte-derived progenitor cells (Cd14+ CD34+) to dendritic cells (Poggi, Tomasello, Ferrero, Zocchi, & Moretta, 1998). According to Poggi et al. (1998) this inhibitory receptor impairs GM-CSF receptor-mediated activation signalling. Therefore, where intracellular free calcium ion levels should rise, they are down regulated when there is cross-linking of the receptor (Poggi et al., 1998). A separate study the stimulation of monocytes and type-2 conventional dendritic cells results in enhanced expression of LAIR1 (Carvalheiro et al., 2020). According to Carvalheiro et al. (2020), the ligation of LAIR1 on monocytes was linked to inhibition of Toll like receptor 4 (TLR4) and interferon alpha signalling. Overall, there was an initial rapid increase in

LAIR1 surface expression during inflammation and a decline during resolution (Carvalheiro et al., 2020). These findings suggest the involvement of LAIR1 in the regulation of inflammation.

LAIR1 alters the expression of B cells, immunoglobulin production and cytokine secretion. The cross-linking of LAIR1 resulted in a reduction of the proportion of IgG+ and IgE+ B cells (Merlo et al., 2005). Additionally, the study established that LAIR1 contributed to down regulation of IgG and IgE production. Merlo et al. (2005) further established that interleukins 8 and 10 (IL-8 and IL-10) as well as the tumour necrosis factor alpha (TNF-α) were reduced. The three down regulated cytokines are crucial for chemoattraction of B lymphocytes (Nielsen et al., 1997). IL-10 is costimulatory in B lymphocyte proliferation in the presence of CD40 system (Rousset et al., 1992). The cytokine is also required to induce activated B cells to secrete immunoglobulin isotypes G, A, M and E (Rousset et al., 1992). Thus, a down regulation of the cytokines culminates in reduced B cell proliferation as well as diminished immunoglobulin secretion from the activated B cells.

Maasho and colleagues in 2005 established that unlike other inhibitory receptors, LAIR1 is highly expressed in naive T cells. The same study reported that TCR cross-linking heightens the surface expression of LAIR1 and a Mitogen-activated protein (MAP) kinases dependent process. They also established that the crosslinking of Cross-linking of the LAIR1 inhibits T cell receptor (TCR) signalling (Maasho et al., 2005). The cell-surface expression of LAIR1 varies with subtypes and CD8+ T cells had greater expression than CD4+ T cells in a study conducted by Saverino et al., (2002). This study established that the cytotoxicity of CD8+ T cells was down regulated by LAIR1. Further, the proliferation of CD4+ T cells was diminished by the inhibitory receptors (Saverino

et al., 2002). Thus, the differential expression of LAIR1 on T cells suggests its involvement in immunomodulation.

2.5.2.3.2 Immunopathogensis involving LAIR1

LAIR1 can mediate cancer development using SH-P in a phosphatase independent manner (Kang et al., 2015). Kang and colleagues (2015) used a mouse model to investigate the mechanism of leukemia development. They established that LAIR1 did not affect the cell cycle status. Instead, the SH-P associated with ITIM domains of LAIR1 recruited calmodulin-dependent protein kinases 1 (CAMK1) (Kang et al., 2015). These SH-P acted as positive mediators that prevented exhaustion of myeloid cells. Consequently, the LAIR1-SHP-CAMK complex was found to increase the lifespan of myeloid cells hence the development of some leukemias.

There is an association between LAIR1 expression and the development of chronic inflammatory conditions. In a case-control study, there was significant reduction both in the proportions of neutrophils that express LAIR1 and the mean fluorescent intensity (MFI) of neutrophils LAIR1 among the *Psoriasis vulgaris* cases relative to the healthy controls (Hammad et al., 2020). When Hammad et al. (2020) compared the severe and the moderate-to-mild cases of *Psoriasis vulgaris* the levels of LAIR1 on neutrophils and monocytes were significantly reduced in the severe cases. Thus, the presence and severity of *Psoriasis vulgaris* is associated with diminished expression of LAIR1 on neutrophils and monocytes. From a mouse model, there was evidence that LAIR1 supressed neutrophil mediated airway inflammation (Kumawat et al., 2019). These findings illustrate that diminished expression of LAIR1 on neutrophils contribute to the pathophysiology of inflammatory conditions such as *Psoriasis vulgaris.*

Achieng' and colleagues investigated the role of LAIR1 in severe malaria anaemia (SMA) among children. They reported close to 2 fold increase in the soluble LAIR1

(sLAIR1) in the SMA group relative to the non-SMA (Achieng et al., 2019). According to the study, the levels of C1q was about 1.2 fold lower in the SMA group relative to the non-SMA group. Further analysis demonstrated that phagocytosis of hemozoins resulted to a reduction in LAIR1 transcripts and protein expression (Achieng et al., 2019). There was also an inhibition of LAIR1 signalling due to diminished phosphorylation of SHP1. Thus, the study provides a molecular interplay of decreased surface expression of LAIR1, increased secretion of sLAIR1 and reduced C1q levels in reducing the inhibitory effect of LAIR1 during severe malaria anaemia.

2.5.2.3.3 LAIR1 bearing antibody

Tan and colleagues performed a mixed agglutination assay on 557 plasma samples using culture adapted Kenyan parasite isolates. 3 of the plasma samples formed mixed agglutinates with at least 6 parasite isolates (Tan et al., 2016). Samples from 2 donors, C and D, formed mixed agglutinates with 8 parasite isolates. Most of the monoclonal antibodies generated from these two donors were broadly reactive and molecular analysis illustrated that the broadly reactive antibodies had an ectopic insert that was about 90% homologous to LAIR1 in the V and DJ segments of the heavy chain (Tan et al., 2016). The LAIR1 insert was mutated with reduced capacity to bind collagen and enhanced binding to infected red blood cells (Tan et al., 2016). Using liquid chromatography-mass spectrometry, it was established that the LBA recognised type A RIFIN (Tan et al., 2016). The **Figure *2.5.1*** suggests that LBA recognises different subsets of RIFINs and the intensity of recognition varies across parasite isolates (Tan et al., 2016). Apart from agglutination, LBA demonstrated a strong capacity for opsonic phagocytosis of infected red blood cells (Tan et al., 2016).

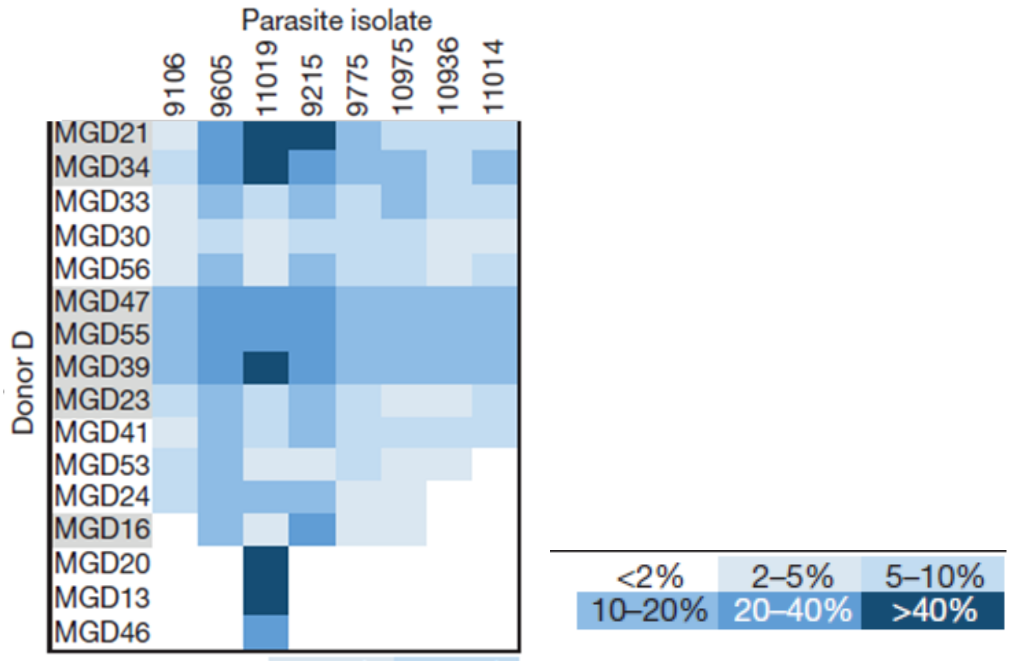

Figure 2.5.1: Percentage of infected red blood cells of 8 parasite isolates recognised by monoclonal antibodies from donor D (Tan et al., 2016)

Along the Y axis are 16 monoclonal antibodies from donor D that were subjected to 8 parasite isolates indicated along the X axis. Most of the monoclonal antibodies recognised multiple parasite isolates while the last 3 only recognised one parasite isolate. The antibodies recognised varied fractions of infected red blood cells across different parasite isolates.

It was later established that 5 to 10% of people in malaria-endemic regions have antibodies to *P. falciparum* that contain LAIR1 insert (Pieper et al., 2017; Tan et al., 2016). The mutated LAIR1 is either flanked by the V and DJ segments or inserted in the switch region of the heavy chain of LAIR1-bearing antibodies (LBA) (Hsieh & Higgins, 2017; Pieper et al., 2017; Tan et al., 2016) **Figure *2.5.2***. The insertion into the switch regions results in either an insertion between JH and CH1 segments or the replacement of the VDJ and the CH1 segments to yield a camel-like immunoglobulin (Pieper et al., 2017). The insertion between then V and DJ segments, as well as the camel-like

antibodies, are broadly reactive. The insertion into the elbow is bi-specific but not as broadly reactive as the former two (Pieper et al., 2017).

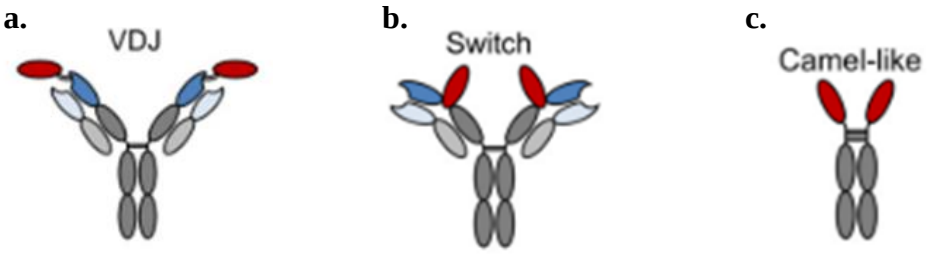

Figure 2.5.2: LAIR1 insertion modalities (Pierper et al., 2017)

*In **a.** LAIR1 is flanked between the V and DJ junctions, **b.** LAIR1 is located in the switch region and **c.** LAIR1 replaces the VDJ and CH1 segments.*

LBA broadly binds to iRBC through the inserted LAIR1 rather than the complementarity determining regions (CDRs). While the insertion between V and DJ occurs at the CDR3 loop of the heavy chain, the LAIR1 insert tends to extend into the groove between the light and heavy chains of the Fab and partially occludes the remaining five CDRs (Hsieh & Higgins, 2017) **Figure *2.5.3***. As a result, the LAIR1 becomes an auxiliary domain that recognises antigens while the Fab fragment links it to the Fc segment.

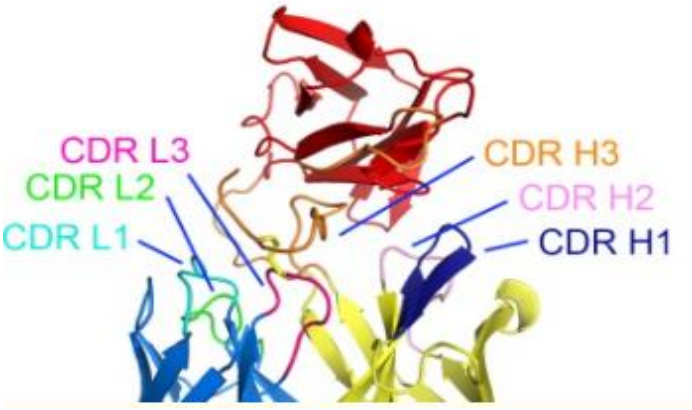

Figure 2.5.3: The antigen binding region (Fab) of LBA (Hsieh & Higgins, 2017)

The schema illustrates the structure of antigen biding site composed of 6 CRDs (Complementarity Determining Regions). Three of them, CDR L1, L2, L3 are light chain Complementarity Determining Regions 1, 2 and 3 while the other three, CDR H1, 2, 3 are the heavy chain CDRs. LAIR1, the red loop, inserts into CDR H3 and partially occluded the remaining 5 CDRs.

This section has presented supporting evidence that people get immune to malaria. Additionally, VSAs are important in the establishment of this immunity. However, the variant-specific nature of antibody development enables for escape of *P. falciparum* from immune surveillance and clearance. Therefore, the broadly reacting LBA can circumvent the immune escape strategy thus leading us closer to interventions against malaria.

CHAPTER THREE

MATERIALS AND METHODS

In this section, a breakdown of the source of the samples used, methods used to establish a high throughput assay for LBA screening, the assays for actual LBA screening and a detailed definition of anti-VSA antibody breadth are provided.

3.1 Study area

This study took advantage of the controlled human malaria (CHMI) studies at KEMRI Wellcome Trust Research Programme (KWTRP) in Kilifi and Ahero Clinical Trials Unit (ACTU) in Ahero. CHMI used plasma samples collected from participants from Ahero (Kisumu County in Western of Kenya), Kilifi North (Ngerenya) and Kilifi South (Pingilikani and Junju). Malaria transmission rate in Ahero averages 108.6 bites/person/year (Degefa et al., 2017). The transmission rate in Kilifi South (approximate range, 0.35 – 0.7 MPF) is higher than that in Kilifi North (a maximum of about 0.35 malaria positive fraction) (Mogeni et al., 2016). From survey data, the transmission rates were estimated as ≥30% in Ahero and less than 30% *Plasmodium falciparum* parasite prevalence ($Pf\text{PR}_{2\text{-}10}$) in most parts of Kilifi (Macharia et al., 2018). The transmission rates ($Pf\text{PR}_{2\text{-}10}$) are approximately 10 – 29% with a small region registering ≥30% in Kilifi South and 5 – 9% in Kilifi North (Macharia et al., 2018). For this study, areas were categorised as high transmission (Ahero and Kilifi South) and low transmission (Kilifi North) (Kapulu et al., 2022a, 2020). The negative controls consisted of plasma samples from European donors with no history of malaria or had never travelled to a malaria endemic region.

3.2 Study design

This study was nested in CHMI as a retrospective analysis. Plasma samples from 142 healthy semi-immune adults at 2 time points recruited into the CHMI study were utilized.

Additional data from anti-schizont antibodies and anti-VSA antibodies responses were used as proxy for prior exposure to malaria and antibody breadth respectively.

3.2.1 Controlled Human Malaria Infection (CHMI)

3.2.1.1 Background

CHMI is an open label, non-randomised trial that recruited 200 healthy adult volunteers over several years in cohorts of between 20 and 60 volunteers (Kapulu et al., 2019). The study aimed to explore how *in vivo P. falciparum* growth rate is altered by pre-existent immunity to blood-stage antigens.

3.2.1.2 Inclusion and exclusion criteria

In the CHMI study, healthy adults (18 - 45 years of age) with informed consent and on effective contraceptive plan (for women). Effective contraception was defined as those products with at most 1% failure rate from the product-label.

Volunteers were disqualified from the study on the following basis:

1) The use of drugs with anti-malarial action within 30 days after PfSPZ challenge was administered.
2) If they were taking part in another clinical trial or had just completed on within 12 weeks of enrolment.
3) Having taken investigational malaria vaccine.
4) Previous inoculation of PfSPZ challenge.
5) Any confirmed or suspected case of immune-suppression was also excluded - even those that had immunosuppressive therapy within 6 months.
6) Use of immunoglobulins or blood and its derivatives within a period of 3 months.
7) Any reported or identified medical condition that would increase risk of CHMI.
8) Any significant biochemistry, haematological, urinalysis or clinical anomaly.
9) Women who were pregnant or intending to become pregnant within the study period.

10) Confirmed PCR-positivity for parasitemia a day prior to challenge.

11) On the day of PfSPZ challenge, a candidate with moderate to severe illness in the presence or absence of fever was excluded.

3.2.1.3 Administration of *Pf*SPZ challenge

During the CHMI study, prior informed consent was obtained, history was taken and laboratory test done to elicit prior exposure to malaria. The diagnostic tests for malaria were quantitative PCR (qPCR) for the detection of *Plasmodium falciparum* deoxyribonucleic acid (DNA) and microscopy. Enzyme linked immunosorbent assay (ELISA) was conducted to quantify anti-Schizonts antibodies and elicit past exposure to malaria parasites among study participants (Hodgson et al., 2014; Kapulu et al., 2020; Osier et al., 2008). Eligible volunteers who were PCR-positive for malaria infection were treated with artesunate and only included once confirmed PCR-negative a week prior to challenge. These individuals were injected with a dose of 3,200 aseptic, purified, cryopreserved sporozoites of *Plasmodium falciparum* laboratory isolate NF4 (*Pf*NF54) (Kapulu et al., 2020). The *Pf*SPZ was administered directly to the veins using needle and syringe by trained clinician - the dose and route was selected to maximise the probability of infection. The injection site was covered with a sterile gauze. The participants were observed for an hour after injection before they were released to an inpatient setting where all volunteers resided for the entire period of the challenge.

3.2.1.4 Monitoring post challenge

A day prior to challenge (C-1) all eligible candidates were reviewed to rule out new medical conditions, or symptoms and for clinical assessments. Baseline data and samples were collected. On the day of challenge - clinical assessment and review of C-1 results was done so that only those that met the inclusion criteria received the *Pf*SPZ challenge. Individuals inoculated with the *Pf*SPZ preparation were monitored for an hour for any

adverse events which were then documented. Post challenge, at day 5 (C +5) a blood sample to assess liver stage immunity was drawn. From days 7 to 21 after challenge (C +7 to C+21) daily review involving clinical assessment was conducted. Venous blood sample for qPCR twice daily at 5, 7, 9, 14 and 21 days post challenge was drawn. Biochemistry was carried out at days 9 and 21 post challenge. Antimalarial treatment at C+21 and clinic review at day 35 (C+35) was done.

3.2.1.5 Treatment and malaria management

Artemether and Lumefantrine (AL) was administered to all volunteers at end-point (C+21) and based on qPCR results. Anti-malarial administration was given in either of the following conditions: i) parasitemia exceeded 500 parasites per µl in the absence of signs and symptoms; ii) volunteer developed signs and symptoms with any level of detectable parasites in the blood films; or iii) the volunteer reached the 21st day of monitoring. When a volunteer developed signs and symptoms of malaria, rapid diagnostic test was done, blood film – both thick and thin was examined at 100 high power filed by experienced microscopists and confirmed by a second one. Volunteers undiagnosed with malaria put on AL at the end and those that could not tolerate oral medication, artesunate was administered parenterally.

3.2.2 Defining CHMI outcomes

The CHMI outcomes were classified based on the progression of infection following the inoculation with *Pf*NF54. The “polymerase chain reaction negative” (PCR –ve) individuals were those participants in whom no parasites were detected by PCR. The “untreated PCR positive” (Untreated PCR +ve) participants, had fluctuating levels of parasite load that never reached the threshold for treatment set at 500 parasite/µL. The “treated”, were treated before the end of the study because they reached the cut off for parasitemia whether or not they had systemic febrile symptoms. For the “febrile”

individuals apart from reaching the threshold of parasitemia requiring treatment, they also developed a fever (temperature >37.5°C) (Kapulu et al., 2019). Of the 142 samples qPCR analysed for CHMI outcomes, 26 (18.3%) were treated febrile, 30 (21.1%) were treated non-febrile, 53 (37.3%) were untreated PCR +ve and 23 (23.2%) were PCR –ve.

Table 1: Number of participants per region and CHMI outcome

	Region/ Area of residence of study participants		
CHMI outcome	**Ahero (N=15)**	**Kilifi South (N=93)**	**Kilifi North (N = 34)**
Treated febrile (Febrile) (N=26)	1	7	18
Treated non-febrile (Treated) (N=30)	2	13	13
Untreated PCR +ve (N=53)	9	46	1
PCR –ve (N=23)	3	27	2

N = number of participants

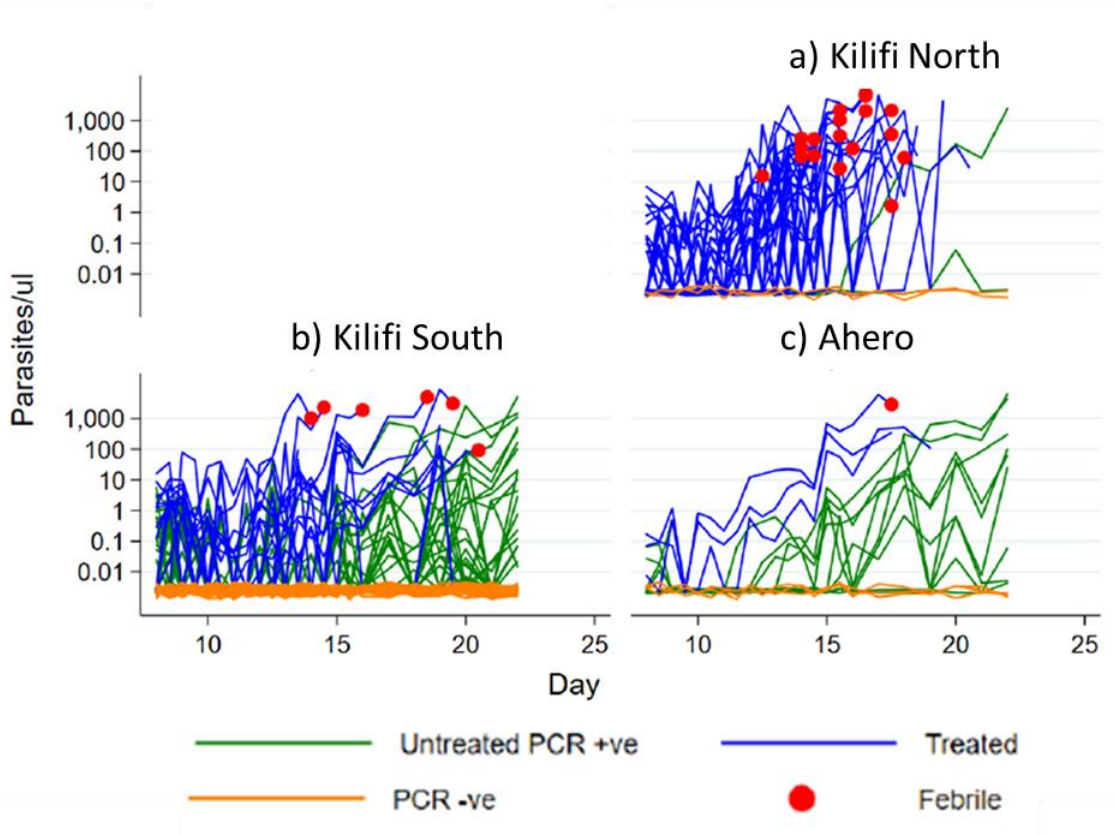

Figure 3.2.1: CHMI outcomes adopted from Kapulu et al., 2020.

The X axis represents the number of days from the day of PfSPZ inoculation. The Y axis represents the number of parasites per microliter of an individual's blood sample. The differently coloured line represent the different outcomes with the fluctuations of parasitemia over time.

3.2.3 Sample size

The study used powerreg facility from STATA version 13 to take power of multivariate model into account. The assumption was r^2 value of 0.3, including 50 variables and 80% power to detect a single variable accounting for 0.15 of the variability in growth rates. The estimated sample size was 200 (Kapulu et al., 2019). Five hundred and four volunteers were recruited and after screening and follow-up, only 161 volunteers completed CHMI with 15, 34, and 112 samples from Ahero, Kilifi North, and Kilifi South, respectively. Out of the 161 participants, 19 were excluded from qPCR analysis

for CHMI outcomes. Seven of them were exclude for having non-*Pf*NF54 infection while the remaining 12 had plasma levels of lumefantrine exceeding a minimum inhibitory concentration of 280 ng/ml. All the excluded participants were from Kilifi South reducing the number to 93 that qualified for qPCR analysis. Consequently, only 142 plasma samples were qPCR analysed for CHMI outcomes.

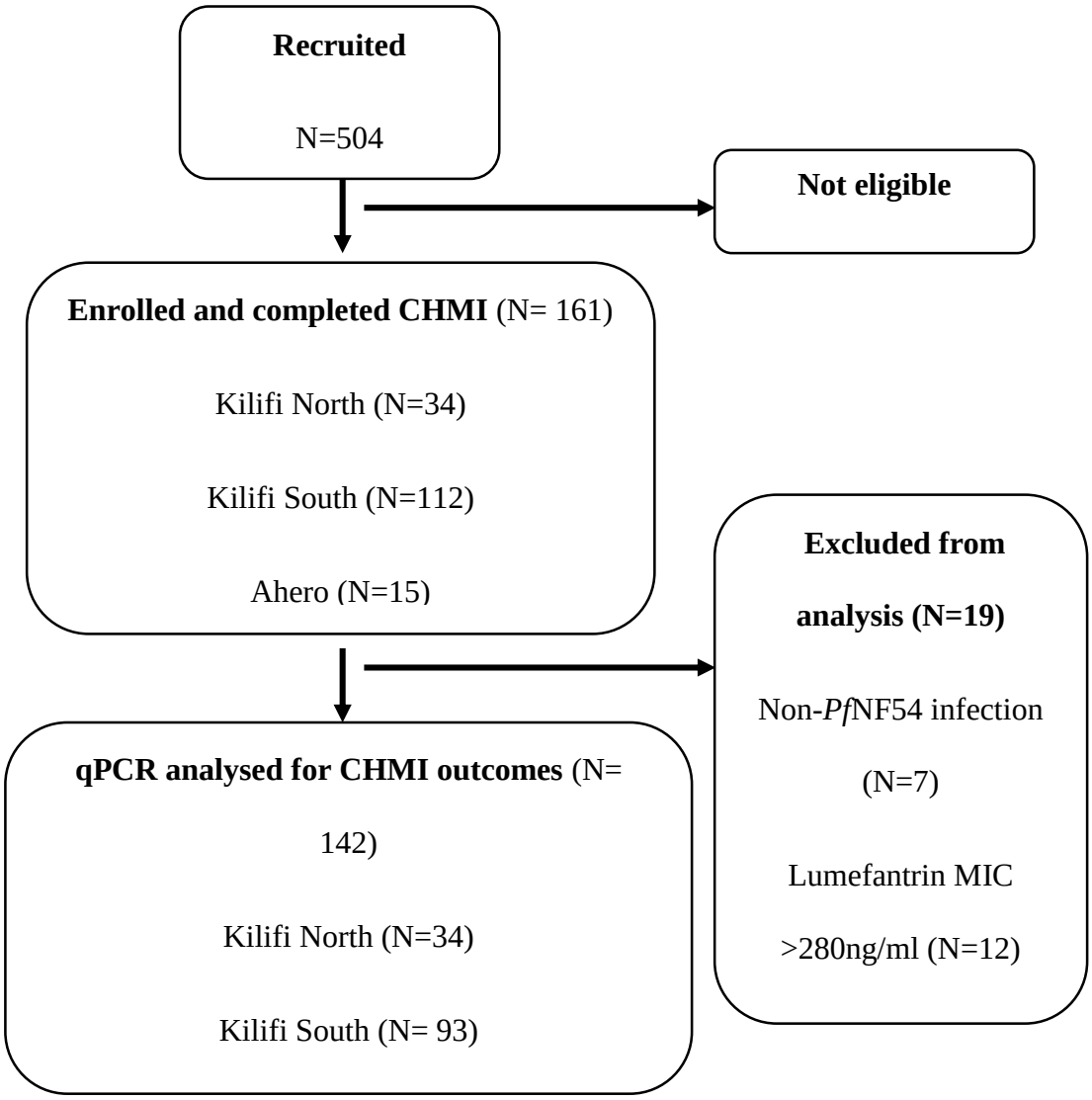

Figure 3.2.2: The number of CHMI participants from recruitment to qPCR analysis for outcomes.

MIC = minimum inhibitory concentration measured in Nano grams per millilitre of plasma (ng/ml)

For this MSc study, the samples size was based on 10% positivity of LBA in malaria-endemic population - Mali and Tanzania (Pieper et al., 2017). Assuming a similar prevalence in the three study sites of Kenya and 95% confidence interval, the estimated sample size using OpenEpi was 139 (Dean AG, Sullivan KM, 2013). Therefore, in this study, all the 142 plasma samples that were qPCR-analysed for CHMI outcomes were included.

3.3 Laboratory methods

3.3.1 Establishing a high-throughput assay for the screening of plasma samples for LBA

3.3.1.1 Developing a flow cytometry assay for the screening of LBA

3.3.1.1.1 Monoclonal Antibodies as Positive and Negative Controls.

The monoclonal used in this study were kind donations from Joshua Tan and Antonio Lanzavecchia. Tan and colleagues performed a mixed agglutination assay on the plasma samples of over 500 individuals and 2 of them, designated donors C and D, were found to form mixed agglutinates. They single-sorted the IgG+ B cells, immortalised them in the Ebstein-Barr virus and generated monoclonal antibodies. A total of 27 monoclonal antibodies with LAIR1 insert were identified from donors C and D (Tan et al., 2016). Ten of those 27 monoclonal antibodies have been published as either LBA positive or negative through protein sequencing and were available for this study (Pieper et al., 2017; Tan et al., 2016). Nine out of the 10 Mabs (MGD21, MGC34, MGD47, MGC29, MGC28, MGD39, MGC26, MGB74 and MGD55) were positive to LBA while only MGD13 lacked LAIR1 insert. Therefore, MGD13 was selected as the negative control for LBA assays.

3.3.1.1.2 Quantification of Monoclonal Antibodies.

Monoclonal antibody (Mab) concentration was measured using the Bradford assay. 250 µl of Coomassie Brilliant Blue G-250 (ThermoScientific) dye was added to 5µl of either Bovine Serum albumin (BSA) which was the standard, or the Mab and mixed by pipetting up and down in a Thermo scientific microplate. The BSA was diluted to concentrations of 2,000, 1,500, 1,000, 500, 250, 100 and 50 ug/ml. The plate was incubated for 5 minutes at room temperature and read at a wavelength of 595nm on the spectrophotometer.

3.3.1.1.3 Thawing of Frozen Plasmodium Falciparum Isolates.

This study utilised frozen *ex-vivo Plasmodium falciparum* parasite isolate 9215 obtained from patients presenting with malaria at the Kilifi County Hospital. The isolate had been cultured in the laboratory and cryopreserved at trophozoite stage in liquid nitrogen using protocols described elsewhere (Rossan, 1985; W Trager & Jensen, 1977; William Trager & Jensen, 1976).

To restore isotonicity, the frozen parasite isolates were thawed by adding decreasing concentrations with increasing volumes of sodium chloride as described elsewhere (Coggeshall, 1939; Kinyanjui, Bull, Newbold, & Marsh, 2003). Briefly, the frozen samples were retrieved from liquid nitrogen storage and thawed by gently rubbing between two gloved hands. 1 ml of the sample was then transferred into a 50 ml falcon tube and 200 µl of 12% sodium chloride was slowly added drop wise. Then the first few drops of 10 ml 1.8% sodium chloride was added slowly to the mixture. Afterwards, 10 ml of 0.9% sodium chloride were added drop-wise while shaking the tube. The mixture was centrifuged at 2000 rotations per minute (RPM) for 5 minutes at room temperature on the Rotanta 460R centrifuge (HettichZentrifugen, Germany). The supernatant was

drawn and discarded. The cells were then washed twice in 200 µl RPMI media without albumax (incomplete RPMI). These are the cells used for flow cytometry assay.

3.3.1.1.4 Preparing Infected RBC Suspension for Flowcytometry.

Early trophozoites at 10% haematocrit was used for this assay. In order to obtain the volume of iRBC required for the reconstitution to 2% parasitemia, the formula below was applied:

$$\%\ parasitemia\ of\ pellet\ x\ Volume\ of\ pellet = Expected\ \%\ parasitemia\ (2\%) x\ expected\ pellet\ volume$$

Thawed iRBC were washed in 1XPBS three times after the initial wash with incomplete RPMI at 450Xg for 5 minutes and at room temperature. The supernatant was aspirated and 1µl aliquots of iRBC prepared (2 µl of parasitized cells in PBS) to which 2µl of SYBR ® Green I nucleic acid gel stain (Thermofisher) at a dilution X 0.625 in 1XPBS was added and incubated for 30 minutes (Somsak *et al*., 2012), at room temperature and in the dark to prevent quenching of the DNA dye. SYBR is a DNA dye that is being taken up by the nucleic acid of the *Plasmodium falciparum* parasite. Since the mature red blood cells lack nucleic material, only the infected red blood cells take up the dye. Stained iRBC were washed using 1XPBS, re-suspend and 5,000 events were acquired on the BD FACS Canto™ II flow-cytometer(Beckman Coulter, UK) (Jang et al., 2014) and read parent (%) as the parasitemia. The focus of these study was trophozoite which can be differentiated from the rings by their higher uptake of the SYBR green dye.

3.3.1.1.5 Screening of Plasma for LAIR1 Bearing Antibodies

A master-mix comprising 2.5µl of 10% hematocrit at 2% parasitemia iRBCs, 10µl of 0.5% BSA/1XPBS and X0.0625 final concentration of SYBR green was added to 1µl of plasma in each well and incubated for 30 minutes. MGD13 was used the negative control for this assay. The preparation was washed three times in 200µl 0f 0.5%BSA/X1PBS per

well at 110XG and room temperature using Rotanta 460R centrifuge (Hettich Zentrifugen, Germany). 50µl of Alexa Fluor 647 (A647) conjugated anti-human IgG (2.5µg/ml) (Jackson Immuno Research Laboratories) and Phycoerythrin (PE) conjugated anti-LAIR1 (1 in 20) (BD Pharmingen ™) were then added and the preparation incubated for 30 minutes in the dark at room temperature. The preparation was washed three times as before and the cells reconstituted to 0.05% haematocrit for the acquisition of 1,000 trophozoite-iRBCs on FACS Canto II flow cytometer.

MGD21 is a known LBA positive monoclonal antibody that recognises VSAs on parasite isolate 9215. Therefore, it was used at different concentrations as the positive control in the detection of LBA from selected CHMI samples. On the other hand, UK is a malaria naïve European sample that was used as the negative control.

Gating strategy

The Side-scatter area (SSC-A) against forward scatter area (FSC-A) were used to gate for the total red blood cell population. The forward scatter height (FSC-H) against the FSC-A are used to distinguish single cells from the duplex. The SYBR is the DNA dye used to segregate infected red blood cells (iRBC) from uninfected red blood cells (uRBC). From the iRBC population, further gating was done using Alexa Fluor conjugated antihuman antibody (A647) against Phycoerythrine conjugated anti-LAIR1 antibody (LAIR1 (PE)). IgG (A647) was used to establish whether antibodies from plasma that bound to iRBC were IgG. On the other hand, LAIR1 (PE) was used to establish that the antibodies contained the LAIR1 insert. Therefore, the second quadrant (Q2) in (D) contains IgG positive LAIR1 bearing antibodies (LBA).

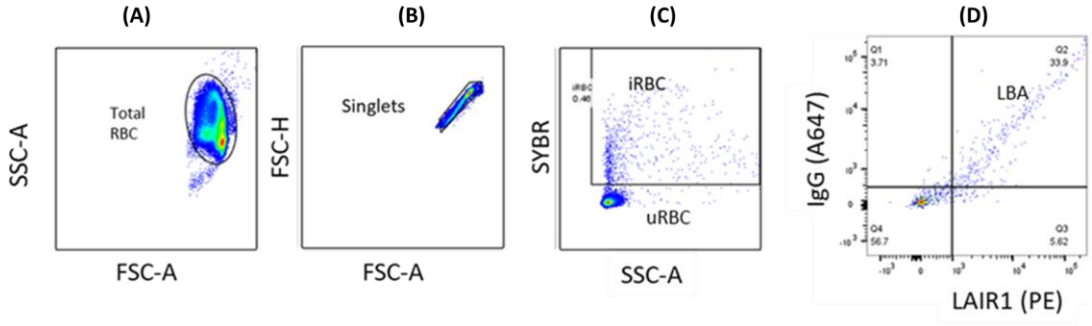

Figure 3.3.1: The stepwise gating from total red blood cells to detect LBA

First, total red blood cell (Total RBC) population was gated in A. Then, the single cells (singlets) was gated in B. From the singlets, further gating for infected RBCs (iRBC) that had taken up SYBR green fluorescent dye in **Figure *3.3.1* C** *was done. Lastly, from the iRBC population, LAIR1 using PE fluorescence and immunoglobulin G (IgG) using Alexa Fluor 647 (A647) fluorescence was gated in D.*

Calculating fluorescent intensity

FlowJo version 10 (Becton, 2019) was used to obtain the median fluorescent intensity (MFI). The concentration of IgG that bears LAIR1 were expressed as MFI of the population positive for both A647 and PE. 3 standard deviations above the MFI of MGD13 was used as the positivity cut-off for LBA.

3.3.1.2 Developing an ELISA assay for LBA screening

This section describes the approach this study took to quantify Mabs using ELISA and to optimise an ELISA assay to measure LBA is described. The assay established for screening CHMI plasma samples is described later.

3.3.1.2.1 Quantification of LAIR1-containing Monoclonal Antibodies

Quantification of LBA in the monoclonal antibody preparations was done using sandwich enzyme-linked immunosorbent assay (ELISA). MGD13 which has been shown

to lack LAIR1 insert was used a negative control while MGD21 was used as the positive control. This assay utilised the following reagents: (i) Coating buffer contained 5µg/ml of anti-LAIR1 antibody (Thermofisher) in carbonate bicarbonate (Sigma-Aldrich). (ii) Wash buffer which was composed of 0.05%Tween-20 in ×1PBS. (iii) Blocking buffer which was prepared by adding 10% skimmed milk to the wash buffer. (iv) Substrate is prepared by first dissolving 1 tablet buffer first that contains hydrogen peroxide (H_2O_2) and adding 1 tablet of 1 o-Phenylenediamine dihydrochloride (OPD) in 20 ml distilled water)

100µl/well of coating buffer was aliquoted into a flat-bottomed microplate (Thermo Scientific) which was incubated overnight at 4°C. The plate was washed four times using 200 µl wash buffer to remove unbound antibodies. 200 µl/well of blocking buffer was added and incubated for 1 hour at 37°C to block non-specific binding. The plate was washed six times to remove excess blocking buffer. To each well, different preparations of 1µg of monoclonal antibodies (Mab) in 100 µl of blocking buffer was added and incubated for 2 hours at 37°C to allow Mab to bind to anti-LAIR1 antibody. The plate was washed 6 times to avoid unspecific binding. 100 µl of rabbit anti-human IgG-HRP diluted at 1:5000 in blocking buffer was added and incubated for 2 hours at 37°C to allow the anti-human IgG to bind to the Mab. The plate was washed six times and banged on folded paper-towels to dry and 100 µl of substrate was added and incubated for 15 minutes to allow for reaction and colour change. Finally, 25 µl of 2M H_2SO_4 was added to stop the reaction and then absorbance was read at 492nm on the spectrophotometer. To avoid cross-contamination, all washes were done manually.

3.3.1.2.2 Calculation of the Sensitivity of ELISA

To calculate the sensitivity of ELISA, a 2 by 2 table was developed. The 2 by 2 table involves a gold standard and the diagnostic test result forming the basis for conventional

determination of sensitivity and specificity (Hajian-Tilaki, 2013). The gold standard establishes the presence or absence of a certain characteristic of interest against which a diagnostic tool's positive and negative test results are compared. In this analysis, protein sequencing data was used as the gold standard against which ELISA LBA status data was matched (Pieper et al., 2017; Tan et al., 2016). From the sequencing data, 9 Mab were LBA positive while only 1 used in this assay was lacked the LAIR1 insert. Although flow cytometry has been done before, we lacked the materials to replicate the assay. Therefore, the alternative was to use sequencing data which was available to us, so were the samples.

Sensitivity, which is also the true positivity fraction and specificity (also termed true negativity fraction) were computed as follows (Hajian-Tilaki, 2013):

$$Sensitivity\ (TPF) = \frac{TP}{TP+FN} \qquad Specificity\ (TNF) = \frac{TN}{TN+FP}$$

3.3.1.2.3 Optimisation of ELISA assay for the screening of LBA

The objective of this assay was to establish the optimal coating concentration of anti-LAIR1 antibody and the corresponding optimal plasma dilution to use in the screening of CHMI samples for the presence of LBA. Table 2 is a template of the plate map used for the assay.

Table 2: Plate map for checkerboard ELISA

		Plasma dilution										Mab (1µg/ml)	Blank
		1/50		1/100		1/200		1/400		1/800			
Coating concentration (µg/ml)	8												
	4												
	2												
	1												
	0.5												
	0.25												
	0.125												
	0.063												

The rows represent the concentration of anti-LAIR1 antibody used in coating the plate while the columns represent the serial dilution of primary antibodies. The assay was conducted on two plates. The coating was the same in both plates, however, different primary antibody preparations were used in the two plates. To the first plate, M58 was used as the plasma sample and MGD21 as the monoclonal antibody (Mab). To the second plate, M158 was the plasma sample and MGD13 the monoclonal antibody. M58 and M158 are LAIR1 positive plasma samples.

All plasma samples were assayed in duplicate except the monoclonal antibodies. All washes were done with 200µl/well of wash buffer. On the first day, coating was done using 100µl of the serial dilution preparation of anti-LAIR1 antibody and the plate incubated overnight at 4^{o}C. On the second day, four washes were done, the plate was blotted on paper towels to dry and blocked with 100µl of 5% skimmed milk in x1PBS in 0.05% Tween20 (blocking buffer) for 5 hours at room temperature. To each well, 50µl of primary antibody preparation was added and incubated overnight at 4^{o}C. On the third day, the plates were washed, 100µl of blocking buffer added and the plates were incubated for 3 hours at room temperature to prevent unspecific binding. The plates were washed and 50µl of antihuman-IgG conjugated to HRP diluted at 1/5000 was added and incubated for 2 hours at room temperature. The plate was washed and OPD added and allowed to develop for 25 minutes before 25µl of 2M sulphuric acid was added to stop the reaction. Absorbance was read at 492 nM.

3.3.2 Screening of CHMI plasma samples for LBA using ELISA

The coating buffer contained 5µg/ml of anti-LAIR1 antibody (Thermofisher) in carbonate bicarbonate (Sigma-Aldrich). The wash buffer was made up of 0.05%Tween-

20 in ×1PBS. The blocking buffer was made by adding 10% skimmed milk to the wash buffer.

The plates were coated with 50µl of 4µg/ml of anti-LAIR1 antibody in coating buffer (Thermofisher) in carbonate bicarbonate (Sigma-Aldrich) and incubated overnight at 4^{o}C. The plates were washed using 200µl/well four times. The wells were blocked with 100µl of blocking buffer for 5 hours at room temperature. After washing, the plates were incubated with 50µl of primary antibody prepared in duplicates at 4^{o}C overnight. The plates were washed and a secondary antibody added and incubated at room temperature for 3 hours. Another wash was done before IgG-HRP was added and the plates incubated for 2 hours to allow them to bind to the IgG in the primary antibody preparations. The final wash was done 4 times, the plate was blotted on a paper towel to dry, OPD was added and developed for 25 minutes. The reaction was stopped with 2M sulphuric acid and the absorbance was read at 492nM.

3.3.3 Defining the breadth of anti-VSA antibody response

Data from a previous yet-to-be-published study was used to define the breadth of anti-VSA antibody response (Kimingi et al *in prep*). The study measured anti-VSA IgG antibodies response to 6 parasite isolates among CHMI study participants using flow-cytometry. Three *ex vivo* isolates (6454, 19462, 19477) were obtained from patients admitted to the High Dependency Unit at Kilifi County Hospital with severe malaria and cultured to mature trophozoites stage before being frozen. The other three (NF54-ICAM1/CD36, SAO75, A4U) were laboratory-adapted. All isolates were cultured in the laboratory per protocol (William Trager & Jensen, 1976) and cryopreserved as mature trophozoites in glycerolyte (Kinyanjui, Howard, et al., 2004).

The median florescent intensity (MFI) was used as the proxy for anti-VSA antibody response. Each MFI was ranked on a scale of 0 to 3 such that 0 meant no response, 1 mild response, 2 moderate and 3 high response. In this study, a plasma sample was considered to have recognised a parasite isolate if it had a score of at least one. Consequently breadth was quantified against the number of isolates recognised yielding a minimum of 1 and a maximum of 6.

3.3.4 Determining prior malaria exposure

Data from anti-schizont antibody response can be used as proxy for prior malaria exposure (Osier et al., 2008). Thus, this study utilized anti-schizont antibody response data from the study conducted by Kapulu and colleagues in 2022. Kapulu et al. (2022) conducted a sandwich ELISA as describe in other studies (Hodgson et al., 2014; Kapulu et al., 2022b; Osier et al., 2008). The study cultured parasite isolate 3D7 to schizont stage, prepared schizont extract and used the schizont extract to coat ELISA plates. The study utilized hyper-immune serum as the positive control and to develop a standard curve against which the Antibody Units (AU) were computed. The study established that anti-schizont antibody response was higher in the high transmission regions (Ahero and Kilifi South) and low in the low malaria transmission region (Kilifi North) (Kapulu et al., 2022b). For this study, we utilised the mean optical density of each of the 142 samples assayed.

3.4 Data storage

Data obtained on FACS Diva was saved in the FCS file format while ELISA data was saved as an Excel spreadsheet. Both sets of data were stored and backed up into a designated KEMRI-Wellcome Trust's data storage servers. All data handling was conducted from within the KEMRI-Wellcome Trust Programme's central server to allow

for automatic backup and prevent loss of data. However, during the COVID-19 pandemic, with limited access to the programme, any additional data was saved on KEMRI's cloud account.

3.5 Data analysis

The preliminary analysis of flow cytometry data was conducted on FlowJo version 10.6.2. Data analysis was performed using R software, version 4.0.3 (R Core Team, 2019), with MFI as the proxy for LAIR1 containing antibodies. Graphs from ELISA output were developed on GraphPad Prism version 8.0.2. The positivity cut-off for ELISA data was 3 standard deviations above the mean OD of negative control samples. To determine the association between LBA levels and the locations of the CHMI participants' residence, the Kruskal Wallis test was used to compare the median OD across Ahero, Kilifi North and Kilifi South. To establish the association between LBA levels and endemicity of malaria of the participants' residence, the Mann-Whitney-U test was used to compare the median LBA OD between the high and low malaria-endemic regions. The Wilcoxon rank-sum test was utilised to assess variation in LBA OD over time (a day before challenge (C-1) and 14 days later (C+14)). To establish the association between LBA and anti-VSA antibody breadth, a linear regression model of mean LBA OD against median MFI of anti-VSA antibody was developed. Spearman's correlation was utilized to establish the correlation between LBA levels and anti-schizont antibody responses.

3.6 Ethical consideration

The CHMI plasma samples used had already received ethical clearance from KEMRI-scientific and ethical review unit (protocol number: KEMRI-SERU 3190) for work that includes this study.

CHAPTER FOUR

RESULTS

4.1 Establishing a high-throughput assay for the screening of plasma samples for LBA

4.1.1 Concentration of monoclonal antibody concentration used as negative and positive controls

For the 11 Monoclonal antibodies assayed, the maximum percentage coefficient of variation (CV) of the optical densities was 20.2%, with only 3 samples having a CV (%) above 15. The mean absorbance of BSA ranged from 1.003 at 2000 µg/ml to 0.367 at 50µg/ml yielding a standard curve illustrated in **Figure *4.1.1*** below. The highest recorded unadjusted mean OD of Mabs was 0.8265 (MGB74) followed by 0.819 (MGD21) and the lowest was 0.5115 (MGD13). The mean OD of the Mabs from these assay were extrapolated against the standard curve to approximate the protein concentration. The estimated protein concentration of the monoclonal antibodies ranged from 127µg/ml (71 – 524, 95% CI) to 794 µg/ml (728 – 869, 95% CI) as summarized in *Table 3* below. MGB74 had the highest protein concentration followed by MGD21, a known LBA positive sample and the lowest was MGD13, a known LBA negative sample. These approximate mean protein concentrations were used when calculating the desired concentrations of the Mab for the remainder of the assay.

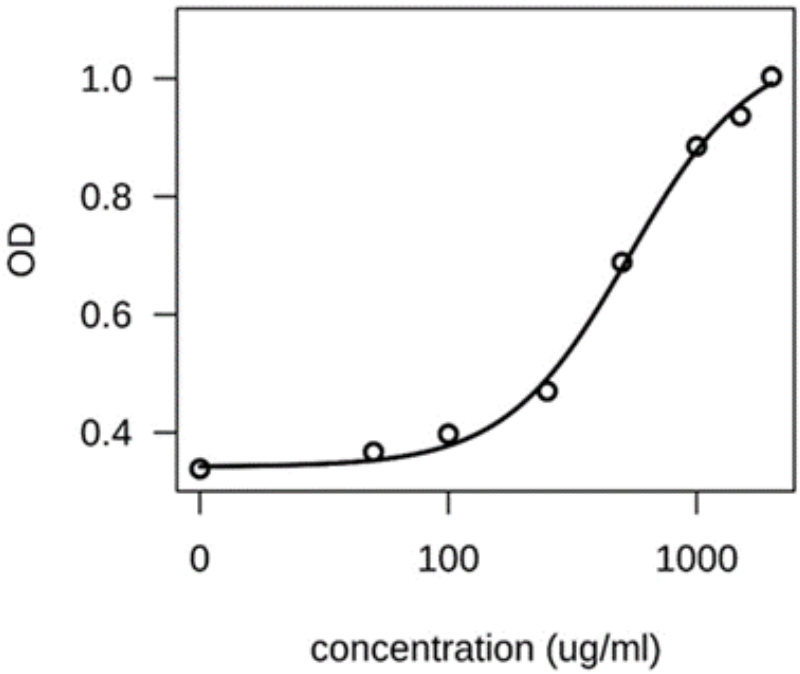

Figure 4.1.1: BSA concentration (µg/ml) against the mean absorbance

Table 3: The mean OD with the corresponding approximate protein concentrations of monoclonal antibodies

Sample ID	**Mean OD**	**CV (%)**	**Concentration (µg/ml) (95% confidence interval)**
BLANK	0.333	0.849378	50 (15 - 121)
MGC26	0.5805	2.314389	367 (336 - 399)
MGB74	0.8265	0.769989	794 (730 - 870)
MGC29	0.5065	18.28845	277 (246 - 308)
MGD39	0.663	5.119325	477 (442 - 515)
MGD21	0.819	7.252377	774 (711 - 847)
MGC28	0.5385	2.494899	316 (285 - 347)
MGC34	0.5295	14.28903	305 (274 - 336)
MGD13	0.3995	1.592982	127 (72 - 168)
MGD55	0.5555	20.23942	337 (306 - 368)
MGD47	0.5585	19.37105	340 (309 - 371)
MGD21 (uca)	0.5115	4.838463	283 (251 - 314)

4.1.2 The sensitivity of flow cytometry in LBA screening

From the assay, when data is normalised by subtracting the LBA positive proportion of UK 5 (Q2 values) from all other Q2 values, monoclonal antibodies (MGD21, and MGB74), and 2 CHMI samples (17K0050 and 17K0003) are positive for IgG that has LAIR1 insert.

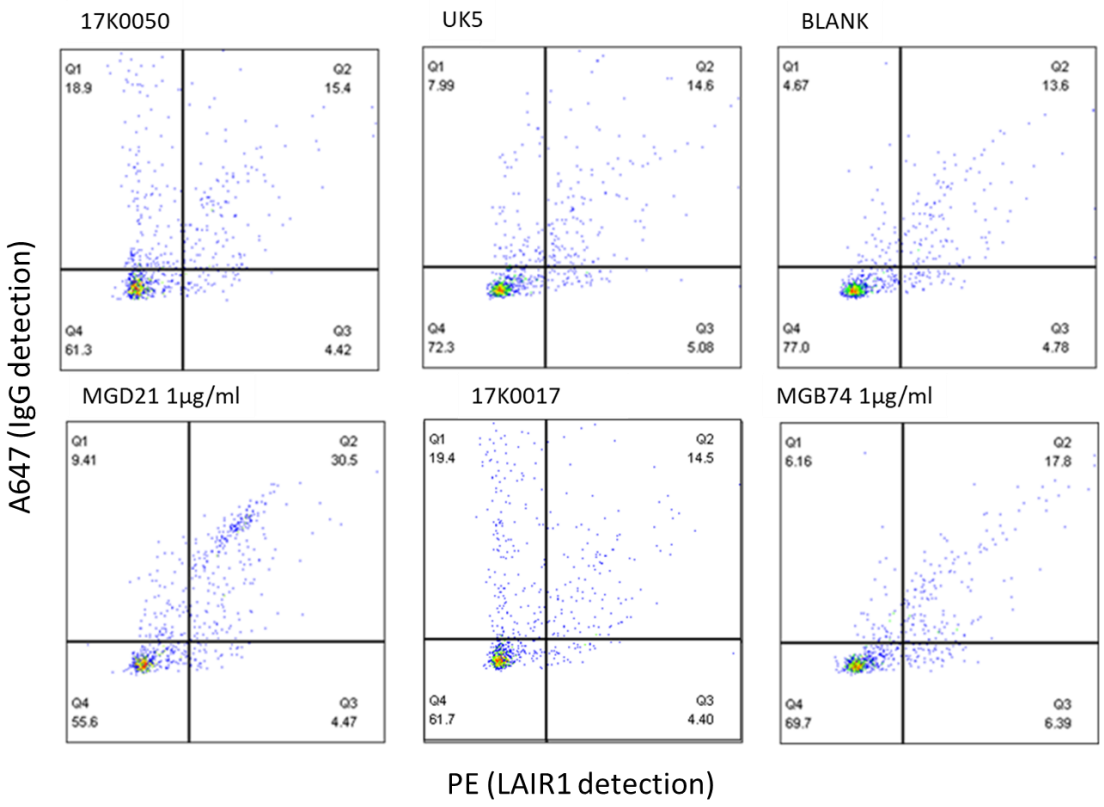

Figure 4.1.2: Gating for IgG that contains LAIR1 insert on flow-jo for selected CHMI sample.

The mid vertical line separates the proportion that lacks the LAIR1 insert to its left (Q1 and Q4) from that with the insert to its right (Q2 and Q3) on the X-axis. On the Y-axis, the mid horizontal line in each box separates the proportion that is IgG negative (below the line, Q4 and Q3) from the proportion positive for IgG (above the line, Q1 and Q2). Therefore, Q2 is the double-positive quadrant composed of IgG with LAIR1 insert.

4.1.3 The sensitivity of ELISA in LBA screening

To test for sensitivity, we used ELISA following optimisation as described in subsection **3.3.1.2**. We found that from the 10 monoclonal antibodies assayed, the CV (%) were all 15% and below. The mean OD of the monoclonal antibodies ranged from 0.15 (MGD55) to 1.7 (MGB74). The positivity cut-off for this assay was 0.2390, only 5 samples were LBA positive while the remaining 5 were LBA negative in this assay (**Figure *4.1.3***)

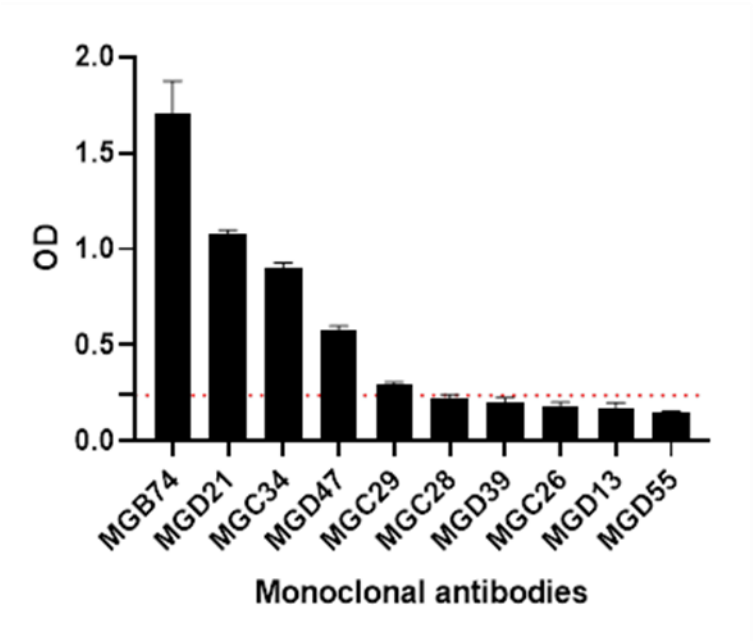

Figure 4.1.3: The mean absorbance in optical density (OD) of monoclonal antibody samples.

The horizontal red dotted line represents the positivity cut off (0.2390).

Four of the LBA negative samples in my assay, MGC28, MGD39, MGC26 and MGD55, have been published before as possessing LAIR1 insert (Pieper et al., 2017; Tan et al., 2016). Thus, this assay yielded 5 true positives, 4 false-positives, 1 true negative and none as false negative as captured in **Table 4** below. Hence, the ELISA assay had a sensitivity of 55.5% and specificity of 100% for monoclonal antibodies.

Table 4: Two by two table for computing sensitivity and specificity

ELISA test results	Sequencing data	
	LAIR-1 insert present (n = 9)	LAIR-1 insert absent (n = 1)
LBA positive (n = 5)	5 (TP)	0 (FP)
LBA negative (n = 5)	4 (FN)	1 (TN)

TP is true positive, FP is false positive, FN is false negative and TN is true negative.

The optimal primary antibody dilution and ELISA plate coating concentration.

In order to establish the best coating concentration, an assay was set with anti-LAIR1 antibody as the coating protein and monoclonal antibodies at 1µg.ml as the primary antibody in subsection 3.3.1.2. The trend in OD between negative control (MGD13) OD and the positive control (MGD21) OD resulted in overt separation of the two lines at 2µg/ml of coating protein and kept increasing as shown in **Figure *4.1.4*** (a). The OD of negative control almost plateaued in coating buffer beyond 4µg/ml. In

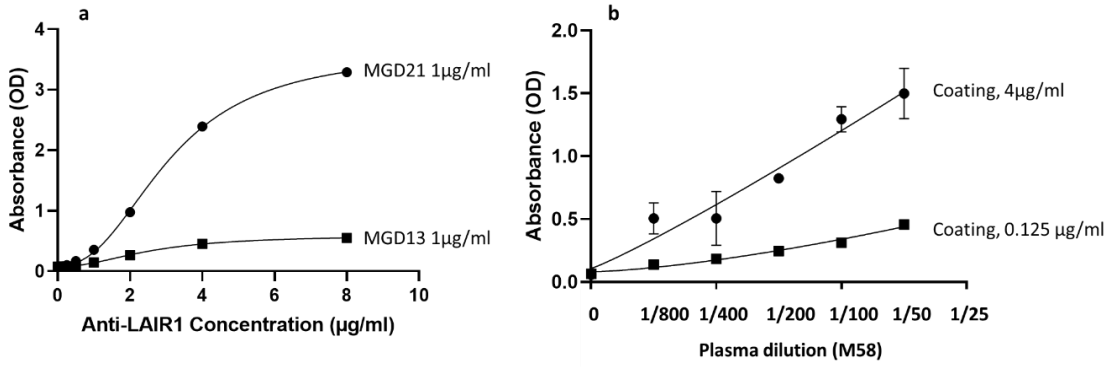

Figure 4.1.4: The optimal coating concentration and the corresponding plasma dilution.

(a) *Association between the mean OD of MGD21 and MGD13 across different concentrations of anti-LAIR1 antibody used to coat ELISA plates. (b) The mean OD of different dilutions of M58 when the plate was coated with either 0.125 µg/ml or 4 µg/ml.*

Plasma negative controls

To screen for the best negative controls for use in the assay, MGD13 was used to establish the positivity cut-off for the malaria naïve samples (mean + 3SD for MDG13, 0.4778) presented in **Figure *4.1.5*** below as the red dotted line. Only 4 out of the 12 malaria naïve plasma samples screened fell below the cut-off line (EU02, SWE7, SWE8, UK7) and were therefore used in the assay as negative controls.

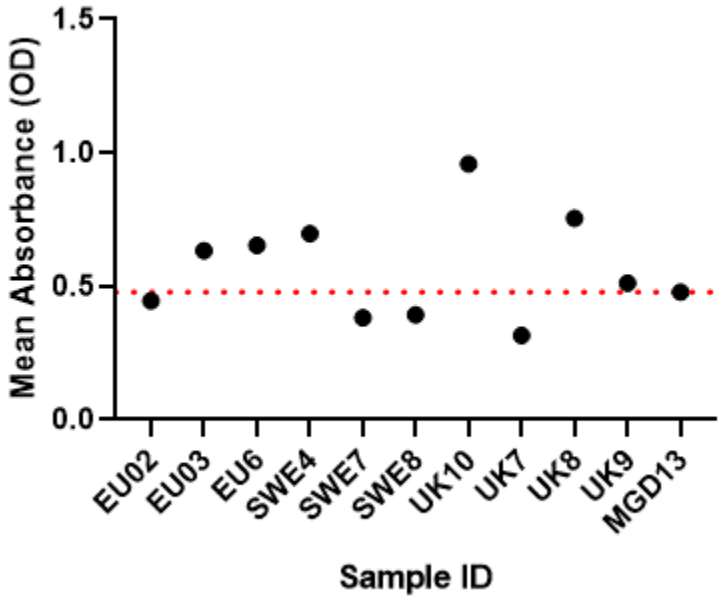

Figure 4.1.5: Malaria naive plasma samples against mean OD

The red dotted line is positivity cut off line computed from the mean +3SD of MGD13 OD. Only 4 of the samples have a mean OD below the positivity cut-off.

The distribution of CHMI LBA data obtained by ELISA

The density plots in **Figure *4.1.6*** were constructed to visualise the distribution type of the data obtained from screening CHMI samples for LBA using ELISA. From the graphs, skewed to the left even when log transformed. Therefore, the data was not normally distributed (Shapiro test, $P<0.0001$).

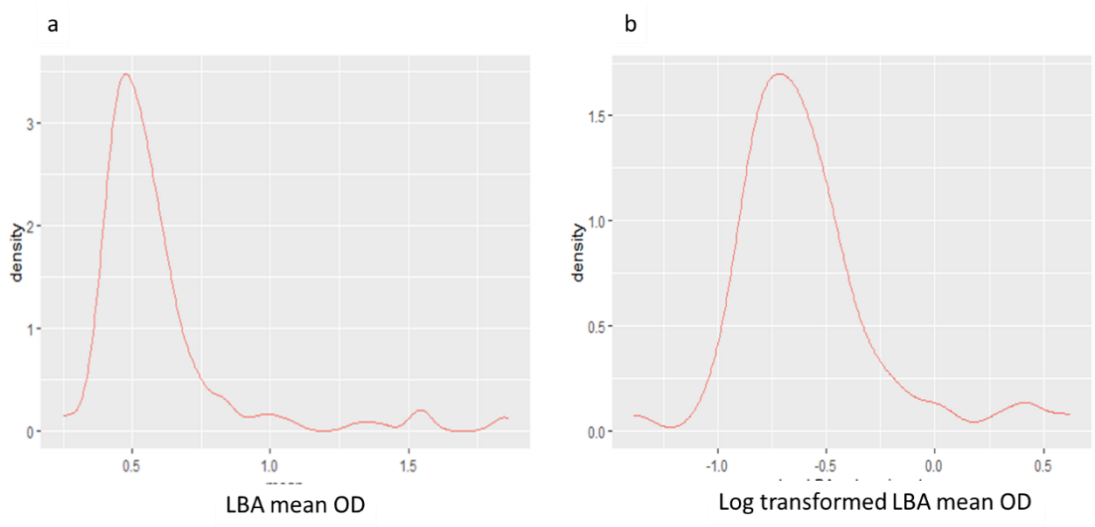

Figure 4.1.6: Density plots

(A – normal, B - log-transformed) of mean OD of the LBA from the screening of CHMI samples. The Y-axis display the concentration of mean OD whereas the X-axis illustrates the intervals of mean OD. The peak of the plot contains the highest number of CHMI samples with a given OD – about 0.5 in both cases.

4.2 Sero-prevalence of LBA among CHMI plasma samples

Positivity cut-off for LBA in CHMI samples was set at three standard deviations above the mean of the selected negative controls (0.6059, the red dotted line in **Figure *4.2.1***). Only 39 (27.46%) out of the 142 CHMI samples analysed were LBA positive.

4.2.1 Association between LBA and location/endemicity of malaria of participants' residence

LBA seropositivity was 13.3% (2/15), 31.2% (29/93) and 23.5% (8/34) in Ahero, Kilifi South and Kilifi North respectively. Therefore, most of the seropositive samples, 31 (79.5%) were from high endemicity areas – Ahero and Kilifi South while only 8 (20.5%) were from Kilifi North which has low endemicity of malaria.

A summary of the established association between LBA seroprevalnce and the area of residence, endemicity are captured in **Figure *4.2.2***. There was no significant difference in the median LBA ODs of participants from across the three locations (Kruskal Wallis test *P*-value = 0.7289) (**Figure *4.2.2* a)**. Similarly, there was no association between LBA (OD) and endemicity (Mann-Whitney test, *P*-value =0.9915) (**Figure *4.2.2* b**). Additionally, we established that the participant's location of origin and LBA status, are independent of each other (Chi-squared test, *P*-value =0.615) (**Figure *4.2.2* c**).

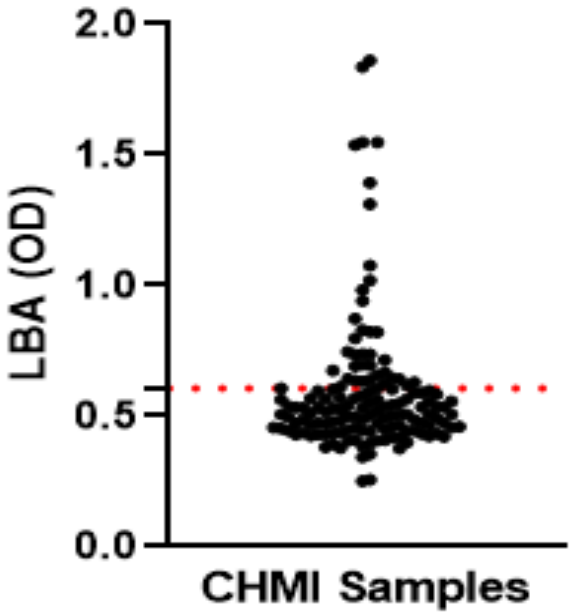

Figure 4.2.1: CHMI plasma samples against mean OD of LBA.

The red dotted line is positivity cut off line computed from the mean +3SD of malaria naïve negative controls.

4.2.2 Association of LBA with CHMI outcomes

Overall, the median OD were almost equal across the 4 CHMI outcome groups: 0.4992 (n=32), 0.5415 (n=27), 0.5422 (n=16), and 0.5060 (n=27) in the PCR-negative, untreated PCR positive, treated non-febrile and treated febrile groups respectively. The median OD score was not significantly different across the CHMI outcome groups (Kruskal Wallis

test *P*-value = 0.5043 and Mann Whitney test, *P*-value = 0.6415) (**Figure *4.2.3*** a and b, respectively).

Twenty-five (64%) of the 39 LBA positive samples were from untreated participants with 17 (43.5%) being in the PCR positive group and 8 (20.5%) were PCR-ve. The treated were equally distributed between the febrile and non-febrile groups having 7 (18%) from each group. There was no association between 4 CHMI outcome group and LBA status (LBA positive and negative groups) (Chi-squared test *P*-value = 0.9610) (**Figure *4.2.3***

c).

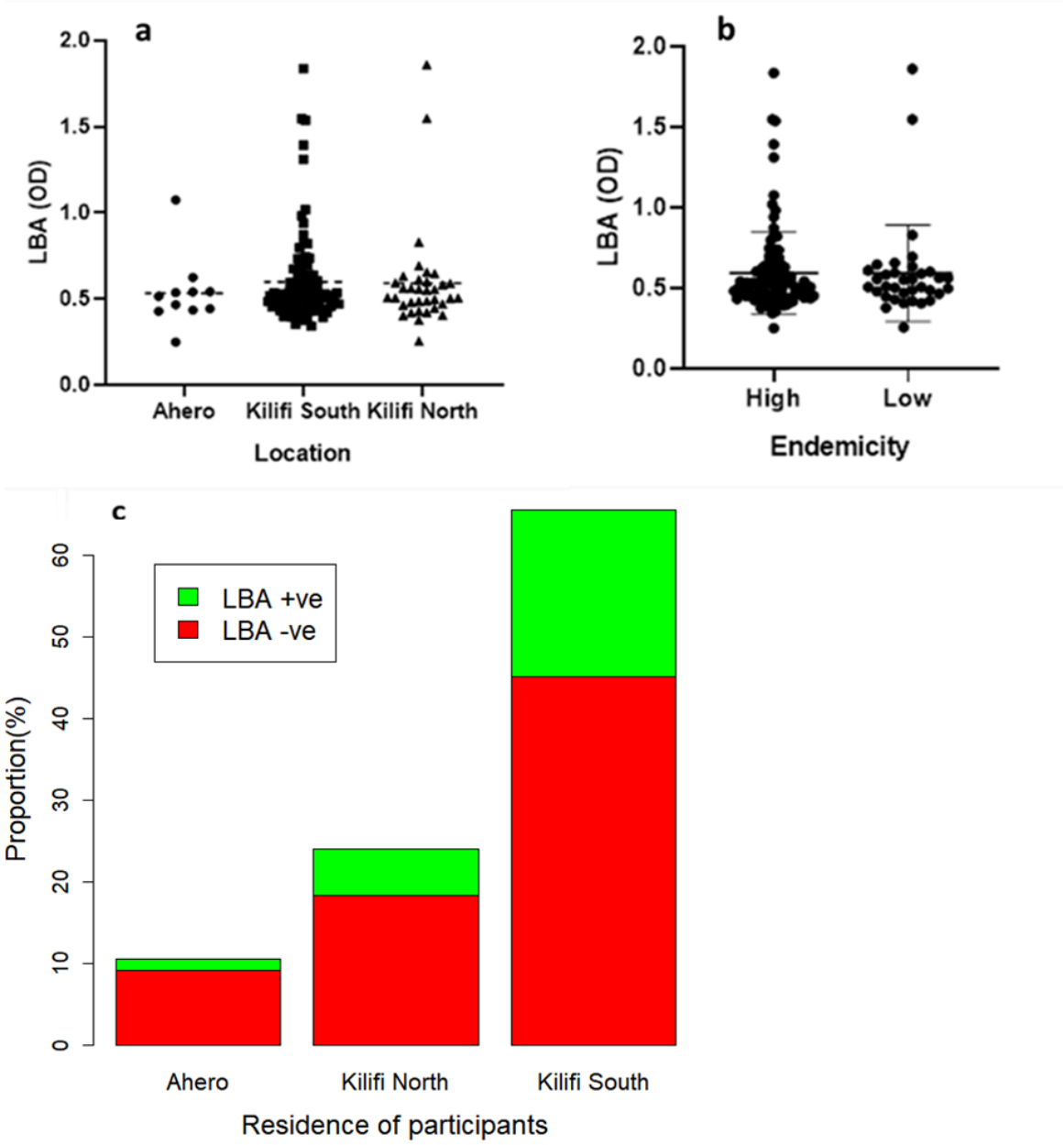

Figure 4.2.2: Association between mean absorbance of LBA and location or endemicity of participants' residence (figures a and b respectively).

Figure C shows the proportions positive or negative for LBA in each location of participants' residence.

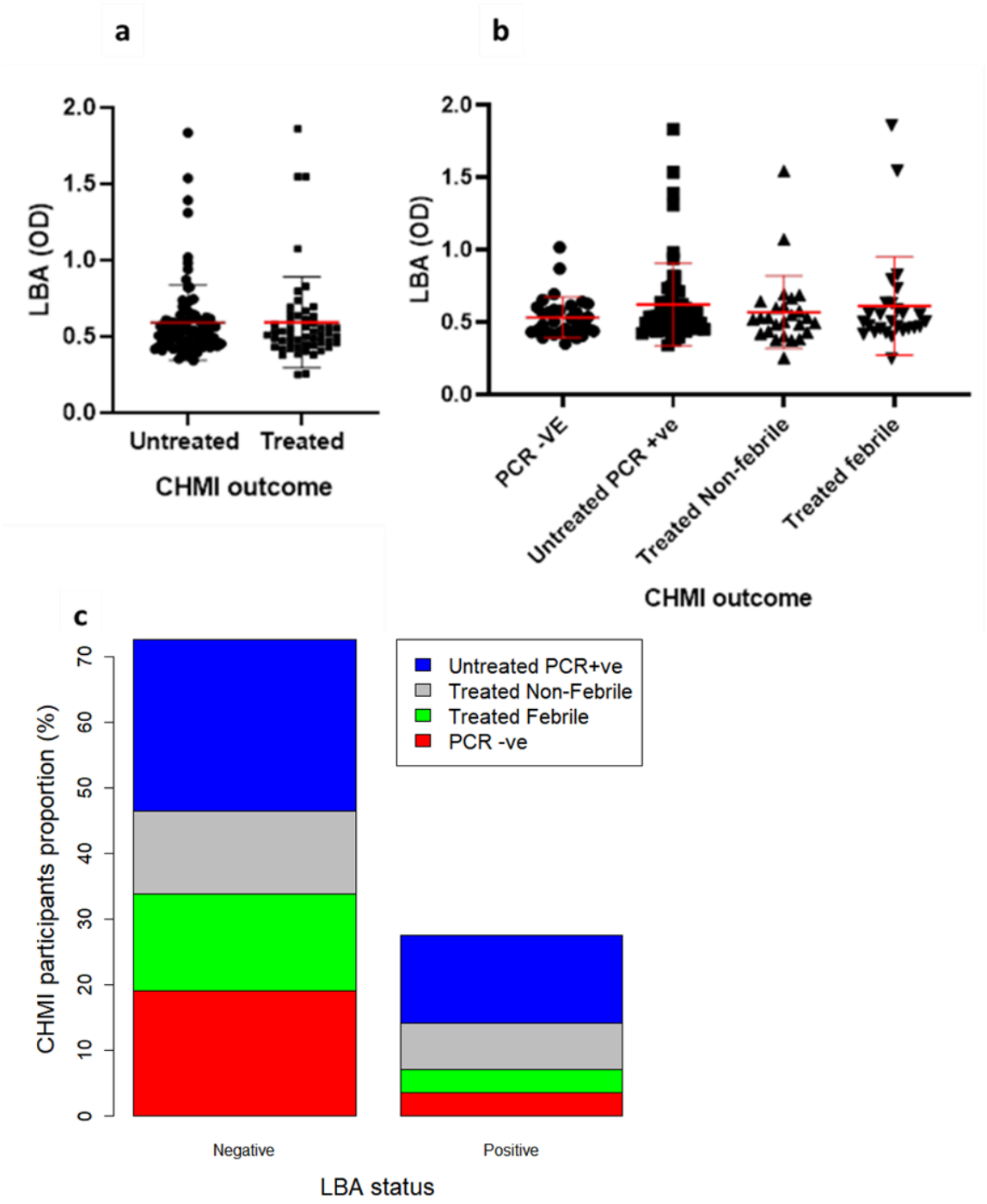

Figure 4.2.3: Association between the mean absorbance of LBA (LBA OD) and CHMI outcomes (figures a and b) as well as the association between CHMI outcomes and LBA status

4.2.3 Change in LBA levels over time

The mean OD of LBA did not change much over time. However, the proportion positive declined from 29.7% (38/128) to 27.3% (35/128) over 14 days. From the 128 samples, 68 had a reduction in OD, 2 maintained while 8 had an increase. Out of 38 LBA positives, 23 had a decline (range, 0.7% to 23.9%) resulting in the loss of 10 seropositive samples to seronegativity. On the other hand, 44 seronegative samples demonstrated boosting (range, 0.8% to 94%) and resulted in 7 of them becoming LBA positive. The median value at C-1 was 0.5218 (IQR, 0.4651 – 0.6200) which slightly increased by the 14th day after the challenge (C+14) to 0.5317 (IQR, 0.4550 – 0.6200). While there was a slight increase in the median absorbance over time, this difference was not significant (Wilcoxon rank-sum test, *P*-value = 0.6852) (**Figure *4.2.4***).

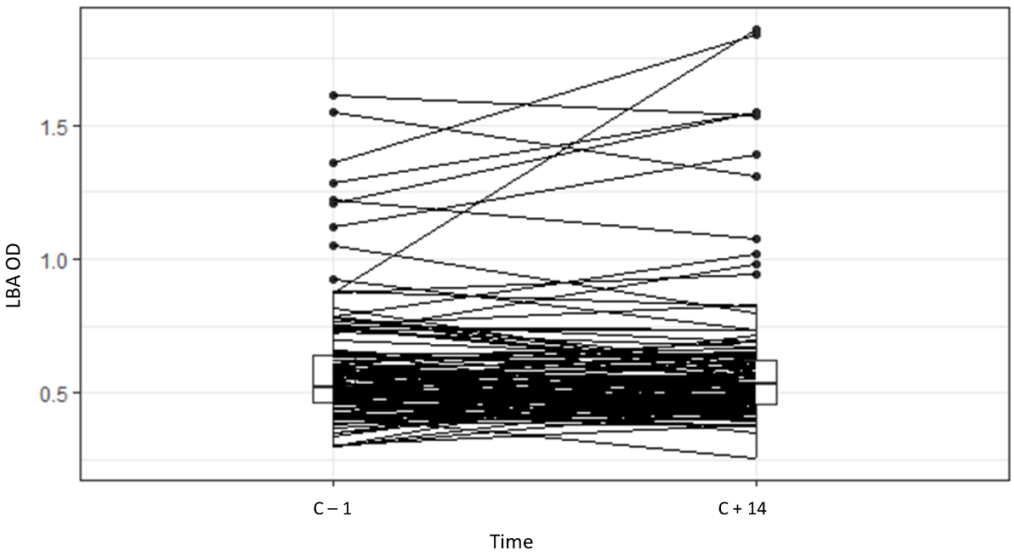

Figure 4.2.4: Change of the mean absorbance of LBA (LBA OD) from a day prior to challenge (C-1) to 14 days post-challenge (C+14).

4.3 Association between LBA and anti-VSA antibody breadth

The median MFI of anti VSA antibodies to 6 isolates was computed and used in a linear regression model to establish the correlation between and individuals mean OD of LBA

and their median MFI. **Figure *4.3.1*** below demonstrates an intercept of 181.14 (± 39.06) with a weak but not significant negative correlation between LBA levels and anti-VSA antibody breadth (slope -41.14 ± 60.24, *P*-value = 0.4957). Further, the median MFI was higher among the LBA negative samples than the LBA negative (89.25 versus 93.66). However, the difference in the median MFI between the two groups was not statistically significant (Mann-Whitney U test, *P*- value = 0.6385) illustrated in **Figure *4.3.2***. Further, the median number of parasite isolates recognised in the LBA positive and negative groups were equal (**Figure *4.3.3***) (median= 5, Mann Whitney-U test, *P value* = 0.2807). Except for the low intensity registered across LBA negative samples with the lowest OD, there was no definite pattern in the intensity of anti-VSA antibody response as the levels of LBA increased (**Figure *4.3.4***). However, there was an increase in the number of parasite isolates recognised as the optical density of LBA increased (Spearman correlation, S = 387725, p-value = 0.02547 and rho = 0.1875) as shown in **Figure *4.3.5***.

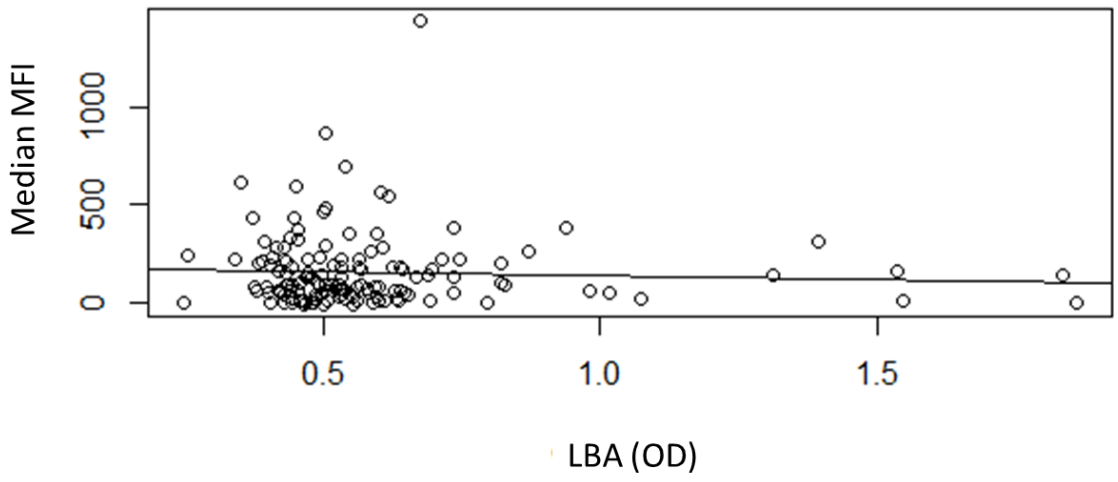

Figure 4.3.1: Correlation between mean absorbance of LBA and the median MFI for anti-VSA antibodies to 6 parasite isolates.

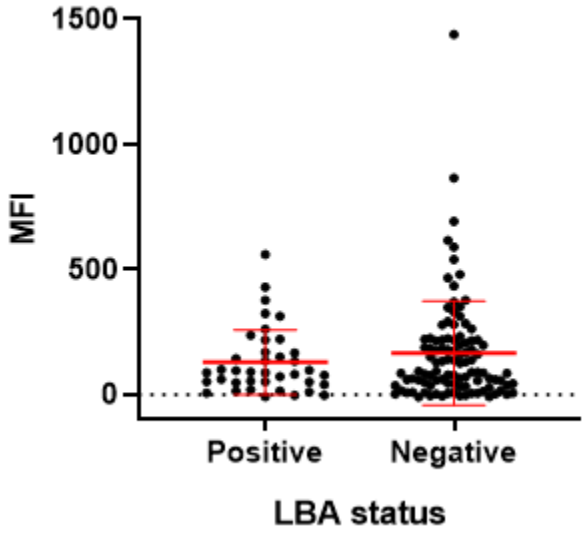

Figure 4.3.2: LBA status against levels of anti-VSA antibody response (MFI)

The CHMI plasma samples were placed into the LBA positive and negative groups based on positivity cut-off on the X axis. The median value of median florescent intensity (MFI) across 6 parasite isolates for each plasma sample was recorded along the Y axis.

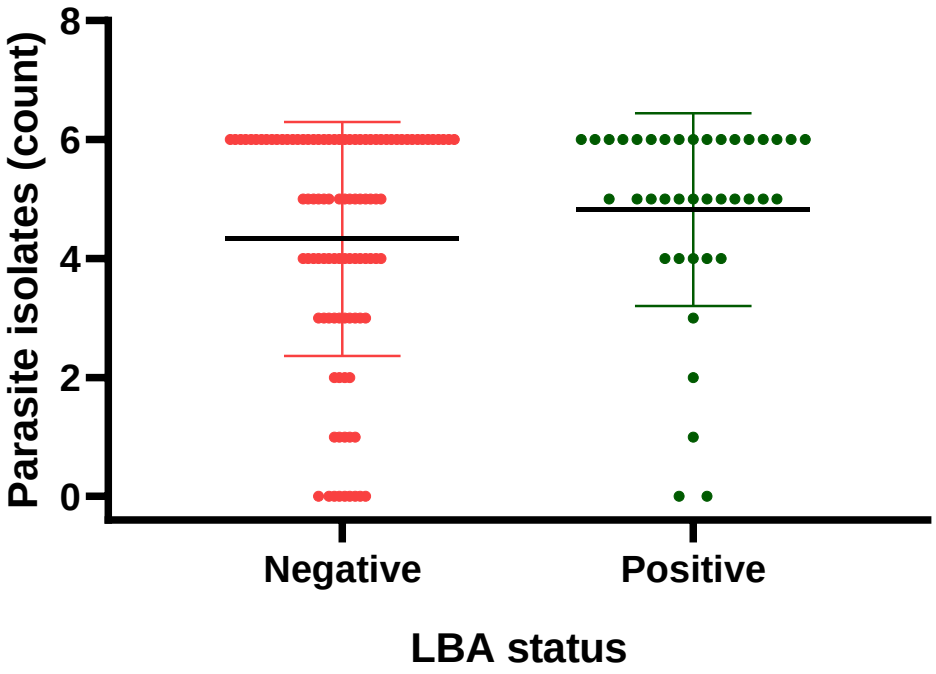

Figure 4.3.3: LBA status against number of plasmodium parasites recognised

Along the X axis, CHMI samples were grouped as either LBA positive or negative based on the set positivity cut-off. The Y axis represents the number of parasite isolates. Thus, each dot on the plot represents a CHMI sample and the corresponding number of isolates it recognised.

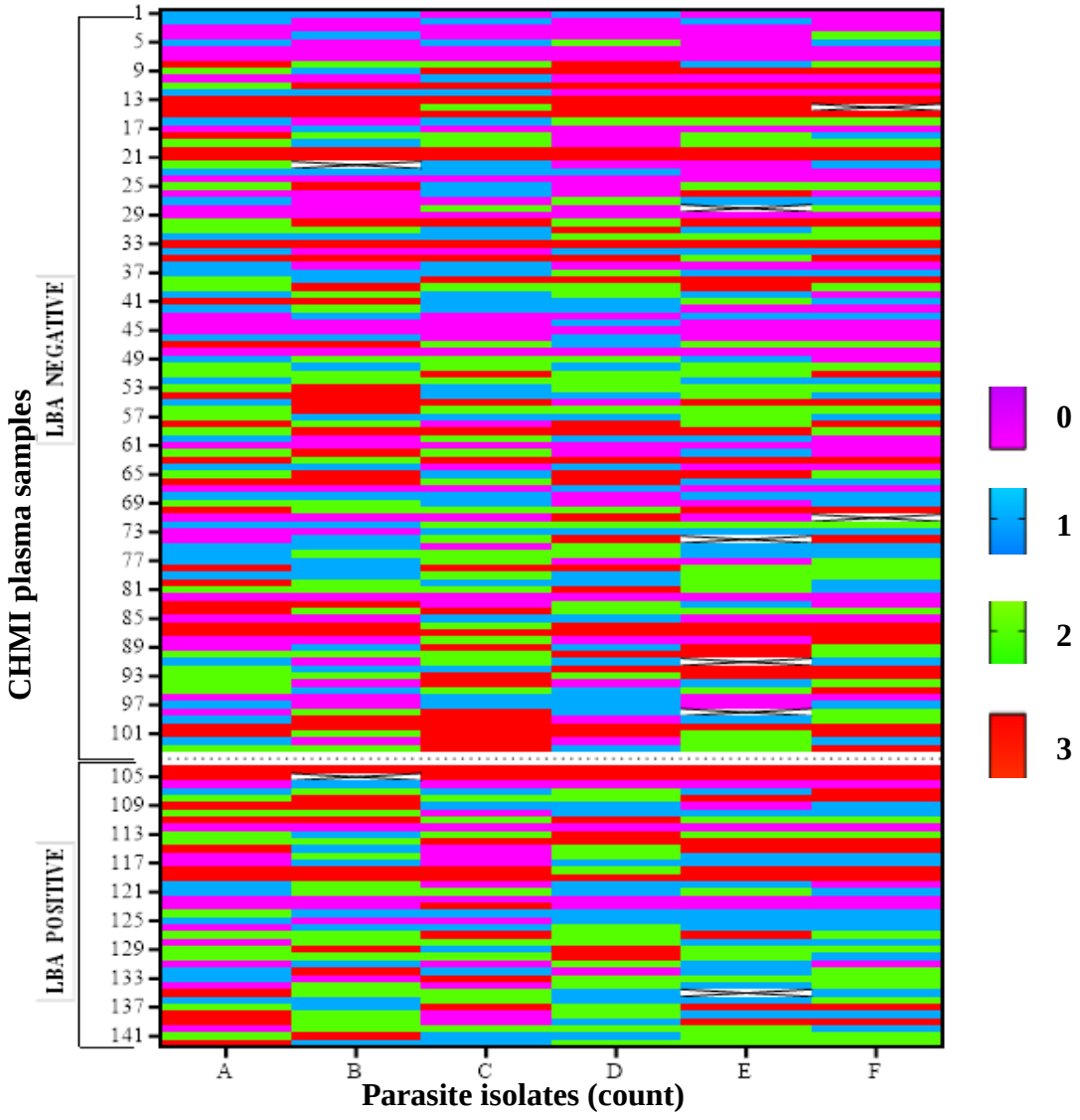

Figure 4.3.4: The intensity of anti-VSA antibody response across 6 parasite isolates

The Y axis represents CHMI plasma samples (N=142) arranged according to the mean absorbance of LBA in descending order (the lowest and highest absorbance are 0.25 and 1.86 for CHMI plasma samples 1 and 142 respectively). The white line is the positivity

cut-off separating LBA negative samples, above from LBA positive samples below the line. The X axis represents 6 parasite isolates screened by flow cytometry. A *to* F *are different parasite isolates: (A=19462 ,B=19477 , C=A4U , D=NF54 , E= 6454 and F= SAO75). The different colors indicate the different intensities of anti-VSA antibody response graded as quatiles. Purple = 0 (no anti-VSA antibody response), Blue = 1 (mild anti-VSA antibody response), Green = 2 (moderate anti-VSA antibody response) and red = 3 (strong anti-VSA antibody response).*

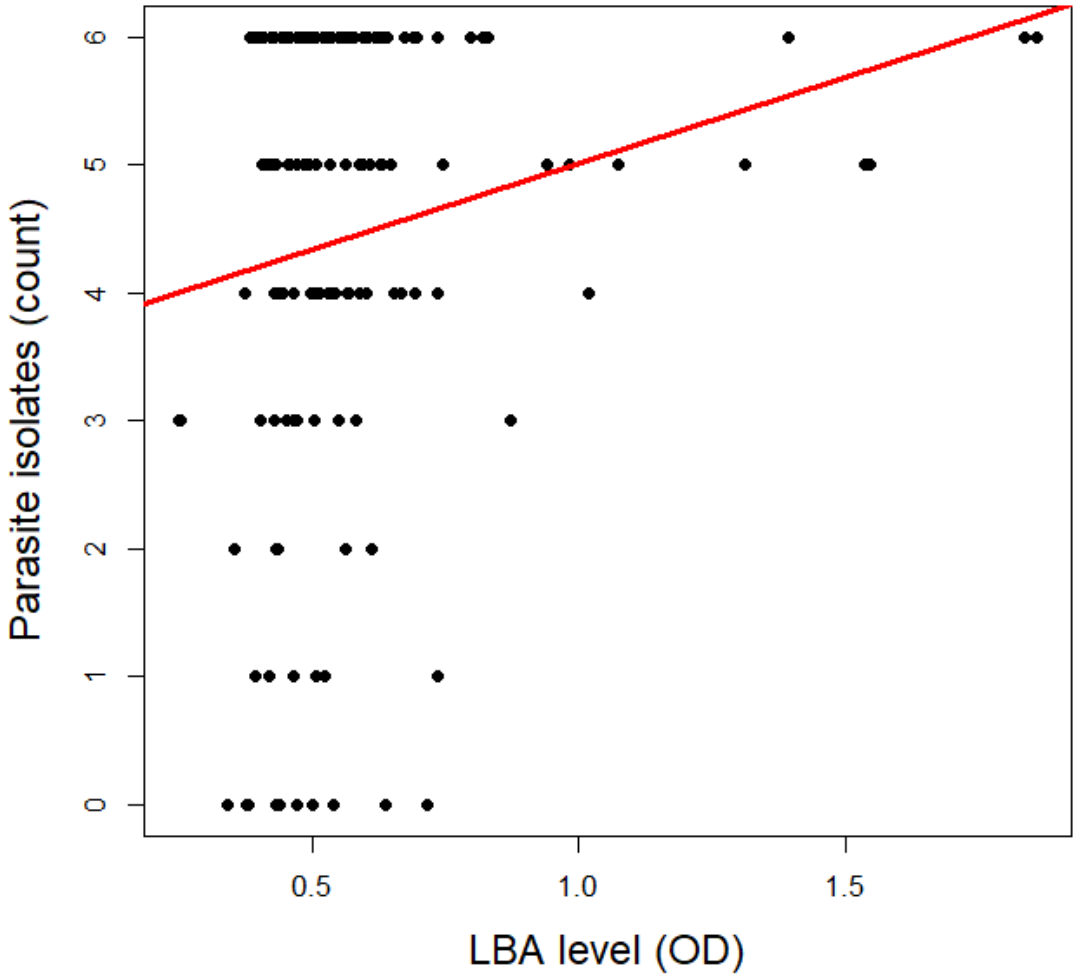

Figure 4.3.5: Correlation between LBA levels and the number of parasite isolates recognised

The red line represents the correlation line.

4.4 Association between LBA and past exposure to malaria

Anti-schizont antibody levels measured by ELISA were used as a proxy for past exposure. There was statistically significant weak positive correlation between participant's anti-schizont antibody levels and mean LBA levels in **Figure *4.4.1***. Spearman (rho) correlation (S = 552730, rho (spearman correlation coefficient) = 0.260821, *P*-value = 0.001718). Thus, this study suggests a positive correlation between prior exposure to malaria and the development of LBA among the study participants

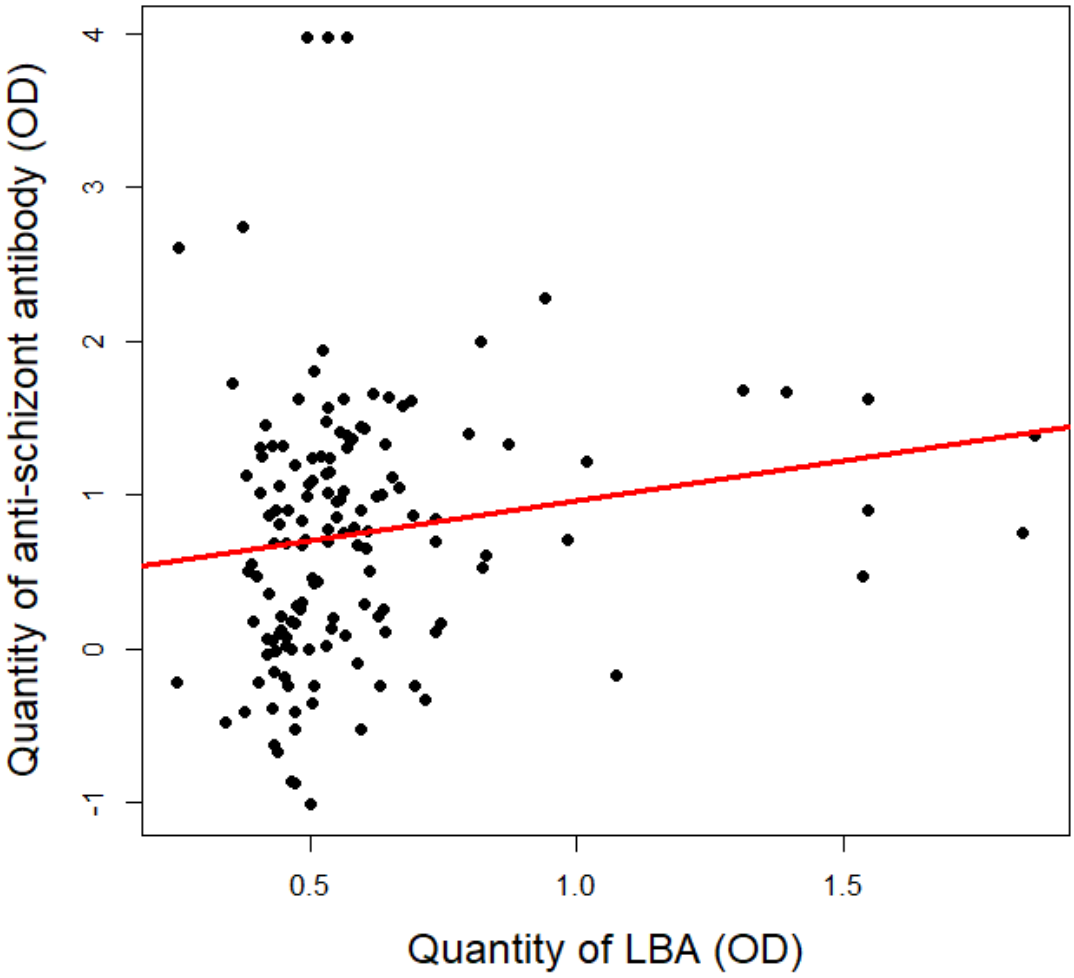

Figure 4.4.1: LBA against anti-schizont antibody levels.

The mean OD of LBA was plotted against the mean OD of anti-schizont antibody response of the same CHMI plama samples.

CHAPTER FIVE

DISCUSSION

5.1 Findings and implications

The overall aim of the study was to establish the role of LBA in immunity against malaria in a CHMI model. It used CHMI plasma samples because the participants who had different natural exposure but treated injected with an equal dose of a known parasite isolate and the outcomes were well-defined. Specifically, this study aimed to examine the association between the LBA antibody and CHMI outcomes, endemicity of malaria of participant's location of residence of study participants, anti-VSA antibody breadth and prior malaria exposure.

Sandwich ELISA was validated as a method for screening for LBA since this method has not been applied before. The assay allowed for high-throughput screening for LBA. A potential challenge with this method is the possibility of steric hindrance interfering with signal generation (Butler et al., 1986). This challenge was circumvented by fact that the anti-LAIR1 antibody binds the LAIR1 insert which is situated in the Fab region while the rabbit-antihuman IgG binds the Fc region of the primary antibody (Hsieh & Higgins, 2017; Pieper et al., 2017).

The data was not normally distributed. Non-normally distributed data is transformed to reduce skewedness. If skewed to the right, the alternatives for transformation are logarithmic transformation. On the other hand, square or cubic transformation is recommended for data skewed to the left (Betty & Sterne A. C. Jonathan, 2003; Lee, 2020). My log-transformed data remained skewed to the left with statistically significant lack of normal distribution. Consequently, the study settled for non-parametric analysis.

The seroprevalence of LBA among the CHMI samples was higher than those reported before. The earlier studies using samples from Mali and Tanzania found a prevalence of

between 5 and 10% with only about 0.3% (3/1043) in the European samples (Pieper et al., 2017). Unlike Kenya, Mali and Tanzania featured among the 11 countries that contribute to 70% of the global burden of malaria (WHO, 2020). While the regions with substantially higher LBA prevalence are malaria endemic, our study demonstrated lack of association between malaria exposure and LBA levels. In addition, this study used sandwich ELISA whereas Pieper et al. (2017) performed flowcytometry using microbeads. The relative sensitivity of these two assays in LBA screening remains unexplored. In order to have comparable results, future studies should consider direct comprison of these two methods.

Our findings suggest that there may be no impact of malaria endemicity on LBA seroprevalence since there was no difference in the median OD of LBA between the high and low endemicity regions. Moreover, there was no association between endemicity of participants' residence and proportions positive for LBA. Since the high endemicity of malaria did not drive LBA development, the study leaves room for the investigation of other factors in malaria exposed regions that result in LBA development.

This study established LBA as a broadly reactive antibody that is not protective against malaria. The study established no association between LBA and CHMI outcomes. While the highest median OD was recorded among the treated non-febrile, the lowest was in the PCR negative group. Noteworthy, more LBA positive samples were from the untreated group than the treated group with the majority being PCR positive. In agreement with Pieper (2017) LBA is not protective against parasitemia or fever in malaria among the CHMI participants. The breadth of antibody responses to blood stage parasites are predictive of protection against malaria (Osier et al., 2008). The study established an increase in breadth with increasing LBA levels. However, there was no definite pattern in the intensity of recognition of parasite isolates by the anti-VSA

antibodies. Overall, the median LBA levels remained the same over 14 days of the CHMI study. However, 10 of the 39 LBA positive samples had a decline in the OD so that they became seronegative while 7 out of the initially LBA negative samples demonstrated boosting such that they shifted to sero-positivity. The 10 samples may have reverted to seronegativity because of a decay in antibody levels over time. On the other hand, the LBA negative samples that became seropositive might be those that recognised the subset of RIFINs on the NF54 parasite isolate used in the CHMI study.

5.2 Limitations recommendations and conclusion

The challenge with flow-cytometry was that the proportion of plasma samples that are positive for LBA varied with parasite isolates since the LBA recognise only a subset of RIFINs which vary from one parasite isolate to another (Tan et al., 2016). Additionally, this recognition pattern would demand different positive controls for each parasite isolate. The alternative, therefore, was to use a method to which a common positive control would be applied across all samples hence sandwich ELISA. The limitation with the ELISA assay is that it did not include parasite antigens. Consequently, there is no evidence that the LBA measured are necessarily specific to *P. falciparum*.

The challenge with how sensitivity was established in the ELISA assay was that only 10 monoclonal antibodies were used because they were the only available LBA confirmed samples. Additionally, the positivity cut-off was arbitrarily set at 3SD above the mean of negative controls (Hajian-Tilaki, 2013). Moreover, there were no reagents for two-determinant microbead assay on flow cytometry, the only LBA screening method published so far (Pieper et al., 2017).

CHAPTER SIX

RECOMMENDATIONS AND CONCLUSION

For validation of ELISA as a tool for the screening for LBA among CHMI samples, flow cytometry using microbeads assay as the gold standard can be done and compare with the ELISA output of the same samples. About 100 CHMI plasma samples can be used for the determination of the accuracy of ELISA using receiver operating characteristics curve to circumvent the effect of positivity cut-off on the measure of accuracy (Hajian-Tilaki, 2013).

To establish whether the LBA detected are parasite-isolate specific, first, the flow cytometry assay described in this study should be optimised. Thereafter, a panel of parasite isolates will be co-incubated with LBA positive CHMI plasma samples for the optimised assay. The success of this assay will require that appropriate positive control is selected for each parasite isolate screened. For instance, MGD47 can be used as the positive control for parasite isolates 9106, 9605, 11019, 9215, 9775, 10975, 10936 and 11014 (Tan et al., 2016). The negative controls will include EU02, SWE07, SWE08 and UK07 which are malaria naïve samples available in our laboratory and established LBA negative in our assay as well as published by Pieper et al (2017).

In conclusion, nearly a third of the CHMI study participants had LBA and there was a significant increase in levels of LBA with exposure to malaria. However, there seems to be no association between LBA positivity with CHMI outcomes, the malaria endemicity of participants' residence and anti-VSA antibody breadth.

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APPENDICES

Appendix 1.0 Funding statement

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Appendix 2.0 Ethics clearance certificate

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KEMRI/RES/7/3/1 May 02, 2019

TO: **PROF. PHILIP BEJON,**
PRINCIPAL INVESTIGATOR

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

DIRECTOR CENTRE FOR GEOGRAPHIC MEDICINE RESEARCH, COAST

Dear Sir,

RE: **SERU 3190(*REQUEST FOR ANNUAL RENEWAL*): CONTROLLED HUMAN INFECTION (CHMI) TO ASSESS HUMAN IMMUNITY TO *P. FALCIPARUM* USING SPOROZOITES ADMINISTERED BY DIRECT VENOUS INOCULATION**

Thank you for the continuing review report for the period **April 24,2018** to **March 29, 2019.**
The Scientific and Ethics Review Unit (SERU) Secretariat acknowledges receipt of the following documents:

1. Continuing review report
2. Current approved protocol: SERU submission version 5.0 dated 09.08.2017
3. The last SERU approval letter (including all study amendment approval letters)
4. OXTREC annual progress report and current approval letters
5. Manuscript submitted for publication

This is to inform you that during the 286th Committee C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **April 17, 2019,** the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from **May 16, 2019** through to **May 15, 2020.** Please note that authorization to conduct this study will automatically expire on **May 14, 2020.** If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU by **April 2, 2020.**
You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

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

Centre for Geographic Medicine Research RECEIVED 07 MAY 2019 Tel: 0709983676/77 P.O. Box 428, Kilifi

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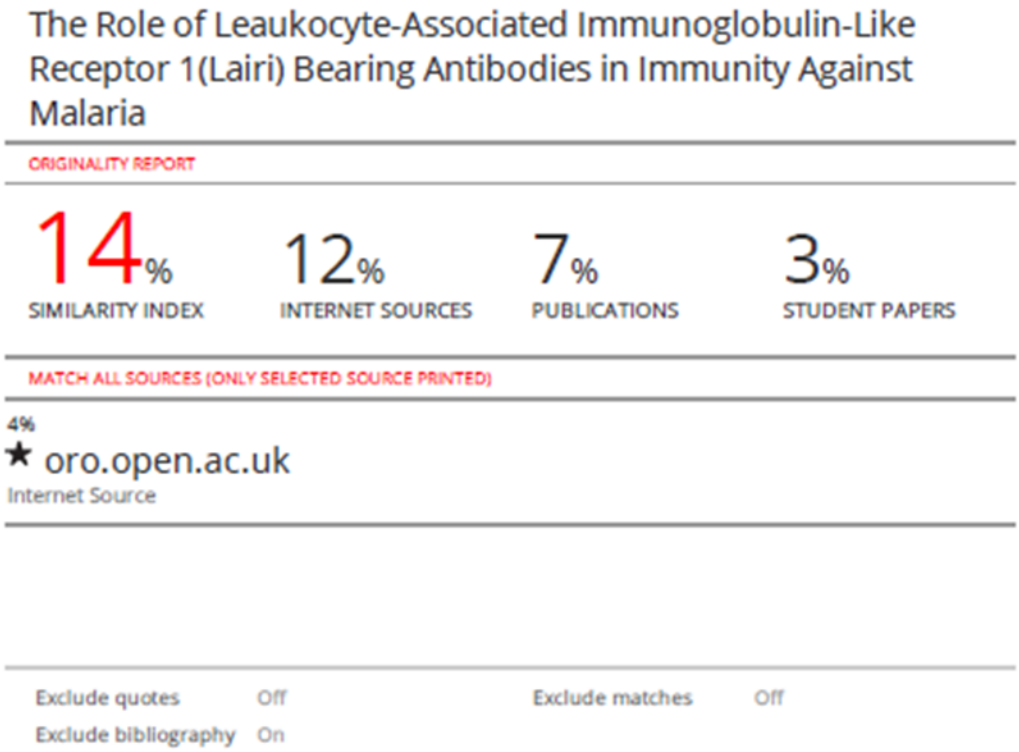

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