Identification and characterization of immunological responses induced by vaccination with R21/Matrix- M, using the controlled human malaria infection (CHMI) model in semi-immune adults.

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Elizabeth Kauchi Kibwana
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Abstract

Malaria remains a public health concern, and the development of a malaria vaccine remains a priority for reducing the global burden. A second-generation vaccine called R21 has recently been licensed by WHO for use in preventing malaria in children. It is a circumsporozoite protein-based particle vaccine with a higher proportion of CSP compared to the first recommended malaria vaccine RTS, S/AS01. I hypothesize that vaccination with R21/Matrix-M will induce improved CSP responses, leading to better controlled human malaria infection (CHMI) outcomes in semi-immune volunteers. The objective was to characterize immune responses to R21 and identify potential correlates of vaccine-induced protection in Kenyan adults using the CHMI model.

An extensive review provided an overview of CHMI research in Africa, focusing on vaccine efficacy and effects of naturally acquired immunity. I then worked on validating an existing qPCR assay for measuring parasite densities, as an outcome measure, following CHMI. Validation work showed that smaller extraction volumes <500µl, improved assay performance but larger volumes of 1000 µl decreased reproducibility.

I conducted several immunological assays to determine the quantity, quality, and durability of antibody responses. Here I demonstrated high, durable antibody titres to different epitopes indicating broad and diverse responses with complement fixing capabilities potentially enhancing neutralization ability against the parasite. Also, vaccination elicited a variety of antibody isotypes. Indicating a multifaceted immune response that could contribute to enhanced protection from infection.

Cellular responses investigation indicated sustained acquisition of memory B cells to both R21 protein and C-terminus. As well as an association between T follicular helper cells and memory B cells to R21 and a predominant Th2/Th17 population. Lastly, I observed that the route of challenge plays a major role in determining protective outcome following challenge.

The findings highlight comprehensive immunogenicity generated by R21/MM which is crucial in developing effective strategies against malaria.

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Dedication

To my parents Miriam and Joseph Kibwana

Author's Declaration

I declare that, except when reference is made to the contributions of others, this thesis is the result of my work and has not been submitted for any other degree at the Open University or any other institution. All work pertaining to the VCA074 clinical trial was conducted by the clinical trial study team, clinicians and research nurses including clinical care, vaccination, sample collection, and sporozoite challenge. The laboratory clinical team conducted sample processing, and qPCR testing. Hilary Kiprono, the study's principal statistician, analysed the qPCR data, which was kindly provided by Prof. Melissa Kapulu (Chapter 6).

List of Publications

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Elizabeth Kibwana, Domtila Kimani, Nick J Edwards, Kelvias Keter, Agnes Mutiso, Lydia Nyamako, Rose Gatheru, Adrian V S Hill, Philip Bejon, Katie J Ewer, and Melissa C Kapulu Quantification of *Plasmodium falciparum*: validation of qPCR assays for detection of parasites in controlled human malaria trials – awaiting submission **(chapter 3)**

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List of Abbreviations

°C - Degree Celsius
95% CI - 95% Confidence Intervals
ACT - Artemisinin Combination Therapy
AMA - Apical Membrane Antigen
APC - Antigen-Presenting Cell
AS - Adjuvant System
ASC - Antibody Secreting Cell
BCR - B cell receptor
BSV – Blood stage vaccine
CD - Cluster of Differentiation
CDC - Centre for Disease Control
ChAd - Chimpanzee Adenovirus
CHMI - Controlled Human Malaria Infection
CoP - Correlate of Protection
COVID-19 - Coronavirus Disease 2019
C+ - challenge plus
CSA - Chondroitin sulphate A
CSP - Circumsporozoite Protein
D - Day
DAPI - 4',6-diamidino-2-phenylindole
DC - Dendritic cell
DNA - Deoxyribonucleic acid
DVI – Direct venous injection
ELISA - Enzyme-Linked Immunosorbent Assay
ELISpot - Enzyme-Linked Immunosorbent Spot
AU – Arbitrary ELISA unit
Fab - Fragment antigen binding
FACS - Fluorescence-Activated Cell Sorting
FC - Fold Change
FCS - Foetal Calf Serum
FFS - Forward Scatter
GC - Germinal Centre
GCP - Good Clinical Practice
GFP - Green Fluorescent Protein
GMT - Geometric Mean Titre
GPI - Glycophosphatidylinositol
HI - Heat-inactivated
ID - Intradermal
IFN - Interferon
Ig - Immunoglobulin
IL - Interleukin

IM - Intramuscular
IQR - Inter-Quartile Range
iRBC - infected red blood cell
ISI - Inhibition of Sporozoite Invasion
ITN - Insecticide Treated Nets
IV - Intravenous
KEMRI - Kenyan Medical Research Institute
Kg - Kilogram
LOD - Lower Limit of detection
LLOQ - Lower Limit of quantification
LLPC - Long-Lived Plasma Cell
LSA-1 - Liver Stage Antigen 1
MAC - Membrane Attack Complex
MBC – Memory B cells
mCoP - mechanistic correlate of protection
ME-TRAP - Multiple Epitope – Thrombospondin Adhesion particle
MHC - Major Histocompatibility Complex
ml - Millilitre
MM - Matrix MTM
MSD - Meso-Scale Discovery
MSP - Merozoite Surface protein
MVA - Modified Vaccinia Ankara Virus
NAI - Naturally Acquired Immunity
NaSCN - Sodium Thiocyanate
nCoP - non-mechanistic correlate of protection
NK - Natural Killer
OD - Optical Density
PAMPs - Pathogen-Associated Recognition Receptor
PBMC - Peripheral Blood Mononuclear Cell
PBS - Phosphate buffered saline
PBST - Phosphate buffered saline with 0.05% Tween 20
PCR - Polymerase Chain Reaction
PD1 - Programmed Cell Death Protein 1
PE - Pre-erythrocytic
Pf - Plasmodium falciparum
PFA - Paraformaldehyde
*Pf*EMP-1 - *Plasmodium falciparum* Erythrocyte Membrane Protein 1
*Pf*SPZ - Cryopreserved *Plasmodium falciparum* Sporozoite Vaccine
PMR - Parasite multiplication rate
pNPP - p-nitrophenyl phosphate
Pv - *Plasmodium vivax*
QC - Quality Control
qPCR - quantitative Polymerase Chain Reaction

R21 - R21 manufactured in SII, India
RAS - Radiation Attenuated Sporozoites
RBC - Red Blood Cell
RDT - Rapid Diagnostic Test
RH5 - Reticulocyte-binding Homologue 5
RNA - Ribonucleic acid
ROS - Reactive Oxygen Species
RT - Room temperature
SD - Standard Deviation
SOP - Standard Operating Procedure
Spz - Sporozoites
SSC - Side Scatter
std – Standards
TBV – Transmission blocking vaccine
TCR - T Cell Receptor
Tfh - T Follicular Helper Cell
TNF - Tumour necrosis factor
TP - Time Point
Treg - Regulatory T Cell
UK - United Kingdom
VAC - Vaccine Trial prefix
VE - Vaccine Efficacy
VLP - Virus-Like Particle
WHO - World Health Organisation Programme
WRAIR - Walter Reed Army Institute of Research
WSV - Whole Sporozoite Vaccines
Yrs - Years
µg - Microgramme(s)
µl - Microlitre

1 CHAPTER ONE – INTRODUCTION

1.1 Malaria Overview

Malaria is a complex infectious disease that continues to pose a significant global health challenge. Malaria is caused by Apicomplexan parasites of the genus *Plasmodium* and is transmitted by the bite of an infected female *Anopheles* mosquito (Sato, 2021; Talapko et al., 2019). Despite significant progress made in the past two decades, malaria remains a leading cause of morbidity and mortality, particularly in sub-Saharan Africa, which accounts for about 95% of global cases (WHO, 2023).

Plasmodium infection can cause a variety of symptoms, and the clinical presentation of the disease is classically defined as uncomplicated or severe. Severe malaria occurs mainly in children under 5 years, pregnant women, and naïve adults and can cause complications such as cerebral malaria, severe anaemia, organ failure, pulmonary complications, and death (Bartoloni & Zammarchi, 2012).

1.2 Malaria burden

The global burden of malaria remains a significant public health challenge. According to the World Health Organization (WHO) malaria report in 2023, there were an estimated 249 million malaria cases worldwide in 2022 (WHO, 2023). This was an increase of approximately 5 million cases reported in 2022; of these, 234 million cases (~95%) occurred in Sub-Saharan Africa. The number of malaria deaths, however, did decline from 864, 000 in 2000 to 608, 000 in 2022. In 2020, there were 55,000 deaths that can partly be attributed to disruptions in malaria control interventions and health systems as a direct consequence of the COVID-19 pandemic. This increase was followed by a marginal decline in 2021 and 2022, to 610 000 and 608 000, respectively (WHO, 2023). It is important to note that the impact of COVID-19 on malaria deaths was not uniform across countries and regions. Countries with already strong malaria control programmes and resilient healthcare systems managed to mitigate some of the negative effects. In contrast, regions with weaker health systems and a high malaria burden may have faced a more substantial impact.

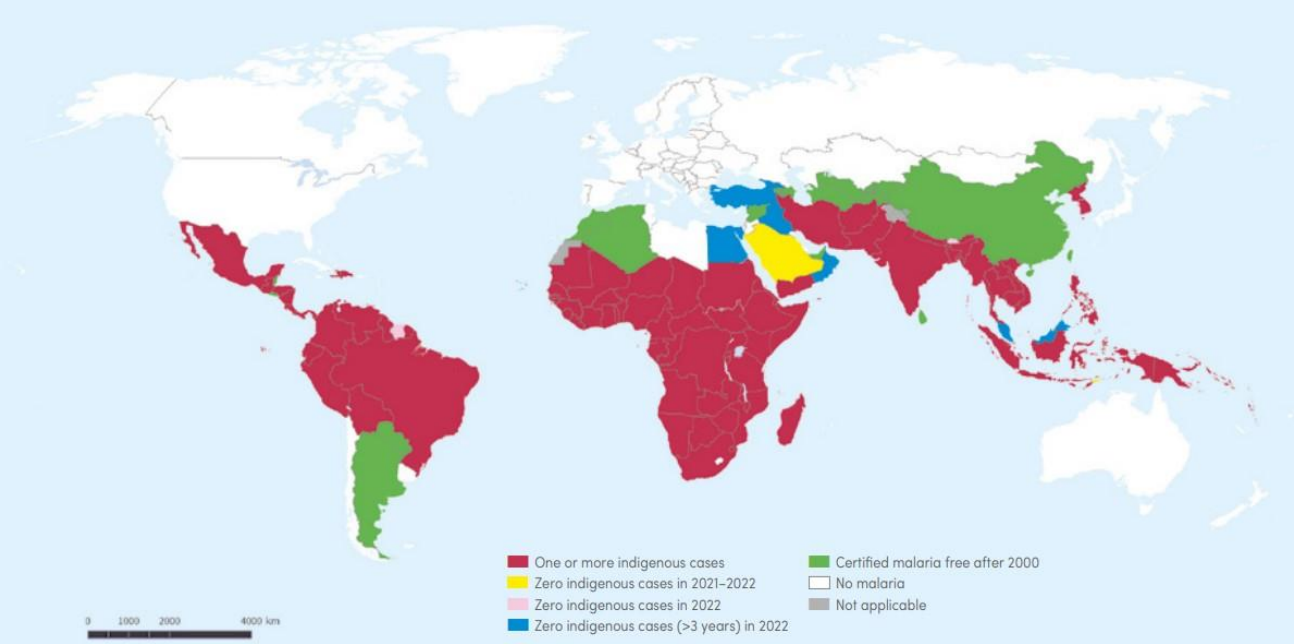


Figure 1. 1: Malaria world map of the current state of malaria cases.
Image taken from World malaria report 2023 (WHO, 2023).

In 2022, approximately 95% of global malaria deaths were attributed to 29 African countries. Four countries recorded more than half of all malaria deaths worldwide: (Nigeria [31.1%], the Democratic Republic of the Congo [12.3%], Uganda [5.1%], and Mozambique [4.2%]. Nigeria accounted for 38.5% of global malaria deaths in children aged under 5 years (WHO, 2023).

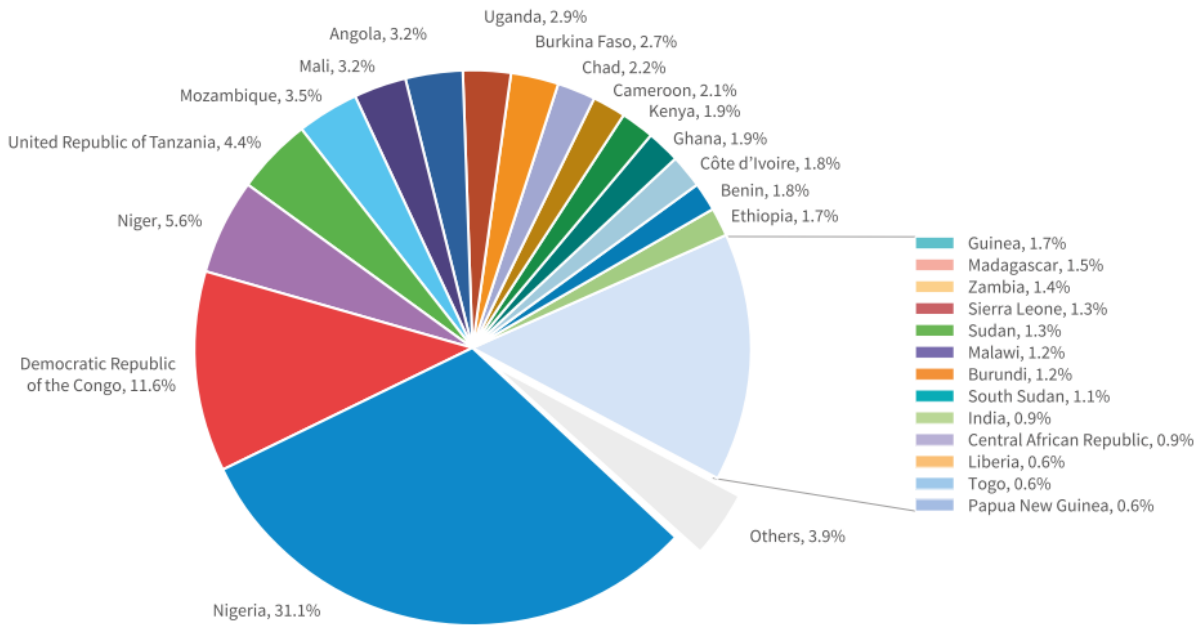


Figure 1. 2: Percentage distribution of *Plasmodium falciparum* malaria deaths in Africa.
Image taken from world malaria report 2023 (WHO, 2023).

1.3 Economical cost of malaria.

Malaria not only causes high mortality, but has a significant economic cost, particularly in regions where it is endemic. The economic impact can be seen through various factors such as the burden on healthcare systems in regions already struggling with healthcare care, loss of productivity and long-term health consequences because of severe malaria (Andrade et al., 2022).

It is challenging to estimate the precise economic cost of malaria globally, as it varies across regions and countries. However, according to Sarma et al., a 10% reduction in malaria incidence is associated with a 0.27% increase in the level of purchasing power parity (PPP) of the gross domestic product per capita (GDPpc) (Sarma et al., 2019).

1.4 The Vector

Plasmodium falciparum is a complex organism that has evolved numerous strategies to evade the human immune system and thrive within its host. *P. falciparum* has a complex life cycle with several different developmental stages, involving both human and mosquito hosts, making it difficult to eradicate. The transmission of malaria through the bite of an infected mosquito was discovered in 1897 by Ronald Ross who was later awarded a Nobel prize for this groundbreaking work (Hagan et al., 1997; Sinden, 2007). Not all mosquitoes can transmit malaria, of the thousands of recognized mosquito species only a fraction of these serve as disease vectors.

Table 1. 1: The most important mosquito vectors in insect-borne diseases.

Species	**Infectious disease transmitted**
Anopheles *sp*	Malaria Lymphatic filariasis
Aedes *sp*	Chikungunya Dengue fever Lymphatic filariasis Rift Valley fever Yellow fever Zika
Culex *sp*	Japanese encephalitis

The primary vector for *P. falciparum*, is the female *Anopheles* mosquito (Sinka et al., 2020). Several species of *Anopheles* mosquitoes can transmit *P. falciparum*, with different species being prevalent in different regions of the world. In sub-Saharan Africa, the most important vector species for *P. falciparum* is the *Anopheles gambiae* complex; which includes *Anopheles gambiae sensu stricto, Anopheles arabiensis, Anopheles funestus,* and *Anopheles coluzzii* (Sinka, ME., 2020; Wiebe et al., 2017). These mosquito species are highly efficient in transmitting the parasite and are responsible for most malaria cases in the region.

1.5 The parasite

The genus *Plasmodium* includes more than 170 different species that infect mammals, reptiles, birds, and amphibians (Sato, 2021). There are five species of *Plasmodium* that infect humans and cause malaria. *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale*, and *Plasmodium knowlesi* (Antinori et al., 2012; Sato, 2021). *P. falciparum* and *P. vivax* are responsible for most of the disease burden caused by malaria. *P. falciparum* is the deadliest and infections can progress rapidly and cause severe complications, including cerebral malaria (Moxon et al., 2020). It is the most prevalent in Africa and is the focus of this thesis. *P.vivax* is the second most common species and has a broader geographic distribution and can cause relapses, as the parasite can remain dormant in the liver (Adams & Mueller, 2017). Table 1.2 describes the prevalence, distribution and characteristics of *Plasmodium* species.

Table 1. 2: The prevalence, distribution, and characteristics of *Plasmodium* species

Adapted from (Antinori et al., 2012; Doolan et al., 2009)

	Regions with the highest incidence	**Prevalence of the disease**	**Risk of severe malaria**	**Relapse**	**Risk of drug resistance**	**Red cells affected**	**Average Parasitaemia (per µL)**	**Pre-patent period (days)**
Plasmodium falciparum	Sub-Sahara Africa and India	High	Yes	No	Yes	All	20,000-500,000	9-10
Plasmodium vivax	Southeast Asia and Western Pacific Regions	High	Yes	Yes	Yes	Reticulocytes	20,000	11-13
Plasmodium malariae	Southeast Asia, Western Pacific Islands, Sub-Saharan Africa & South America	Low (Pooled prevalence 2.0%) (Hawadak et al., 2021))	No	No	No	Mature erythrocytes	6000	15-16
Plasmodium ovale	Sub-Saharan Africa, Western Pacific islands & Southeast Asia	Low-Rare (Pooled prevalence 0.7%) (Hawadak et al., 2021)	No	Yes	No	Reticulocytes	9000	10-14
Plasmodium Knowlesi* *Natural host macaque monkey	Southeast Asia	Medium-low (Pooled prevalence 19%) (Kotepui et al., 2020))	Yes	No	No	All	600-10,000	9-12

1.6 Life cycle of *Plasmodium falciparum*

In humans, the life cycle can be divided into three stages: (i) pre-erythrocytic stage (liver stage) (ii) erythrocytic stage (blood-stage), (iii) sexual stage.

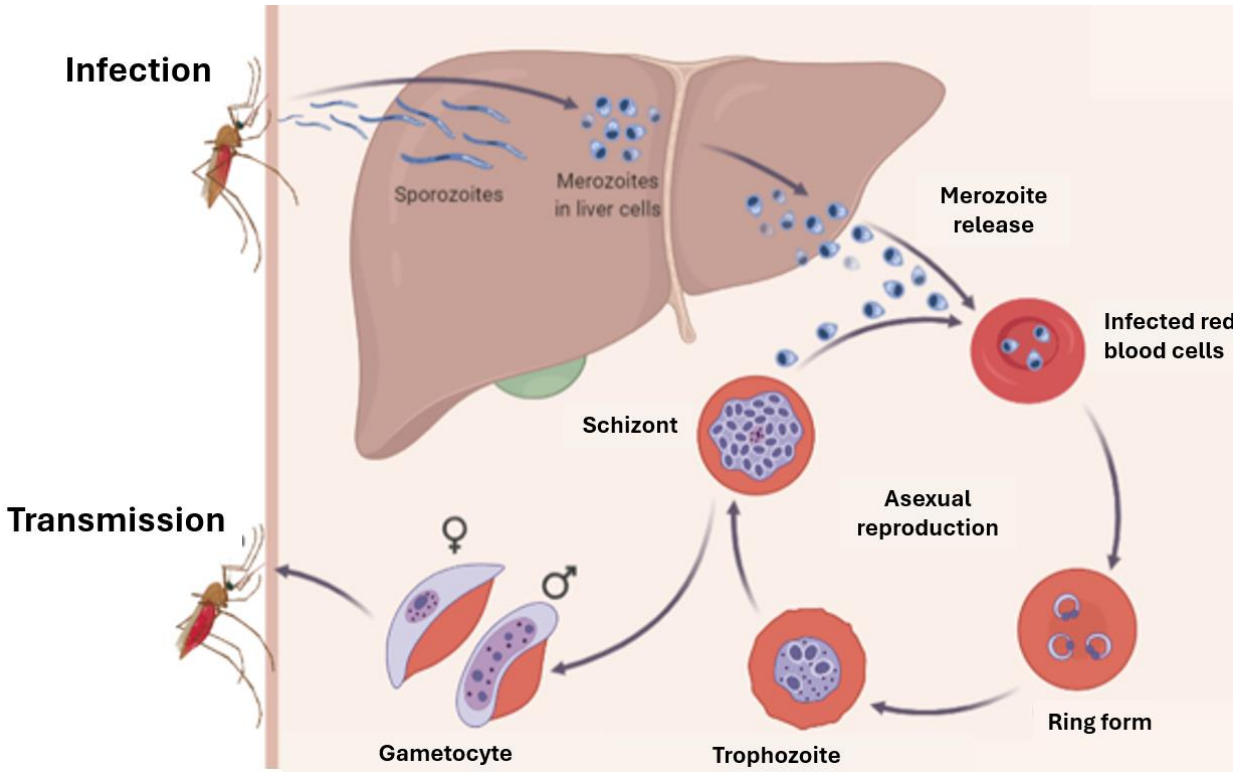


Figure 1. 3: *P. falciparum* life cycle in the human host.
Image created in Biorender by E. Kibwana.

1.6.1 Pre-erythrocytic stage

Infection in humans begins when an infected *Anopheline* mosquito injects sporozoites into the dermis. The average number of sporozoites injected into the dermis varies enormously. It is estimated that majority of mosquitoes inject a medium of 8-39 sporozoites during probing, generally less than 1% of the sporozoites in their salivary glands (Graumans et al., 2020; Ménard et al., 2013). Although some studies have shown a minority of mosquitoes can inject an average of 125 sporozoites (Sinnis & Zavala, 2012). This early stage of the life cycle is a bottle neck for the parasite and represents a good strategy for controlling the disease as only a few sporozoites are injected into the host. The exact duration for which sporozoites remain in the skin after a mosquito bite can also vary, but studies suggest that it can be a

couple of hours and the sporozoites 'trickle out' from the injection site (Amino et al., 2006; Yamauchi et al., 2007). Sporozoites are highly motile and move through the dermis by gliding motility using the actin/myosin-based motor (Hopp et al., 2015). A proportion of these sporozoites will leave the dermis through a blood vessel, whilst others remain in the skin where they are destroyed by host immune responses and around 30% end up in the draining lymph nodes (Amino et al., 2006). Once the sporozoites enter the bloodstream, they are rapidly carried to the liver, where they traverse the sinusoidal endothelial cells comprising of endothelial cells and macrophage like Kupffer cells to access the liver parenchyma (Loubens et al., 2021). Sporozoites have been reported to transverse through several hepatocytes before arresting in one and establishing infection in a hepatocyte. Within hepatocytes, the parasites form a specialized membrane-bound compartment parasitophorous vacuole (PV) derived from the host cell membrane that separates the parasites from the host (Goldberg & Zimmerberg, 2020; Loubens et al., 2021). The PV is crucial for the survival and progression of the parasite within the host. The parasite matures, differentiates and undergoes several cycles of asexual multiplication releasing up to thousands of merozoites per hepatocyte within merosomes into the bloodstream, between 7-10 days after infection (Prudêncio et al., 2006). In the blood, merosomes release individual merozoites which invade red blood cells and initiate the erythrocytic stage of the life cycle.

Several proteins are instrumental in the process of cell traversal, and each of these proteins presents as a potential candidate for vaccination. The most studied are the circumsporozoite protein (CSP), which is the major antigen on the sporozoite surface (V. Nussenzweig & Nussenzweig, 1985), and thrombospondin-related anonymous protein (TRAP), a micronemal protein (Paoletta & Wilkowsky, 2022). CSP is the most abundant protein on the parasite's surface and is essential for sporozoite formation, motility, and invasion (Coppi et al., 2011). The development of immunity to sporozoites is a critical vaccine target for preventing clinical malaria and transmission.

1.6.2 Erythrocytic stage

Once released, merozoites quickly infect erythrocytes and undergo synchronous cycles of asexual replication, progressing through to the ring stage. The parasite rings rapidly grow by ingesting the host's cytoplasm and utilizing the haemoglobin into a trophozoite before finally replicating into 16 to 32 daughter merozoites in the schizont stage (schizogony), causing erythrocytes to burst approximately every 48 hours as new merozoites are released into circulation (Venugopal et al., 2020). This cycle of invasion, replication and bursting of the blood cells results in the symptomatic peaks of fever and anaemia associated with malaria (Cowman et al., 2016; Moxon et al., 2020).

1.6.3 Sexual stage

After 7-15 days in circulation, a small proportion of trophozoites will commit to sexual reproduction, a process known as schizogony to produce sexual progeny that becomes male and female sexual forms, known as gametocytes (Kuehn & Pradel, 2010). The signals inducing this developmental pathway are still unclear, but gametocyte production is increased by factors that negatively impact asexual multiplication such as drug treatment, anaemia, or parasite density (Grange et al., 2015; Kuehn & Pradel, 2010). The infected red blood cells (iRBCs) carrying the immature gametocytes sequester in the bone marrow, avoiding the immune system until they mature. After 7-10 days, the mature gametes enter the systemic circulation where they are subsequently taken up in a mosquito blood meal (Chawla et al., 2021; Cowman et al., 2016; Kuehn & Pradel, 2010). In the mosquito midgut, the gametocytes differentiate into gametes and emerge from the erythrocytes. Male and female gametes fuse to form a zygote, which is further divided, resulting in motile ookinetes. The ookinetes penetrate the gut wall and develop into mature oocyst within which the parasite asexually replicates, forming several thousands of infective sporozoites that migrate to the salivary gland which will be released when the mosquito takes up its next blood meal malaria initiating a new human infection (Cowman et al., 2016; Kuehn & Pradel, 2010).

1.7 Pathogenesis of malaria: disease symptoms

The pre-erythrocytic stage of malaria infection is asymptomatic and not associated with pathology. Clinical symptoms develop during the blood stage. The clinical symptoms can broadly be divided into two presentations: uncomplicated and severe malaria (Cowman et al., 2016; Moxon et al., 2020).

1.7.1 Uncomplicated malaria

The accompanying symptoms for uncomplicated malaria are non-specific and often include rigors, headache, nausea, and muscle pains and these typically appear 10-14 days after infection. The most common symptom is a cyclic fever and chills which occurs in synchrony with the rupture of merozoites from infected blood cells. This releases parasite proteins, toxins and parasite debris including hemozoin (malarial pigment) and glycophosphatidylinositol (GPI) (Cowman et al., 2016; Mawson, 2013). This activates the innate immune cells like gamma delta T cells (γδ T cells), monocytes and dendritic cells (DCs) resulting in the release of proinflammatory cytokines and chemokines like tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and reactive oxygen species (ROS), these mediate immune responses and serve as important effector molecules (Bucşan & Williamson, 2020; Ezema et al., 2023). Uncomplicated malaria in malaria exposed individuals can resolve naturally or treated during the

symptomatic episodes with over-the-counter anti-malarials, and majority of patients will clear the infection with proper compliance (Cowman et al., 2016; Milner, 2018). In some cases, or when left untreated uncomplicated malaria can progress to severe malaria, which may end in death or long-term health complications.

1.7.2 Severe malaria

The pathophysiology of severe malaria is a complex multisystem disorder and can affect many tissues in the body. Nearly all severe cases of malaria and malaria-related deaths are caused by *P. falciparum*. The clinical manifestation of severe malaria depends on age and can include severe anaemia, coma and hypoglycaemia, which are more common in children (Bartoloni & Zammarchi, 2012; N. J. White, 2022). While adults mainly present with acute kidney injury. Acute respiratory distress, metabolic acidosis and/or cerebral malaria occurs in patients of all ages. Severe malaria typically occurs in children and/or malaria naïve individuals who have low protective immunity (Cowman et al., 2016).

Severe malaria anaemia (SMA) is a major cause of mortality in children under 5 years of age (Trampuz et al., 2003). According to WHO, SMA is defined as a haemoglobin level of <50 g/L or haematocrit <15% in children; <70 g/L and <20% in adults The pathogenies of SMA is complex and involves several different factors which are still not well understood (Cowman et al., 2016; Moxon et al., 2020; N. J. White, 2022). These include the destruction of iRBCs during parasite replication, complement- or antibody-binding triggers immune-mediated haemolysis, sequestration of infected and uninfected red blood cells removes them from circulation, dyserythropoiesis due to hemozoin affecting bone marrow production of red blood cells (Moxon et al., 2020).

The most severe form of *P.falciparum* malaria is cerebral malaria, multiple complications can occur causing some of the highest mortality rates (Schiess et al., 2020). CM is defined by deep coma (Blantyre Coma Scale score ≤2 or Glasgow Coma Scale score ≤10) (Moxon et al., 2020). It occurs when iRBCs sequester to the micro-vessels in the brain, causing obstruction and reducing the blood flow resulting in ischemia and neurological damage (Idro et al., 2010; Moxon et al., 2020). The presence of iRBCs also triggers a strong inflammatory response and the release of pro-inflammatory cytokines which can then lead to edema, tissue damage and intracranial pressure (Idro et al., 2010; Schiess et al., 2020).

1.7.3 Placental malaria

Placental malaria is a specific form of malaria that affects pregnant women, characterized by the presence of malaria parasites in the placenta and is commonly seen in *P. falciparum* infections. *P.falciparum* iRBCs sequester in the placental intervillous spaces, due to the parasite ligand *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) expressing a variant receptor called VAR2CSA that has an active Duffy binding like protein which adheres to the chondroitin sulfate A (CSA) receptors in the placental intervillous spaces (Chua et al., 2021; Sharma & Shukla, 2017). This in turn leads to the obstruction of blood flow and the disruption of maternal-foetal nutrients and oxygen exchange system as well as triggering a proinflammatory response (Chua et al., 2021). Placental malaria can cause preterm birth, low birth weight, miscarriage and infant and maternal mortality.

1.8 Malaria Diagnosis

The most common initial approach for diagnosis is based on clinical symptoms, such as fever, chills, and bodily pain. However, this is methods is prone to misdiagnosis as several infectious diseases present with the symptoms especially in malaria endemic areas. Usually, this approach is followed by more accurate laboratory methods such as microscopy or rapid diagnostic tests. WHO guidelines state that all suspected malaria cases should be confirmed with a parasitological test either using light microscopy and/or rapid diagnostic tests (RDTs) (WHO, 2023).

1.8.1 Microscopy

Microscopic examination of blood smears is considered the gold standard method of malaria diagnosis (Murphy et al., 2013). It involves the involves the detection of parasites in blood films that have been stained with Giemsa stain, it allows identification of the species, stage of development and a quantification of parasites (Payne, 1988). It is relatively cheap as it requires very little equipment, highly specific and accurate when performed by trained personnel. The sensitivity of blood smear detection is about ~4-50 parasites/µl of blood depending on the number of fields examined (Mpina et al., 2022; Seilie et al., 2019). However, the method does have its limitations it requires trained personnel with experience in malaria microscopy to ensure accurate interpretation of the blood slides, it is time consuming and laborious depending on the amount of samples coming into the diagnostic lab and the sensitivity of the test in cases with low levels of parasitaemia or mixed infection is low (Mpina et al., 2022; Okell et al., 2009).

1.8.2 Rapid diagnostic Test

RDTs are increasing being used in clinical settings. The test detects malaria blood stage antigens in the blood by immunochromatography of monoclonal antibodies (mAb) directed against the target antigen (Wongsrichanalai et al., 2007). The most common RDTs detect Histidine Rich Protein 2 (HRP2), *Plasmodium* Lactate Dehydrogenase (pLDH) which detect all species of human malaria (Wilson, 2013) or Aldolase (Kattenberg et al., 2011; Moody, 2002). HRP-2 is an abundant protein expressed on the surface of iRBCs of *P.falciparum* at asexual and young gametocyte stages (Moody, 2002). RDTs provide a simple and fast way to diagnose malaria as results are usually obtained in 5 – 20 min. However, they too have their limitations, the sensitivity of RDTs are approximately 100 - 200 parasites/µl (Picot et al., 2020) making it difficult to use in cases with low parasitaemia and it is not always species specific (Moody, 2002). Additionally, there is a risk of false positives when using HRP-2 designed kits, as HRP-2 can persist for weeks in the blood even after treatment and parasite clearance (Moody, 2002; Wongsrichanalai et al., 2007)

1.8.3 Quantitative polymerase chain reaction (qPCR)

PCR is the most sensitive method for detecting parasites in the blood, the limit of detection can be as low as is 1 parasite per microliter (Kamau et al. 2011, 2014). Most molecular approaches target the detection of conserved regions in *Plasmodium* genomic DNA that encode the 18S ribosomal RNA (rRNA) or the asexual form of 18S rRNA itself (Kimura et al., 1997; Seilie et al., 2019). This method is sensitive, specific, and reliable in detecting *Plasmodium* species. The major drawback is that it expensive as it requires highly specialized equipment and hence is limited to well-equipped centres that are not found in many remote endemic areas.

1.9 Treatment

WHO set up treatment guidelines for the management of malaria (WHO, 2023). Artemisinin-based combination therapies (ACT) are recommended as the first line treatment for uncomplicated *P. falciparum* malaria in children and adults. While treatment for severe malaria in adults and children (including infants and pregnant women) is intravenous or intramuscular artesunate for at least 24 hr (WHO, 2023). ACTs combine fast acting artemisinin derivatives like artesunate, artemether and dihydroartemisinin with a partner drug from a different class (like amodiaquine, piperaquine and pyronaridine, mefloquine or lumefantrine). The combination of the two drugs provides a synergistic effect, to enhance efficacy and reduce the emergence of drug resistance. However, there is a growing resistance to ACTs, with the emergence and spread of resistance to both the artemisinin component and the partner drugs used in ACT

particularly in South East Asia (Dondorp et al., 2009; Kyaw et al., 2013; Phyo et al., 2012). The potential for the spread of resistance remains a global public health issue that further promotes the need for new intervention strategies such as new treatments and vaccines.

1.10 Malaria Immunity

1.10.1 Innate immunity in malaria

The innate immune system plays a crucial role in malaria infections by initiating early responses allowing the host sufficient time to develop specific adaptive immune responses. It involves the activation of various immune cells including DCs, macrophages, and natural killer cells (NK) (Stevenson & Riley, 2004). The complement system also plays a role in innate immune responses.

The first step leading to the activation of the innate immune system is sensing of infection by the host. Antigen presenting cells (APCs) such as monocytes, DCs and macrophages recognize pathogens through receptors called pathogen-recognition receptors (PRRs) (Gowda & Wu, 2018). These receptors, such as the Toll-like receptors (TLRs) and C-type Lectin Receptors (CLRs) play a key role in recognizing conserved molecules of pathogens called pathogen-associated molecular patterns (PAMPs) from *Plasmodium* parasites (Gazzinelli et al., 2014). These PAMPs include GPI, hemozoin and plasmodial DNA (Langhorne et al., 2008; Stevenson & Riley, 2004), after recognition of PAMPs by PRRs, the innate immune system is activated through the initiation of specific signalling pathways, producing pro-inflammatory cytokines and chemokines that recruitment other innate immune cells e.g. neutrophils, and macrophages (Gowda & Wu, 2018).

In the skin, the damage brought about by the bite from a mosquito leads to cutaneous mast cell degranulation, leading to fluid extravasation and the recruitment of neutrophil, DCs, and macrophages (Demeure et al., 2005; Hopp & Sinnis, 2015). These immune cells can kill eliminate sporozoites either by engulfing them through phagocytosis followed by destroying them via intracellular killing mechanisms after ingestion (e.g. through the like reactive oxygen species and enzymes). However some sporozoites escape destruction, partly due to the rapid cell transversal which outpaces the neutrophils (Ezema et al., 2023; Hopp & Sinnis, 2015) and make their way to the liver. Once the sporozoites reach the liver, infected hepatocytes express type I interferons (IFNs) (Gowda & Wu, 2018; Stevenson & Riley, 2004) this triggers the recruitment of NK cells and natural killer T cells (NKT) which are important sources for IFN-γ, these effector cells target and kill the parasite-infected hepatocytes through mechanisms such as perforin and

granzymes which are key components of the cytotoxic immune response or the production of nitric oxide and other reactive species target intracellular pathogens (Gazzinelli et al., 2014; Stevenson & Riley, 2004). In the early stages of blood stage infection, DCs produce cytokines and chemokines and effectively play a key role in bridging the gap between the innate and adaptive immune system (Gowda & Wu, 2018). Macrophages and DCs can phagocytose free hemozoin or infected RBCs. DCs have also been shown to activate Vγ9Vδ2 T cells, a subset of gamma delta (γδ) T cells, via a T cell receptor (TCR) dependent manner to release cytotoxins like granulysin, and IFN-γ to inhibit the parasites (Ezema et al., 2023; Osii et al., 2020). Also, as the parasite multiples and grows within the red blood cells it modifies the structure of the cells making them more ridged and thus infected red cells end up being cleared by the spleen. These responses contribute to the clinical symptoms observed in malaria.

The complement system is part of the innate immune system and serves as an effector arm of adaptive humoral immunity. It consists of over 30 serum proteins that work in a complex cascade of reactions that lead to a variety of immune responses including cell opsonization for phagocytosis, recruitment of immune cells, and induction of inflammation and formation of the membrane attack complex (MAC) for targeted microbial lysis (Kurtovic, Boyle, et al., 2019; Nesargikar et al., 2012). The complement is system can be activated through three main pathways (i) the classical pathway which is initiated by C1q binding to antigen-antibody (IgG or IgM) complexes, (ii) the lectin pathway, which is activated by the binding of mannose-binding lectin (MBL) to carbohydrates on microbial surfaces and lastly (iii) the alternative pathway which can be activated by spontaneously hydrolysis of native complement component C3 in blood or C3b protein directly binds a microbe, foreign material or damaged tissue (Kiyuka et al., 2020; Nesargikar et al., 2012).

1.10.2 Naturally acquired immunity.

Although it is slow to develop, there is natural acquired immunity (NAI) to malaria. Adults who have grown up in an endemic area and are exposed to repeated infections with *P.falciparum* develop resistance, first to severe malaria and then to clinical disease (Doolan, Doban et al., 2009). This immunity is acquired relatively quickly (after as few as three or four exposures) (Gupta et al., 1999; Marsh & Kinyanjui, 2006). The first scientific evidence of NAI was reported by Robert Koch in the 1900s, where he observed adults living in a high malaria endemic region developed a reduced risk to severe malaria as they are repeatedly exposed to the parasite. (Doolan et al., 2009; Marsh & Kinyanjui, 2006). Field studies conducted in Africa collaborated on these early findings from Koch *et al.,* also describing age-dependent immunity, with

children under 5 years of age being more susceptible to severe malaria and death (Marsh & Kinyanjui, 2006). A typical picture of naturally acquired immunity in areas with stable transmission- is depicted below, showing that immunity increases with age.

In general, in areas of high transmission, the risk of severe disease is mainly seen in children under 5 years of age, visitors, and pregnant women, whereas in areas of low transmission almost all individuals are at risk of severe disease (Doolan et al., 2009).

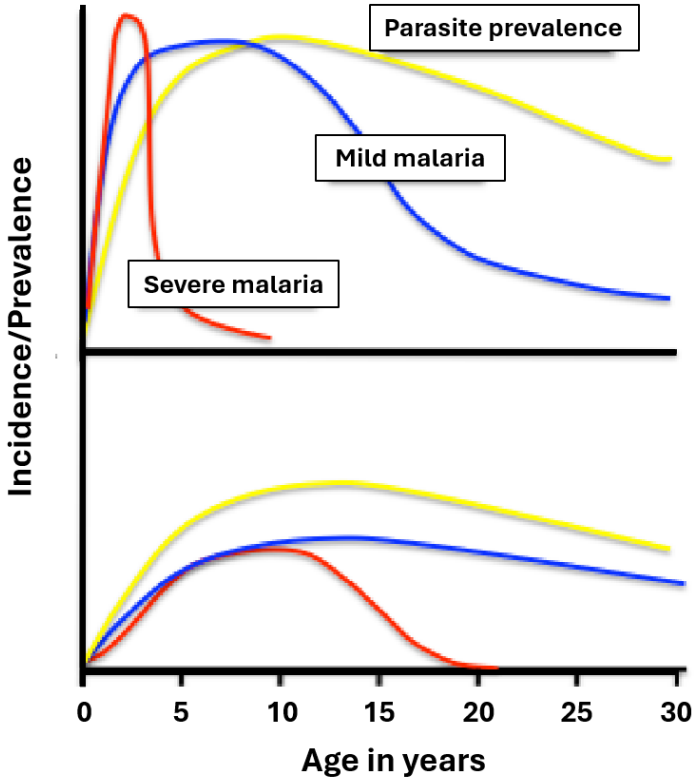


Figure 1. 4: Evidence of naturally acquired immunity increasing with age.

Graph shows the incidence of severe and mild malaria and the prevalence of asymptomatic malaria infection in an area of high (upper graph) and low (lower graph) malaria transmission. Image adapted from (Kinyanjui, 2012).

One of the explanations that has been proposed for the slow acquisition of NAI is antigenic diversity in *P. falciparum*. This was first observed in the late 1890s in Sierra Leone when a medical doctor noted that soldiers from Sierra Leone suffered less attacks from malaria with milder symptoms compared to soldiers who arrived from the West Indies, who suffered severe and often fatal malaria. He went on to conclude that immunity to one *Plasmodium* species does not confer to protection against another *Plasmodium*

species (Smith, 1898). Since then, several studies have been published that describe antigenic diversity and have shown that many antigens of *P. falciparum* exhibit extensive genetic diversity with polymorphisms and allelic variation (Gardner et al., 2002; McKenzie et al., 2008). Overall, antigenic diversity in *P. falciparum* enables the parasite to evade immune recognition and clearance, leading to challenges in developing long-lasting and fully protective immunity.

Pregnant women are increasingly susceptible to malaria infections and may experience more severe symptoms compared to non-pregnant individuals, particularly in their first and second pregnancy. This susceptibility is attributed to changes in the immune response and the altered placenta that can facilitate parasite sequestration. Infections with both *P.falciparum* and *P.vivax* can lead to adverse outcomes to both the mother and foetus, such a anaemia and low birth weight (Chua et al., 2021; Moya-alvarez et al., 2014).

Immunity to malaria can also wane over time in the absence of reinfections. NAI is typically dependent on continuous exposure, when individuals are no longer exposed to the parasite, such as when they move to non-endemic areas (Färnert et al., 2015) or when malaria transmission is interrupted their immune system may gradually lose its ability to mount a robust immune response against the parasite. Although they still remain with some protection against severe disease and death (Doolan et al., 2009; Langhorne et al., 2008; Roestenberg et al., 2011).

In summary, the process of acquiring natural immunity to malaria is complex and multifactorial but the key features of NAI are (i) immunity to malaria increases with age and exposure (ii) it can control parasitaemia and clinical symptoms but not necessarily protect against infection (iii) immunity can wane over time if an individual is no longer exposed (iv) species specific – immunity to one species does not confer strong cross-protection (v) antibodies play a key role (Doolan et al., 2009; Langhorne et al., 2008; Marsh & Kinyanjui, 2006; Roestenberg et al., 2011).

1.10.3 Adaptive immunity

The adaptive immune system can be broadly split into two component 1) humoral immunity and 2) cellular immunity. Both these responses play role in malaria protection, the hallmark feature of the adaptive immune system is in the generation of immunological memory which enables a more rapid and robust response during a secondary infection.

1.10.3.1 Cellular mediated immunity

1.10.3.1.1 CD8 T cells in malaria

Naïve CD8 T cells are activated during malaria infection by APCs, such as DCs. DCs process and present parasite peptide on major histocompatibility complex (MHC) class I molecules DCs express costimulatory molecules required for naïve T cell proliferation and differentiation into effector cells (Yap et al., 2019). It has been suggested that during the liver stage of malaria, CD8 T cells target infected hepatocytes by releasing pro-inflammatory cytokines such as IFN-γ (Kurup et al., 2019). However, this priming of CD8 cells in the liver seems unlikely due to the small number of infected liver cells following mosquito infection (Kurup et al., 2019). During blood stage infections the role of CD8 T cells is limited due to the absence of MHC molecules on the surface of red blood cells (Corradin & Levitskaya, 2014). The underlying mechanisms of priming and function of CD8 T cells in malaria infection is an area that is still not fully understood and remains an important area of research.

1.10.3.1.2 CD4 T cells in malaria

CD4 T cells are known to play a pivotal role in the control of *Plasmodium* infection. Naïve CD4 T cells are activated by APCs, like DCs, presenting *Plasmodium* peptides on MHC class II molecules. Once activated the CD4 T cells proliferate leading to clonal expansion, some which become memory T cells that provide long term immunity (Kurup et al., 2019). Activated CD4 T cells can also produce cytokines that activate other immune cells such as B cells to produce antibodies. Additionally, they can differentiate into various effector subsets like (i) Type 1 T helper (Th1) cells – these produce cytokines like IFN-γ and TNF-α, (ii) Type 2 T helper (Th2) cells – these produce cytokines like interleukin (IL-4, IL-5 and IL-13) and support humoral immunity, (iii) Type 17 T helper (Th17) cells – these produce IL-17 and IL-22 and contribute to inflammatory responses and lastly, (iv) Regulatory T cells (Treg) – which regulate immune responses, promote tolerance and prevent autoimmunity (Kurup et al., 2019; Perez-mazliah & Langhorne, 2015). Th1 cells in blood stage infection triggers strong IFN-γ and TNF responses that stimulate macrophages to produce ROS and reactive nitrogen intermediates (RNI) that kill iRBCs (Kumar et al., 2019).

1.10.3.2 T follicular helper cells

T follicular (Tfh) cells are a specialized subset of CD4 T cells found in the secondary lymphoid organs like the lymph nodes and spleen (Crotty, 2019). They play a critical role in supporting B cell responses by interacting with B cells within the germinal centres (GC). Germinal centres are specialized structures that form within secondary lymphoid organs (e.g. lymph nodes and the spleen) during an immune response. Tfh cells support the differentiation and maturation of B cells, guide antibody class-switching, and promote the formation and maintenance of GCs, which are essential for generating high-affinity antibodies and long-lived plasma cells and memory B cells (Crotty, 2019; Deenick & Cindy, 2011). Tfh cells are characterized by the expression of CXCR5 and programmed cell death 1 (PD1), inducible T cell costimulatory (ICOS), CD40 ligand (CD40L), OX40 as well as the transcription factors B cell lymphoma (Bcl6) (Deenick & Cindy, 2011; Vinuesa et al., 2016).

Tfh cells are activated when a naïve CD4 T cell is presented antigen on DCs or other APCs. This interaction occurs through the TCR and MHC class II molecules on DCs. Along with TCR signalling, co-stimulatory signals are crucial for full activation (Crotty, 2019; Deenick & Cindy, 2011). This usually involves binding between CD28 on the T cells and B7 molecules (CD80 or CD86) on the APCs along with other co-stimulatory signals like ICOS and CD40L. These interactions lead to the expression of CXCR5 (a B cell zone homing marker) and the downregulation of CCR7 which enables them to migrate out of the T cell zone to the T-B border of the lymph node where they encounter B cells (Crotty, 2019). Cognate B cells will also present antigen and provide co-stimulatory signals allowing ongoing signalling to the T cells which maintains the Tfh cell phenotype. At the same time, Tfh cells provide help to B cells in the form of CD40L stimulation and IL-21 (Deenick & Cindy, 2011). These signals drive the formation and maintenance of GCs. The primary function of the GC reaction is to produce high affinity antibodies through somatic hypermutation and affinity maturation as well as plasma cells and memory B cells (Gatto & Brink, 2010). Due to the central role of Tfh cells in antibody production, studying these cells may give an indication on the quality of antibodies produced following infection or vaccination.

1.10.3.3 Humoral Immunity

1.10.3.3.1 B cells

B cells play a crucial role in producing both short-term and long-term humoral antibody responses. B cells can be activated in a T cell dependent manner or a T-cell independent manner. B cell development starts in the bone marrow after which the immature B cell migrates to the spleen for more development and activation. The primary role of B cells is the generation of antibodies; however, they also contribute to immunological memory, can serve as APCs, and produce regulatory and proinflammatory cytokines (Hoffman et al., 2016).

T-independent activation of B cells occurs when antigens stimulate B cells without the involvement of helper T cells (Allman et al., 2019). This activation can be triggered by T-independent type 1 (TI-1) antigens, which directly engage TLR, or by T-independent type (TI-2) antigens, characterized by repetitive structures that cross-link multiple B cell receptors (BCRs) for example bacterial polysaccharide capsules (Allman et al., 2019; Vos et al., 2000). This cross-linking provides the initial activation signal, which is further enhanced by innate immune signals. This process primarily leads to the production of IgM antibodies, resulting in a quick but less robust immune response without memory B cell formation.

A T cell dependant activation occurs when a B cell encounters a cognate antigen, they bind to it with BCRs that are on their surface, internalize it, and present fragments of the antigen on MHC II molecules. This triggers their migration to the B cell follicle border to receive CD4+ T cell help, which provides additional activation signals (Rogers et al., 2021). When B cells receive these signals, they start to differentiate and proliferate. During this process the B cells genes undergo somatic hypermutation, introducing random mutations that increase antibody diversity, they also go through rounds of positive selection by interacting with Tfh cells and antigens on the follicular DCs in the GC, a process called affinity maturation, which leads to production of B cells with higher-affinity antibodies (W. Hoffman et al., 2016; Küppers, 2021). Those with lower affinity or self-reactive antibodies are typically destroyed. At this time B cells may also undergo class-switch recombination, switching from IgM to other classes like IgG or IgA, without altering the specificity for the antigen (Küppers, 2021; Ly & Hansen, 2019). This change is critical for allowing the immune system to utilize different mechanisms for fighting various types of pathogens.

Ultimately, the B cells that have undergone somatic hypermutation and affinity maturation, and potentially class-switch recombination, differentiate into long-lived plasma cells (LLPC) that secrete high-affinity antibodies or into memory B cells that provide rapid and robust responses upon re-exposure to the specific

antigen (Akkaya et al., 2020). These LLPCs and memory B cells are crucial for the success of vaccines and long-lived protection.

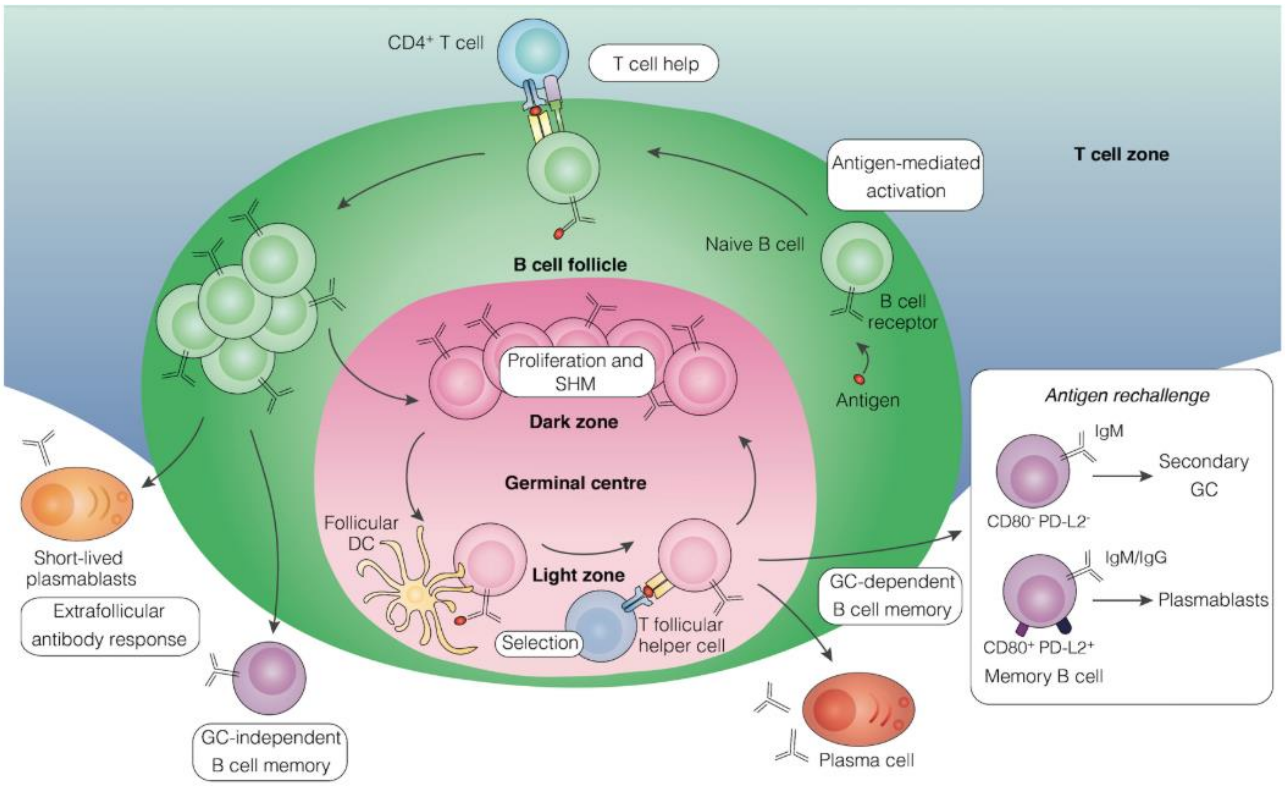


Figure 1. 5: B cell memory development.

Activated B cells in secondary lymphoid tissues receive helper signals from CD4+ T cells at the border of B and T cell zones. Some differentiate into short-lived plasmablasts for an initial antibody response, while others form early memory B cells independently of germinal centres (GCs). Remaining B cells migrate into the follicle to establish a GC, where they proliferate, undergo somatic hypermutation, and undergo affinity-based selection with support from T follicular helper cells and follicular dendritic cells. The GC reaction produces long-lived plasma cells and memory B cells. Upon re-exposure to the antigen, IgM memory B cells without CD80 and PD-L2 primarily seed new GCs, while memory B cells expressing these markers, including IgM and IgG isotypes, mainly generate plasmablasts for a rapid antibody response. Image adapted from (Ly & Hansen, 2019).

1.10.3.3.2 Antibodies

Antibodies, also known as immunoglobulins, are specialized proteins produced by the immune system that recognize foreign pathogens and bind to them with high affinity ultimately leading to their destruction (Hoffman et al., 2016). They are found on the surface of B cells where they act as BCRs or secreted into circulation. Antibodies act as soluble effector molecules in humoral immune response (Hoffman et al., 2016; Leitner et al., 2020). Antibodies are typically Y shaped proteins (figure 1.6) consisting of four polypeptide chains; two identical light chains (LC) and two identical heavy chains (HC) connected by disulfide bonds. The HC and LC have fragment antigen binding (Fab) region containing two antigen-binding domains at the N-terminus that is unique to each antibody and allows it to bind specifically to a particular antigen and a fragment crystallizable (Fc) region (Chiu et al., 2019; Hoffman et al., 2016). Antibodies bind to antigens through their Fab region while the Fc region mediates various effector functions, through binding to Fc receptors on certain immune cells (James, 2022). Once bound to an antigen, antibodies can activate various immune mechanisms: i) Opsonization: antibodies coat pathogens, enhancing their uptake and destruction by phagocytic cells like macrophages, ii) Complement Activation: binding of antibodies to antigens can trigger the complement system, leading to the lysis of pathogens and iii) Neutralization: antibodies can block toxins or prevent viruses from entering and infecting host cells (Forthal, 2015; Hoffman et al., 2016; Opi et al., 2021).

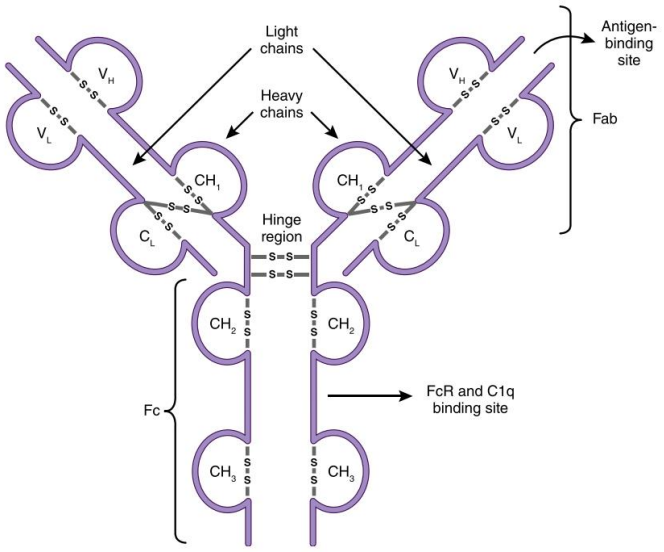


Figure 1. 6: Antibody structure.
Image adapted from (W. Hoffman et al., 2016)

Five isotypes, or classes, of antibodies (IgM, IgD, IgG, IgA, and IgE; summarized in table 1.3) exist and are brought about by class switching recombination which results in production of antibodies with different constant regions (Fc regions) while the variable region remains the same, which alters the effector functions of the antibodies (Hoffman et al., 2016; Stavnezer et al., 2008).

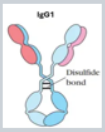
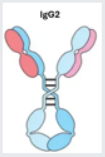
Table 1. 3: Antibody Isotypes
Table adapted from (Aryal, 2018)

	IgG Monomer	IgM Pentamer	Secretory IgA dimer	IgE Monomer	IgD Monomer
					
Heavy chain	γ	μ	α	ε	δ
Number of antigen binding sites	2	10	4	2	2
Molecular weight (Daltons)	150,000	900,000	385,000	200,000	180,000
Percentage of total antibody in serum	80%	6%	13%	0.002%	1%
Fixes complement	yes	yes	no	no	No
Fc binds to	phagocytes			Mast cells and basophils	
Function	Main blood antibody of secondary responses, neutralizes toxins, opsonization	Main antibody of primary responses, best at fixing complement; the monomer form serves as the B cell receptor	Secreted into the mucus, tears, saliva, colostrum	Antibody of allergy and antiparasitic activity	B cell receptor

IgG is the most abundant immunoglobulin in the blood and can be further divided into four isotypes (IgG1, IgG2, IgG3 and IgG4) each with distinct structural features, biological functions, and roles in the immune response (table 1.4) (Doolan et al., 2009). IgG1 and IgG3 are widely found to be associated with protection while IgG2 and IgG4 are the non-cytophilic subclasses (Dobaño, Santano, et al., 2019). IgG1 is effective in opsonization and phagocytosis through FcγRIIa engagement while IgG3 is superior at activating NK cells via FcγRIII, and is particularly important for mediating antibody dependent cellular cytotoxicity (ADCC) against *Plasmodium*-infected cells (Dobaño, Santano, et al., 2019; Gaoqian Feng et al., 2021; Opi et al., 2021)

Table 1. 4: Properties of IgG subclasses

Table adapted from (Vidarsson et al., 2014 and Kuby Immunology)

IgG Isotype	General structure	Percentage of total IgG	Main Function	Complement Activation	Fc receptor Binding	Response
IgG1		60 – 65%	Versatile & effective in binding to a wide range of antigens	High	High	Involved in antibody dependent cellular cytotoxicity (ADCC) & opsonization
IgG2		20 – 25%	Recognizes carbohydrate antigens e.g. bacterial polysaccharides	Moderate	Moderate	Important in immune responses to encapsulated bacteria
IgG3		5 – 10%	Strong in complement activation	Very high	High	Prominent in ADCC & opsonization
IgG4		3 – 6%	Limited inflammatory function	Low	Low	Associated with tolerance & chronic antigen exposure

1.10.3.4 Antibody responses to *Plasmodium* infection

The complex life cycle of the parasite offers the host with multiple potential stages to which antibodies can respond. Cohen et al., was the first to identify the role of antibodies in malaria protection. They demonstrated that passive transfer of purified adult immunoglobulins (IgG) into *P.falciparum* infected children resulted in a marked reduction of parasites and severity of disease (Cohen et al., 1961; McGregor & Carrington S.P, 1963). This seminal work supports the role of antibodies during blood stage infection.

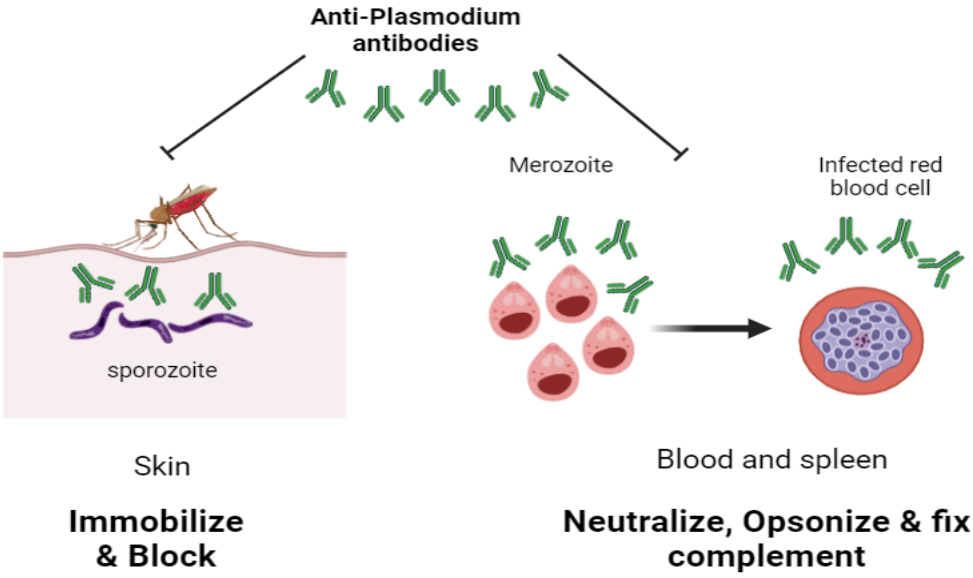


Figure 1. 7: Antibody targets in response to *Plasmodium* infection.

Infection or vaccine induced antibody responses can mobilize sporozoites in the skin or neutralize, opsonize, and activate complement against merozoites or iRBCs. Figure adapted from (Rogers et al., 2021) and designed by E.Kibwana in Biorender.

During pre-erythrocytic stage, sporozoites are injected into the skin, which provides the first step at which antibodies, from either previous exposure or vaccination can respond to *Plasmodium* infection. Studies have demonstrated the present of anti-sporozoite antibodies from natural exposure and that acquisition of these antibodies is age dependent (Offeddu et al., 2012). Recently, Barry *et al.* showed functional naturally acquired antibodies against pre-erythrocytic immunity with anti-CSP antibodies strongly correlated with *in vitro* sporozoite gliding assays and inhibition of hepatocyte invasion in children in Burkina Faso (Barry et al., 2019). The reason for low anti-sporozoite responses is thought to be due to the mosquito injecting small numbers of sporozoites into the skin, and the short amount of time that the sporozoites have in the skin (Amino et al., 2006; Yamauchi et al., 2007), which does not provide the immune system enough time to mount a significant response. As a result, complete protection/sterile protection to the pre-erythrocytic stage is rarely achieved naturally. CSP is the major *P. falciparum* sporozoite surface protein and ever since its identification, anti-CSP antibodies have been considered candidates for mediating protective immunity (V. Nussenzweig, 1989). A vaccine that targets the sporozoite stage effectively could prevent the parasite from infecting the liver cells and progressing to the blood stage where it causes clinical symptoms.

Animal studies and vaccine studies have established that antibodies, particularly IgG, play a crucial role in targeting *Plasmodium* sporozoites through various mechanisms (Beeson et al., 2019). Including impairing sporozoite motility at the inoculation site in the dermis (Flores-Garcia et al., 2018; Hopp et al., 2021) and blocking invasion into the liver (Triller et al., 2017; Wang et al., 2020) through the interaction with Fcγ receptors (FcγR) on various immune cell types. These mechanisms include (i) opsonic phagocytosis, where IgG antibodies bind to the sporozoite and engage Fc receptors (FcγRIIa and FcγRII) on neutrophils and monocytes leading to phagocytosis (Leitner et al., 2020; Musasia et al., 2022), (ii) interacting with serum complement protein, C1q, to activate complement activation, which leads to the lysis and destruction of sporozoites (Behet et al., 2018; Kurtovic, Agius, et al., 2019; Kurtovic, Drew, et al., 2021), and (iii) antibody dependent cellular cytotoxicity (ADCC), where antibodies engage Fc receptors on NK cells, leading to the direct killing of infected cells (Gaoqian Feng et al., 2021). Additionally, antibody isotypes such as naturally acquired anti-CSP IgM has been show to activate the classical complement pathway through recruitment of C1q in malaria exposed adults (Kurtovic et al., 2018). More recently Tan et al., demonstrated that natural exposure and immunization to *P. falciparum* sporozoites induces functional IgA antibodies that were able to inhibit sporozoite invasion into the liver cells *in vitro* and reduce liver parasite burden *in vivo* (Tan et al., 2021).

As demonstrated by Cohen et al., blood stage immunity is mainly driving by antibodies to merozoite antigens (Cohen et al., 1961), additionally to *P. falciparum* variant surface antigens (VSA) that are displayed as knobs on the iRBCs (Julien & Wardemann, 2019). Several merozoite antigens have been identified and show some association with protection (Mensah-Brown et al., 2019; Murungi et al., 2013; Osier et al., 2007). Mechanism of immunity are pre-dominantly Fc driven which include opsonization and complement activation, antibody- mediated agglutination, as well as the recruitment and activation of innate immune cells, promote the lysis and phagocytosis of infected (Julien & Wardemann, 2019; Teo et al., 2016).

1.11 Malaria Vaccines

The Malaria Vaccine Roadmap's strategic goal is to licence vaccines with a minimum efficacy of 75% against both *P. falciparum* and *P. vivax* by 2030 (Malaria Vaccine Funders Group, 2013). However, the complex life cycle of *Plasmodium*, involving both human and mosquito hosts presents a challenge to malaria vaccine development, as the parasite expresses various antigens at each stage of its life cycle, and all serve as potential vaccine candidates. Malaria vaccines can be divided into three main groups (i) pre-erythrocytic vaccines (ii) blood-stage vaccines (iii) transmission-blocking vaccines. However, despite over 100 years of research, a highly effective malaria vaccine that can induce long-lasting protective immunity has yet to be developed (El-Moamly & El-Sweify, 2023; Stanisic & Good, 2023). This thesis will focus on pre-erythrocytic vaccines, specifically targeting CSP.

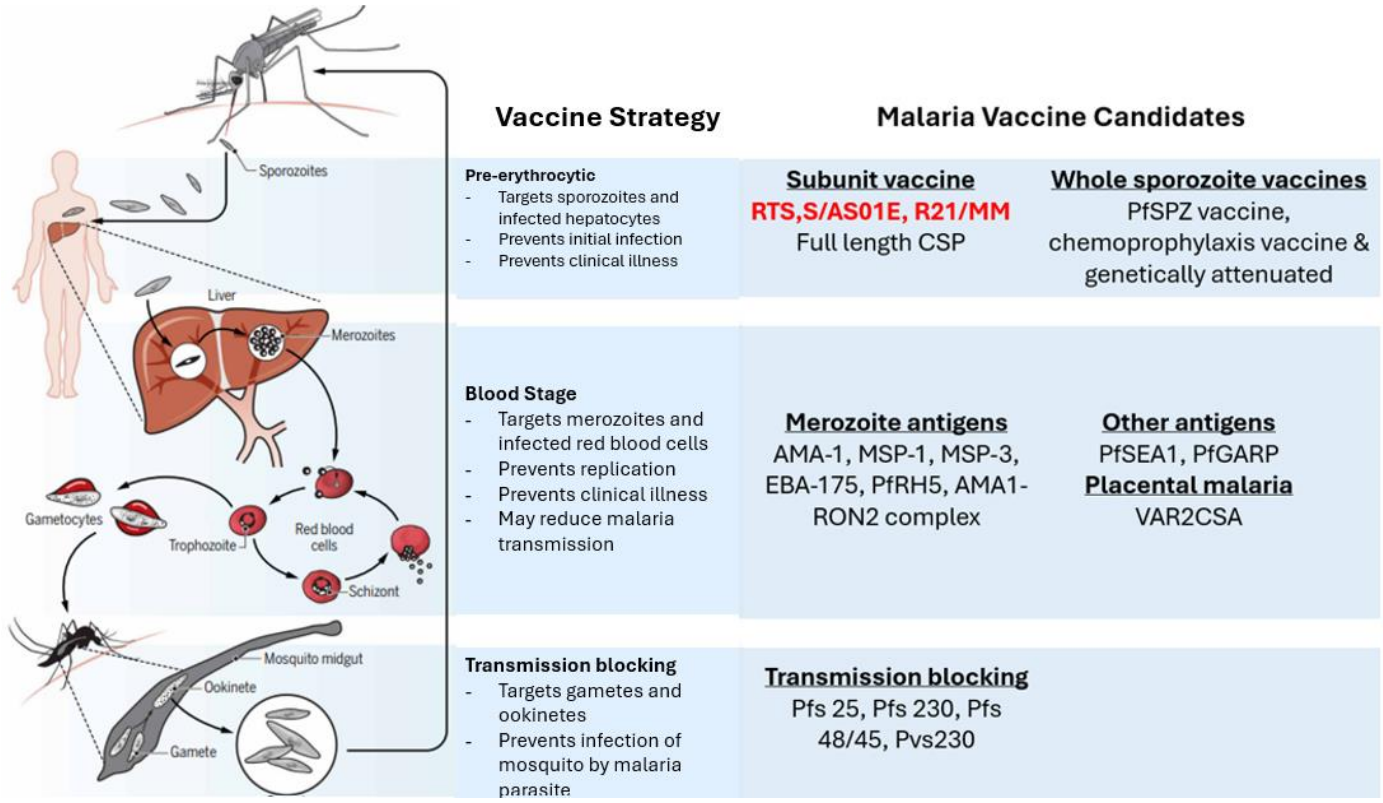


Figure 1. 8*: Plasmodium* life cycle, targets for vaccination and malaria vaccine candidates.
Image adapted from (Beeson et al., 2019; Duffy et al., 2020).

1.11.1 Pre-erythrocytic vaccines (PE)

PE vaccines target the sporozoites and/or infected liver cells. These vaccines aim to induce antibodies that can neutralize sporozoites in the skin and circulation, block hepatocyte invasion by sporozoites and/or induce cell-mediated immune responses that target infected hepatocytes. Vaccines that can prevent pre-erythrocytic development would prevent blood stage infection and hence prevent the pathology. The rationale for pre-erythrocytic malaria vaccines was based off early experiments that demonstrated complete protection in rodents immunised with X-irradiated sporozoites prior to challenge with live parasites (R. Nussenzweig et al., 1969). This work led to human studies that established immunizing naïve adults with X-irradiated mosquitoes infected with *P.falciparum* protected them against challenge with infectious sporozoites (Clyde, McCarthy, et al., 1973; Clyde, Most, et al., 1973; S. L. Hoffman et al., 2002) and in some cases offered long term protection (Roestenberg et al., 2011). Implying immunity to pre-erythrocytic stage was possible and could result in lasting term protection. However, as mentioned the immune system has a small window to amount an efficient response to sporozoites, therefore PE vaccines would need to induce high antibody responses of good quality, to prevent parasite invasion.

1.11.1.1 Whole sporozoite vaccines (WSV)

As previously mentioned, studies in mice and humans confirmed that whole sporozoites can protect against challenge with infectious mosquitoes (Clyde, McCarthy, et al., 1973; Clyde, Most, et al., 1973; R. Nussenzweig et al., 1969). Over the last decade, there has been renewed interest in using WSV, based on the theory that the immune system will generate a boarder response against the sporozoites range of antigens. WSV involve injecting sporozoites that have undergone radiation, so that they can infect hepatocytes, partially develop, and then self-arrest in the liver without causing illness or further transmission (Chattopadhyay et al., 2009).

More recently, Sanaria have also developed a PfSPZ vaccine, composed of aseptic, purified, live (metabolically active), radiation attenuated, cryopreserved PfSPZ (Sanaria) (S. L. Hoffman et al., 2010). They can induce a broad immune response, including antibodies, and T cell responses (Richie, Billingsley, et al., 2015). This vaccine is safe and well-tolerated in naive adults (Epstein et al., 2007; Lyke et al., 2015) with a VE of more than > 90% against CHMI with homologous *P. falciparum* parasites (Richie, Epstein, et al., 2015; Seder et al., 2013) and 83% against CHMI with heterologous *P. falciparum* parasites (Mordmüller et al., 2022). However, when the vaccine was tested in malaria endemic areas, its efficacy was modest, VE was 51% when tested through homologous challenge (S. et al., 2014; Sissoko et al., 2017).

Other attenuated vaccines have been developed, including PfSPZ-CVac, which has been chemically attenuated *in vivo* by anti-malarial drugs (Bastiaens et al., 2016; Roestenberg, McCall, et al., 2009). PfSPZ-CVac involves the administration of PfSPZ challenge together with prophylactic chloroquine (CQ). The PfSPZ-CVac model has shown to be safe and well-tolerated in healthy naïve adults and it prevented infection in 100% of volunteers who underwent CHMI after three doses (Mordmüller et al., 2017; Roestenberg, McCall, et al., 2009). However, when tested in Mali VE to natural infection was only 33.6% (Coulibaly et al., 2022). Further studies are underway to overcome the low VE and immune responses seen in endemic areas by optimizing doses and varying the timing (Coulibaly et al., 2022). Further research is being conducted to improve the low VE and immune responses seen using PfSPZ and PfSPZ-CVac models in endemic areas by optimizing doses and varying the timing.

Lastly, there are genetically attenuated vaccines, so-called PfSPZ-GA1 in which PfSPZ are attenuated through the deletion of essential genes (Richie, Epstein, et al., 2015). A study using PfSPZ-GA1 vaccine, in naïve participants demonstrated that the vaccine was safe and immunogenic, with 3 out of the 25 volunteers showing sterile protection following challenge. Additionally, the vaccinees had a longer patency period than the controls, demonstrating the clinical potential of genetically attenuated PfSPZ vaccines (Roestenberg et al., 2020).

Although WSV are a promising vaccine tool, the practical challenges of the potential cost of implementing WSV like the need for liquid nitrogen cold chain, intravenous dosing, efficacy in pre-exposed populations and the scale-up of manufacture, remain a problem (Duffy et al., 2020).

1.11.1.2 RTS, S

RTS, S/AS01 (Mosquirix), is the first malaria vaccine recommended by WHO in 2021, for the prevention of malaria in children. RTS,S vaccine was created in 1987 as part of a collaboration between GlaxoSmithKline (GSK) and the Walter Reed Army Institute of Research (WRAIR) (Laurens, 2019). RTS, S is based on a large segment of the *P.falciparum* CSP, which is present on the surface of sporozoites, first discovered by Ruth and Victor Nussenzweigs in 1980 (Yoshida et al., 1980). CSP is also expressed by early liver forms and exported into the cytoplasm of hepatocytes (S. L. Hoffman et al., 2015). It is a GPI anchored protein composed of a central repeat region of NANP and NVDP flanked by two conserved regions, the N-terminal domain and the C-terminus α-thrombospondin repeat (α-TSR) domain (Plassmeyer et al., 2009).

The RTS, S vaccine is a subunit vaccine comprising multiple copies (18) of the central Asn-Ala-Asn-Pro (NANP) repeat region, which represents the 'R', and the C-terminus domain of the CSP protein which contains T- and B- cell epitopes (designated T) fused to the N-terminal of hepatitis B surface antigen (HBsAg) (Laurens, 2019). This fusion protein is co-expressed in *Saccharomyces cerevisiae* yeast cells, resulting in a virus like particle (VLP) that expresses both CSP and HBsAg on its surface. The second 'S' in the vaccine is an unfused HBsAg that spontaneously fuses with the RTS portion (Laurens, 2019). The ratio of CSP to unfused HBsAg was 1:4, so the CSP fusion comprises 20% of the molecule in the VLP (Laurens, 2019; Sinnis & Fidock, 2022). The adjuvant AS01 is a liposome-based adjuvant that contains two immunostimulants: 3-O-desacyl-4′-monophosphoryl lipid A (MPL) and the saponin QS-21(a proprietary adjuvant), and is used to further enhance the immunogenicity (Didierlaurent et al., 2017).
Although RTS, S has been recommended for use it does have some limitations. In Phase III trials, the vaccine efficacy (VE) in infants is low, 3 doses of RTS, S/AS01 induced protective efficacy against clinical malaria of 46% in 5–17-month-old children, and 27% in 6–12-week-old infants. This protective efficacy declined during the 38–48 month follow up and at the end of the trial was 28.3% in children and 18.3% for infants (The RTS S Clinical Trials Partnership, 2011).

1.11.1.3 R21

In 2017, the University of Oxford developed R21, a second generation RTS, S vaccine (Collins et al., 2017). This new vaccine is comprised of 19 NANP repeats from the central repeat region and the C-terminus portion of CSP from *P. falciparum* strain NF54 fused to the N-terminus of HBsAg (Collins et al., 2017). They used the *Pichia pastoris* yeast strain as their expressing platform, as it has the advantage of being able to grow to high densities. The virus-like particle (VLP) produced displayed a significantly higher proportion of CSP compared to RTS, S, (figure 1.9) thus enhancing the immune responses to CSP (Collins et al., 2017). Mouse studies demonstrated that R21 is immunogenic and elicits minimal antibodies to HBsAg (Collins et al., 2017). Furthermore, Phase I studies in U.K malaria-naïve and malaria-exposed adults also demonstrated high anti-NANP antibodies following vaccination and an increase in sporozoite inhibition activity with weak anti-HBsAg responses (Venkatraman et al., 2019).

In Phase III trials in children in Africa, vaccine efficacy to R21/Matrix-M (R21/MM) was significantly higher in the younger age group, 5 to 17 months than the older age group 18 to 36 months. VE was 78% (95% CI: 73–82) than in the older age group 70% ([95% CI: 64–74]; p=0·024) respectively. The study was conducted in two seasonal sites and three perennial sites (i.e., where malaria transmission occurs

throughout the year). Overall, 12-month VE was 75% (95% CI 71–79; p<0·0001) at the seasonal sites and 68% (61–74; p<0·0001) at the standard sites for time to first clinical malaria episode (Datoo et al., 2024). In addition to R21's high efficacy and safety, R21/MM also induces robust anti-NANP antibody responses which correlate with VE (Datoo et al., 2024). R21 is also more affordable with a single dose at US$ 2 – US$ 4, vs US$ 10 for RTS, S (WHO, 2023). Mathematical modelling has shown implementing a four-dose regimen of R21/Matrix-M vaccine based on age could prevent 181,825 (range 38,815-333,491) clinical cases per 100,000 fully vaccinated children in perennial settings and 202,017 (29,868-405,702) clinical cases per 100,000 fully vaccinated children in seasonal settings (Schmit et al., 2024).

In October 2023, R21 became the second malaria vaccine to be endorsed by WHO for the prevention of malaria in children (WHO, 2023).

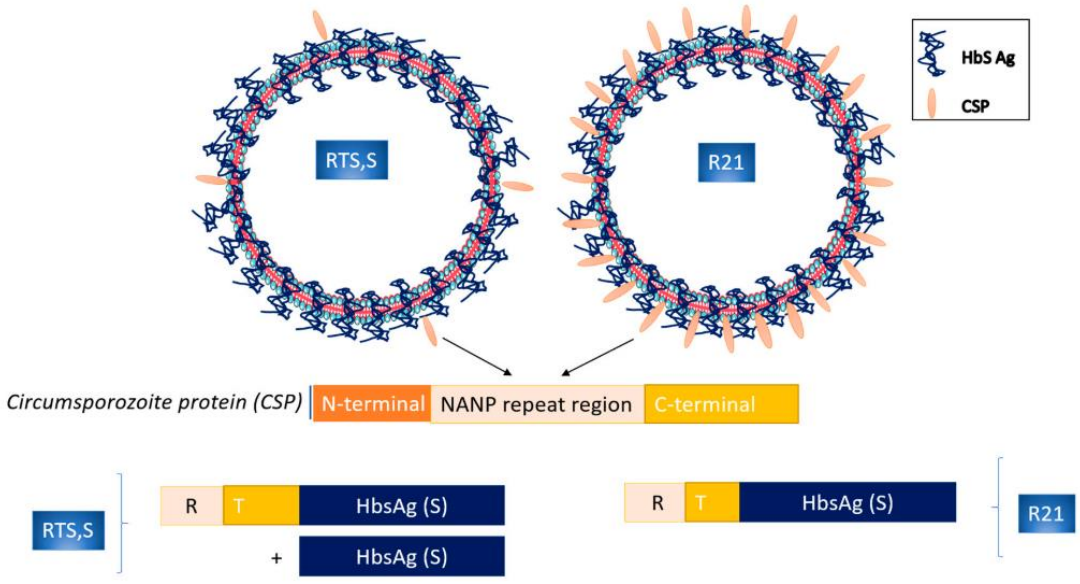


Figure 1. 9: R21 VLP design compared to RTS, S.

Both vaccines consist of the C-terminus portion of CSP from NF54 *P. falciparum* strain fused to the N-terminus of HBsAg. However, RTS, S has extra unbound HBsAg, R21 displays more CSP on the surface. Image from (Tsoumani et al., 2023)

Adjuvants are commonly used in vaccine practice as they enhance and modulate the body's immune responses creating stronger and longer lasting responses, hence improving a vaccines effectiveness (Didierlaurent et al., 2017; Stertman et al., 2023). R21 is coupled with Matrix-M (Novavax), this consists of purified saponin molecules (from the bark of the *Quillaja saponaria* tree) organized into nanoparticles. These nanoparticles form a matrix-like structure with cholesterol and phospholipids. Matrix-M primarily stimulates the immune system by promoting antigen uptake by DCs and macrophages, which enhances antigen presentation and initiates a strong immune response (Stertman et al., 2023).

AS01 used in the RTS, S vaccine is a liposome-based adjuvant system consisting of two immunostimulatory components, the saponin QS-21 (also derived from *Quillaja saponaria*) and the TLR4 agonist monophosphoryl lipid A ((MPL) derived from *Salmonella* LPS) (Didierlaurent et al., 2017). QS-21 and MPL that are encapsulated within liposomes, which helps to stabilise these molecules and delivers them effectively. AS01 stimulates the immune system by a synergistic mechanism. QS-21 stimulates DCs, enhancing antigen presentation, and MPL requires TLR4 signalling on APCs for mediating innate immune responses, including cytokine induction. This dual action enhances antigen presentation but also it directs the immune response to a Th1 type response (Zhao et al., 2023).

1.11.2 Blood stage vaccines (BSV)

These vaccines target merozoites when released from either the liver or iRBCs. Their goal is to block invasion and prevent clinical disease (Miura, 2016). The concept of BSV has been recognized for years because of passive transfer trials in the 1960s that indicated parasite clearance in children from purified adult IgG (Cohen et al., 1961). However clinical trials using blood stage antigens have been disappointing. Development of BSV is very challenging due to multiple reasons, including (i) the brief period of time (seconds) that merozoites are available for antibodies to act between bursting and re-infection of red cells, (ii) antigenic polymorphism of the merozoite protein, which reduces their efficacy in fields studies, (iii) redundant invasion pathways and (iv) the large amount of merozoites that are released and need to be targeted, meaning a potential vaccine would need to induce very high levels of high quality antibodies to neutralize the parasite (Beeson et al., 2019; Duffy et al., 2020; El-Moamly & El-Sweify, 2023; Stanisic & Good, 2023). Clinical trials have been conducted against several immunodominant merozoite antigens, including apical membrane antigen-1 (AMA-1) (Sagara et al., 2009), merozoite surface protein (MSP-1), MSP-2, and MSP-3 (Ogutu et al., 2009; Sheehy et al., 2012; Sirima et al., 2009), erythrocyte-binding antigen-175 (EBA-175) (Koram et al., 2016) and glutamate-rich protein (GLURP) (Bélard et al., 2011).

However, the findings have not be favourable (Beeson et al., 2019; Duffy et al., 2020; El-Moamly & El-Sweify, 2023; Stanisic & Good, 2023).

Due to these disappointing results, other novel merozoite antigens have been identified. *P. falciparum* reticulocyte-binding protein homolog 5 (RH5), has emerged as malaria vaccine candidate. RH5 binds the essential red cell receptor basigin, and is necessary for invasion into erythrocytes, furthermore it is conserved among different parasite strains (Crosnier et al., 2011; Wright et al., 2014). RH5 has shown to induce broadly neutralizing antibodies in mice and shows protection following *P. falciparum* challenge in Aotus Monkeys (Douglas et al., 2015). In humans RH5 has shown to induce high antibody titres and significantly reduced blood-stage parasite growth rate in vaccinated participants (Minassian et al., 2021). Various vaccine delivery platforms and dosing regimens are currently being tested in efforts to improve RH5 as a vaccine candidate. Recently, delayed fractional dosing schedule of RH5 adjuvanted with AS01B demonstrated improved B cell responses in the vaccinees (Nielsen et al., 2023).

1.11.3 Placental Malaria Vaccines

VAR2CSA is a leading vaccine candidate for placental malaria, as it targets interactions between infected erythrocytes and the placental receptor chondroitin sulfate A (CSA) (Sharma & Shukla, 2017). A VAR2CSA-based vaccine aims to induce antibodies to block this interaction, preventing erythrocyte accumulation in the placenta (Duffy et al., 2020). Clinical trials are assessing the safety, immunogenicity, and efficacy of various VAR2CSA-based formulations and have been shown to be safe and well-tolerated, and they induce functional antibodies that inhibit the binding of infected erythrocytes to CSA. However, challenges remain regarding the breadth of protection due to the high genetic diversity of VAR2CSA among different *P. falciparum* strains (Fried & Duffy, 2015; R. Ma et al., 2024).

1.11.4 Transmission blocking vaccines (TBV)

TBV target the sexual stages of the parasite's life cycle with the aim of inducing immune responses that kill or block the parasite before a mosquito blood meal, hence preventing transmission. They specifically target the antigens on gametocytes, zygotes and ookinetes (Miller et al., 2022). They do not prevent infection or clinical symptoms, nonetheless in malaria endemic areas with high gametocytes carriers, TBVs may potentially protect through of herd immunity (Carter, 2001). Several vaccine candidates have been identified, the leading candidates are Pfs230 and Pfs48/45 which are gametocyte antigens, and ookinete surface antigens (Pfs25 and Pfs28) (Duffy et al., 2020). The two leading vaccine candidates are

Pfs25 and Pfs230, antibodies against Pfs25 have shown to stop parasite growth in the mosquito, however these antibodies wane rapidly (Stowers et al., 2000), while antibodies against Pf230 bind to the parasite within the human host and prevent infection to the mosquito (Rausch et al., 2023). Based off preclinical evidence, combing Pfs25 with other antigens was hypothesized to increase its immunogenicity, studies are being carried out using a combination of Pfs25 and Pf230. A recent study found Pfs230 was significantly more potent than Pfs25 as a vaccine, and the addition of Pfs25 did not appear to improve its efficacy (Healy et al., 2021). Studies are underway looking at different formulation and adjuvants in hopes of increasing the immunogenicity of TBV. Challenges with TBV include achieving sufficient antibody titres as well as widespread coverage to accomplish herd immunity (Duffy et al., 2020).

1.12 Controlled Human Malaria Infection (CHMI)

Controlled human infection (CHI) models have been developed for a range of infectious diseases including influenza, cholera, shigella, salmonella and malaria (Choy et al., 2022). They help contribute to our understanding of the pathogenesis, transmission, immune responses and in advancing drug and vaccine development.

Historically, infecting humans with malaria was used to treat neurosyphilis in the 1920s, by psychiatrist Julius Wagner-Jauregg (Tsay, 2013). In 1986, researchers at Walter Reed Army Institute of Research (WRAIR) conducted the first well documented human malaria challenge study. Six malaria naïve volunteers were successfully infected with laboratory cultured *P. falciparum* sporozoites by the bites of laboratory-reared female *Anopheles* mosquitoes (Chulay et al., 1986). With the development of cryopreserved, infectious NF54 *P. falciparum* sporozoites (PfSPZ) from Sanaria, which provides a standardized and reproducible means of performing CHMI research, the number of institutes using the CHMI model has grown. CHMI has been used safely and successfully in thousands of volunteers worldwide and is routinely carried out in the US Military Malaria Vaccine Program (Spring et al., 2014); the University of Maryland, USA (Friedman-Klabanoff et al., 2019); Radboud University Nijmegen Medical Centre (RUNMC), the Netherlands (Sauerwein et al., 2011); and the University of Oxford, UK (Duncan & Draper, 2012). There also several research centres in the African continent using the CHMI model (reviewed in chapter 2).

Parasite challenge can be achieved using infectious mosquito bites (Epstein et al., 2007; Verhage et al., 2005), or by the direct inoculation of sporozoites (Billingsley et al., 2014; Mordmüller et al., 2015) or direct inoculation of *Plasmodium*-infected red blood cells (IBSM) (Duncan & Draper, 2012). The most common *Plasmodium* strains used in CHMI trials are, *P.falciparum* NF54 (an isolate of West African

origin), 3D7 (a clone of NF54), 7G8 (a cloned line of the Brazilian IMTM22 isolate), NF135.C10 (a clone derived from a Cambodian isolate), and HMP02 (an isolate from Ghana), with the latter only available for a blood-stage challenge (Moser et al., 2020; Stanisic et al., 2018).

In CHMI vaccine efficacy studies, volunteers are given one or several doses of the experimental vaccine before being challenged with a known number of sporozoites. Parasitaemia is monitored by microscopy or qPCR, for typically for 21 days prior to treatment. VE is determined by analysing liver to blood inoculum and the parasite kinetics. For example, pre-erythrocytic VE may give sterile protection or partial protection reflected by a reduction in LBI in the vaccinated group relative to controls and a delay to parasitaemia (Draper et al., 2018).

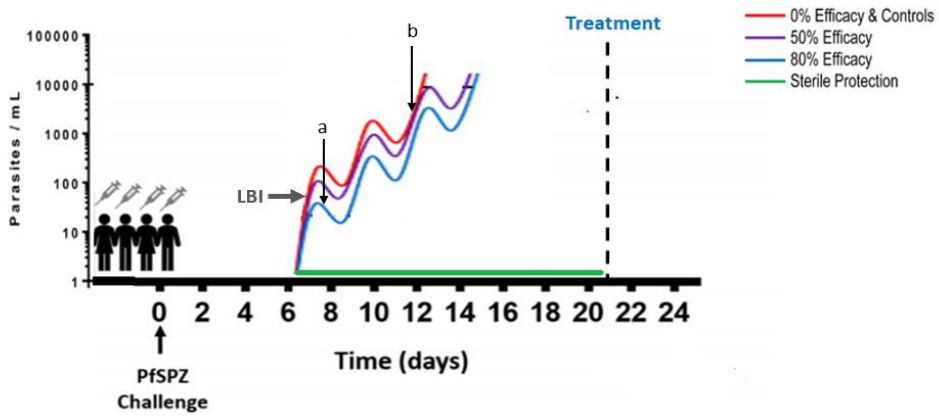


Figure 1. 10: CHMI model for vaccine efficacy.

Image adapted from (Draper et al., 2018)). Cyclic rise in parasitaemia due to asexual reproduction in erythrocytes. (a) parasites become detectable at around 20 parasites/ml for qPCR detection (b) and, 2000 parasites/ml for thick blood smear.

CHMI models are undoubtedly a powerful and valuable tool for accelerating vaccine and drug development by providing rapid and controlled evaluation of the efficacy of the new malaria intervention. It also allows researchers to investigate parasite and host immune responses, offering insights on what immune markers may confer protection. The model does have its limitations and challenges, first, there is a limited number of strains available for challenge, whereas there are antigenically diverse heterologous parasites in the field, secondly it does not mimic the natural route of infection, through the bite of an infected mosquito in the dermis. This is the first stage of infection and is critical for the establishment of

infection as well as induction of the hosts immune response (Sinnis & Zavala, 2012). Finally, in terms of ethics and safety, CHMI studies must be structured to minimize risk to participants, as such they are conducted in adults. Additionally, strong regulatory structures must be in place and adhered to, with oversight by a strict regulatory body that differs by country.

1.13 Correlates of protection

A correlate of protection (CoP) as defined by Plotkin, is a specific immune response to a vaccine that is closely related to and predictive of protection against infection, disease, or other defined end point (Plotkin S., 2008). CoP can additionally be described based on causality summarized in the table below.

Table 1. 5: Terminology for correlates of protection

Table adapted from (Plotkin & Gilbert, 2012)

Term	Definition
Correlate of protection	an immune response/marker that is statistically correlated with protection
Mechanistic CoP (mCoP)	A marker that is mechanistically and causally responsible for protection – i.e. immune markers that play a direct role in the mechanisms that confer protection
Non-mechanistic (nCoP)	a marker that is not mechanistically causal of protection – i.e. immune markers that statistically correlate with protection against a disease but lack a direct functional role in conferring immunity

An example of these two definitions of CoP can be seen with immune responses to the meningococcal vaccine. Immune responses can be assessed using serum bactericidal assay (a functional assay that measures the level of antibody in serum required to kill bacteria by complement activation) or by ELISA (which measures the quantity of antibodies). SBA activity and in turn bactericidal antibodies, are highly correlated with immunity to meningococcal disease and are responsible for vaccine efficacy (Borrow et al., 2005). Conversely, total IgG antibody levels against meningococcal (measured by ELISA) may be induced without causing protection. Thus, bactericidal antibodies are a CoP and an mCoP, they function as mCoP by directly killing bacteria and preventing infection. Whereas total ELISA antibodies are a CoP and an nCoP. They indicate an immune response to the bacteria but they do not necessarily guarantee functional bactericidal activity or protection (Plotkin & Gilbert, 2012). However, vaccine induced

responses are multifaceted and most often involve more than one part of the immune system, and more than one response may be a CoP.

CoP determination is an important part of vaccinology, it allows researcher to assess the efficacy as well as contribute to the design of novel vaccines without necessarily needing large scale clinical trials and from a manufacturers point it helps in licensure of vaccines (Plotkin, 2020; Stanisic & McCall, 2021).

The complex life cycle of the malaria parasites coupled with our incomplete understanding of malaria immunology has made the identification of CoP from both natural infection and vaccine induced immunity extremely difficult. This complexity is compounded by the presence of naturally acquired immunity in individuals living in malaria endemic areas.

For the pre-erythrocytic vaccine, like RTS, S, antibodies against CSP have consistently emerged as potential CoP (Bojang et al., 2001; Kester et al., 2009; Moris et al., 2018; Regules et al., 2016). Additionally, other immune markers have come up as potential CoP such as high avidity antibodies against CSP C-terminus (Chaudhury et al., 2021). Another study found combinations of antibody subclasses, specifically IgG1 and IgG3, and avidity against CSP emerged as CoP (Seaton et al., 2021). While Kurtovic and colleagues measured multiple functional Fc-dependent CSP-antibodies and found those that interact with complement and Fc receptors may play a role in vaccine induced protection (Kurtovic et al., 2020). It is likely multiple immune responses involving both adaptive and innate cells, to multiple antigens are involved with protection.

Using the CHMI model in regions where malaria is prevalent has the potential to aid in the identification of CoP by providing a controlled environment, with known quantities of sporozoites used in challenge, known timing of infection, and known parasite strain used for infection, to carry out detailed analysis of immune responses and markers following vaccination and challenge in individuals with varying levels of pre-existing immunity.

1.14 Aim

R21/MM has demonstrated high efficacy and elicits robust anti-NANP responses to IgG. However, the exact mechanism that confers protection are still unclear. Vaccine induced immunity to malaria is multifaceted and other immune responses are likely to also play a role alongside IgG antibodies. R21 induces mainly humoral responses, in this study I aimed to identify, characterise, and understand the humoral immune responses elicited by vaccination with R21/MM. Additionally, I aimed to see whether any of these responses are involved in protection using the CHMI model in pre-exposed adults.

Due to the higher proportion of CSP being displayed in the R21 VLP, I hypothesize that vaccination with R21/MM will induce improved CSP responses, leading to a better CHMI outcome in the vaccinees.

1.14.1 Chapter Overview

In chapter 2, I present an extensive overview of the current state of research on CHMI studies in the African continent. Here, I explored the establishment, development, and the challenges of using CHMI studies in Africa, with the aim of identifying and analysing the existing gaps. My review sheds lights on the diversity of vaccine candidates that have been tested, the methodology used for parasite challenge and the complex interplay and impact of naturally acquired immunity when using this model in malaria endemic regions.

In chapter 3, the objective was to validate an existing qPCR assay for the quantification of *P. falciparum* malaria parasites used to monitor outcomes in CHMI studies. A validated assay is vital for data accuracy, sensitivity, and reliability of the data, as it can influence clinical decision regarding the administration of treatment to participants. The assay was validated in compliance with the ICH Harmonised Tripartite Guideline on the validation of analytical procedures with the aim of establishing an assay that was fit for purpose.

In chapter 4, I undertook a comprehensive analysis of antibody responses induced by vaccination with R21/MM. My aim was to gain insights into the kinetics of antibody production, the various isotypes of antibodies produced, and the specific antigens targeted. Additionally, I sought to evaluate the durability of these antibody responses as well as their functional properties, such as their ability to activate complement and their avidity for the antigen. I also analysed antibody responses to schizont extract and MSP-1, which serve as markers of naturally acquired immunity, to better understand the role of NAI on vaccine induced responses.

Chapter 5 describes cellular responses to vaccination, specifically I characterized Tfh cells and examined their role in the formation of memory B cells. I also investigated whether additionally vaccine doses influence the total Tfh pool and/or Tfh subsets. Additionally, I measured antigen specific memory B cells to determine the long-term immunogenicity of R21/MM.

In chapter 6, my aim was to identify whether any of the immunological responses measured had an impact in CHMI outcome.

Lastly, in chapter 7, I summarize my findings and provide recommendations for future work.

2 CHAPTER TWO – A review of CHMI studies in Africa

2.1 Introduction

CHMIs are experimental studies in which human volunteers are deliberately infected with malaria parasites, the study endpoint is the detection of a pre-determined density of blood-stage parasites (de Jong et al., 2021; Roestenberg et al., 2017; Stanisic et al., 2018), at which point a study subject is treated with a curative regimen of an anti-malarial drug. In recent years, there has been a shift towards using more sensitive quantitative polymerase chain reaction (qPCR) methods based on 18S ribosomal RNA gene transcripts (Hermsen et al., 2001; Murphy et al., 2012) for monitoring the infection in CHMI studies. This approach offers improved accuracy and efficiency in detecting and quantifying blood-stage parasites compared to traditional microscopy.

There are two main ways that CHMI can be initiated: the sporozoite challenge; this can be carried out by the traditional inoculation of sporozoites by lab-reared mosquito bites (Epstein et al., 2007; Verhage et al., 2005) or by the direct inoculation via needle and syringe of a known amount of sporozoites (Billingsley et al., 2014; Mordmüller et al., 2015; Sheehy et al., 2013) and *Plasmodium*-infected red blood cells, so-called induced blood-stage malaria (IBSM) (Duncan & Draper, 2012).

CHMI models have been extensively used in high resource settings to assess the efficacy of anti-malarial drugs and vaccines. These studies provide a controlled environment where the timing and inoculum dose are well-defined, allowing for the observation of immune responses and the subsequent protection against malaria infection (Roestenberg et al., 2017; Stanisic et al., 2018). Additionally, CHMI models aid in understanding the precise mechanisms of host/parasite interactions in the context of malaria infection. The implementation and impact of CHMIs studies in the Africa are still limited, however, efforts are being made to bridge this gap and leverage the potential of these models in the African context i.e. in malaria endemic areas. One of the limitations in conducting CHMIs in Africa was the logistical challenges brought about by rearing infective mosquitoes and the need for specialized insectary facilities. However, Sanaria Inc., a biotechnology company in Rockville, Maryland, have developed a method that produces aseptic, purified, cryopreserved, infectious NF54 *P. falciparum* sporozoites (PfSPZ) for injection that complies with regulatory standards (Billingsley et al., 2014; Roestenberg et al., 2013; Roestenberg, Mccall, et al., 2009; Vanderberg, 2007). This offers a standardized and reproducible method of conducting CHMI studies, because of this CHMI by injectable PfSPZ is replacing the traditional method of mosquito bite challenge, allowing the model to be more accessible to researchers worldwide.

The use of CHMI models in Africa not only contributes to the evaluation of anti-malarial interventions but also provides valuable insights into the regional differences in immune responses and protection against malaria. Understanding these variations is crucial for the development and implementation of effective malaria control strategies tailored to the specific characteristics of populations in different regions of Africa.

2.2 Aim

The aim of this chapter, was to review and assess the establishment and utilization of *P. falciparum* sporozoite CHMI models in Africa, highlighting progress, recent advances, and challenges in their use in malaria exposed populations.

2.3 Methods

Search Strategy: I conducted a comprehensive search using electronic data bases which included PubMed, Scopus, google scholar, Web of Science and clinicaltrials.gov. I searched using keywords of "controlled human malaria infection," "CHMI models," "*Plasmodium falciparum*," "PfSPZ," "Sanaria," "malaria endemic" and "malaria-exposed individuals," in combination with terms such as "Africa," and "African countries."

Inclusion Criteria: inclusion criteria encompassed any publication or study that described CHMI studies in Africa regardless of study design or publication status carried out using Sanaria's sporozoites. Relevant studies published up to 30th November 2022 were included. Exclusion criteria were applied to exclude studies conducted outside of Africa.

Data Extraction: relevant data including study design, location, participant characteristics, methodology, outcomes, and challenges were extracted from the identified studies. The identified studies provided valuable insights into the establishment and utilization of *P. falciparum* sporozoite CHMI in the African context.

Data Analysis: Relevant studies from the provided sources were analysed for key findings related to CHMI models, naturally acquired immunity and vaccine efficacy assessment. I analysed the data descriptively and identified key themes and trends.

2.4 Results

During my search, I identified 171 studies from various databases. After filtering out duplicates and further screening by title and abstract, I was left with 12 studies that met the selection criteria. Of the 12 studies, three were conducted in West Africa (Mali and The Gambia), four in Central Africa (Gabon and Equatorial Guinea), and five in East Africa (Tanzania and Kenya). All studies used Sanaria's NF54 PfSPZ. The majority of studies (10/12) challenged participants through direct venous injection with 3200 sporozoites. One study from Tanzania challenged participants intradermally (Shekalaghe et al., 2014) and a study in Kenya challenged participants intramuscularly (S. Hodgson et al., 2014). All studies have demonstrated that CHMI is safe and infectious in these populations, despite the route of administration and varying degrees of parasite exposure.

The first modern CHMI study in Africa using cryopreserved PfSPZ was conducted in 2012 (Shekalaghe et al., 2014). The study was conducted in 30 healthy adult males living in Dar es Salaam, Tanzania. The volunteers in this study were considered semi-immune as they had not had a clinical episode of malaria for five years; however, they had a positive *P. falciparum* lysate serology before CHMI, indicating a previous exposure. Volunteers were divided into two dose groups, with one group receiving 10,000 PfSPZ ID and the other receiving 25,000 PfSPZ ID. The study demonstrated that PfSPZ challenge was safe, well-tolerated, and infectious in adults with a previous history of malaria exposure, with 21 out of 23 volunteers being parasite-positive by day 21. Malaria was diagnosed using a thick blood smear, and qPCR was performed retrospectively. Table 2.1 and 2.2 summarizes CHMI studies in Africa.

Table 2. 1: Summary of CHMI studies in Africa

Country	Reference	Year	Study Type	No. of volunteers	Route of CHMI administration	Assay used to measure malaria outcome	Pre-patent period (days [min-max])
Tanzania	(Shekalaghe et al., 2014)	2012	Safety, infectivity & effect of prior malaria exposure to CHMI (NCT01540903)	30	ID	TBS & qPCR*	11-13.5
	(Said A. Jongo et al., 2018)	2014 - 2015	Safety, Immunogenicity, and Efficacy of PfSPZ Vaccine (NCT02132299)	67	DVI	TBS & qPCR*	7.5-9.7
	(Said A. Jongo et al., 2020)	2015 - 2017	Safety and Immunogenicity in Age De-Escalation of PfSPZ Vaccine and efficacy (NCT02613520)	30	DVI	TBS & qPCR*	11.1-12.6
Gabon	(Lell et al., 2018)	2014 - 2016	Safety & Infectivity to CHMI (NTC02237586)	25	DVI	TBS	7.9-10.6
	(Dejon-Agobe et al., 2019)	2015	Safety, Immunogenicity, and Efficacy of GMZ2 Vaccine Pan-African Clinical Trials: PACTR201503001038304	50	DVI	TBS & qPCR	NA
Gambia	(Achan et al., 2019)	2018	Safety, infectivity & effect of prior malaria exposure to CHMI (NCT03496454)	19	DVI	TBS & qPCR*	9-11
Equatorial Guinea	(Said A. Jongo et al., 2021)	2016 - 2018	Tolerability, safety, immunogenicity, and efficacy against CHMI of PfSPZ Vaccine versus PfSPZ-CVac (NTC02859350)	52	DVI	qPCR	8-18 (Group 1: PfSPZ vaccine) 8-16 (Group 2: PfSPZ-CVac)

Table 2. 2: Summary of CHMI studies in Africa

Country	Reference	Year	Study Type	No. of volunteers	Route of CHMI administration	Assay used to measure malaria outcome	Pre-patent period (days [min-max])
Equatorial Guinea	(Said Abdallah Jongo et al., 2022)	2018 - 2019	Safety and Vaccine efficacy of PfSPZ vaccine and regimen optimization (NCT03590340)	104	DVI	qPCR	18 by TBS 14.5 by qPCR
Mali	(Coulibaly et al., 2022)	2017	Safety and efficacy of PfSPZ-CVac (PfSPZ challenge under chemoprophylaxis (NCT02996695)	62	DVI	qPCR	NA
	(Sissoko et al., 2021)	2017	Safety and efficacy of a three-dose regimen of PfSPZ vaccine in adults against homologous CHMI and natural infection	56 (pilot, safety study) 120 (main study)	DVI (Pilot study cohort)	TBS & qPCR*	NA
Kenya	(S. Hodgson et al., 2014)	2013	Safety, infectivity, and effect of prior malaria exposure to CHMI Pan African Clinical Trial Registry (PACTR20121100033272)	28	IM	qPCR*	11.8-16.4
	(Kapulu et al., 2020)	2016, 2017 & 2018	Controlled Human Malaria Infection in Semi-Immune Kenyan Adult (NCT02739763)	142	DVI	qPCR	NA

*qPCR measured retrospectively.
Abbreviations: DVI, direct venous inoculation; IM, intramuscular; ID, intradermal; qPCR, quantitative PCR; TBS, thick blood smear; NA, not available

2.4.1 Impact of natural exposure on CHMI outcomes

Among the 12 studies analysed, I selected those that either had a direct comparison arm examining CHMI outcomes in naïve participants or had sub-divided the study populations into high, medium, and low exposure based on seroprevalence or previous malaria episodes to investigate the effects of NAI on CHMI. Five studies met these inclusion criteria. A study conducted in Tanzania (Shekalaghe et al., 2014) compared the CHMI outcomes of Dutch malaria-naïve (Roestenberg et al., 2013) and semi-immune participants challenged intradermally with varying doses of sporozoites, including a low dose of 10,000 and a high dose of 25,000 sporozoites. They observed that in the low-dose group, five out of six naïve individuals were parasite positive 3 days earlier than the 11 out of 12 semi-immune counterparts, whereas those in the high-dose group (10/11) were parasite positive 1.5 days earlier than the semi-immune individuals (5/6), as measured by qPCR.

A second study compared UK malaria-naïve participants (Sheehy et al., 2013) challenged with 25,000 sporozoites intra-muscularly with Kenyan adults (S. H. Hodgson et al., 2014) with varying levels of exposure, including minimum and definite exposure. All volunteers were diagnosed with malaria in the Kenyan study, except one volunteer who was asymptomatic and negative by microscopy but qPCR-positive at 18 days post-CHMI. In this study, the researchers found no significant difference between the UK naïve participants and Kenyan adults with minimum exposure, as both groups had detectable parasites by day 13 post-challenge. However, there was a significant difference in the time to parasite positivity between the "definitely" exposed group and the naïve and minimum exposure group, with the former being parasite positive 3.5 days (at day 16.5 post-challenge) later than the "minimum" exposed group. A third study in Gabon found that 17 of 19 volunteers challenged by 3200 sporozoites through DVI were parasite-positive by day 28 of follow-up by microscopy. All those in the low exposure group were parasite positive (10/10) by day 9 (SD 1.6), while in the high exposure group, 7 out of 9 were qPCR positive 2 days later, that is, on day 11 (SD 6.3) (Achan et al., 2019).

Lastly, in a more recent study conducted in Kenya, individuals were grouped according to their transmission intensity areas (low, medium, and high), and it was observed that those from the high transmission areas had a longer time to parasite positivity than those from the low and medium transmission areas, and out of 142 participants, 33 had sterile immunity, suggesting that NAI can suppress parasite growth (Kapulu et al., 2022).

Furthermore, several studies have explored the impact of genetic factors on CHMI outcomes. Lell and colleagues grouped adults from Gabon as semi-immune and semi-immune with sickle cell traits and discovered that semi-immune individuals with sickle cell traits had a longer time to parasitaemia compared to naïve participants and compared to the semi-immune without sickle cell traits. Specifically, those with sickle cell trait (7/9) experienced an average of 19.1 days before developing parasitaemia, while the naïve participants (5/5) showed an average of 12.6 days and the semi-immune (9/11) showed an average of 16.9 days till parasite positivity. Furthermore, they demonstrated that although there was no significant effect of the sickle cell trait on the likelihood of infection, there was a significant difference in the development of clinical disease. Only one of nine volunteers with sickle cell traits developed clinical malaria (Lell et al., 2018). Table 2.3 summarizes the findings of studies examining NAI and its effect on CHMI outcomes.

2.4.2 Impact of naturally acquired anti-*Plasmodium* immune responses on CHMI outcomes.

There were four studies that analysed immune responses pre- and post-CHMI. In the Gambia, researchers measured antibody responses to blood stage and sexual stage antigens; apical membrane antigen 1 (AMA-1); early transcribed membrane protein (Etramp5. Ag1); gametocyte exported protein (GEXP18); glutamate-rich protein (GLURP.R2); merozoite surface protein 1.19 (MSP-1.19) and 9 reticulocyte-binding protein homologue (Rh2.2). Here, they observed anti-Rh2.2 ($r = 0.5357$, $p = .018$) and AMA-1 ($r = 0.4959$, $p = .031$) were most predictive of pre-patent period. They also showed a higher growth inhibition assay (GIA) in individuals with high exposure median 21.8% [IQR 8.15 – 29.65%] compared to those with low exposure, median 5.6% [IQR 5.6 – 10.23%] (Achan et al., 2019). In Kenya, Hodgson and colleagues assessed antibody responses to three key blood-stage merozoite antigens MSP1, AMA1, and RH5 and functional activity using GIA and the antibody-dependent respiratory burst activity (ADRB) assay. They observed that antibody responses to schizont extract, and AMA-1 pre-CHMI correlated with parasite multiplication rate (PMR) ($p = 0.044$ and $p = 0.018$ respectively). They also noted a significant difference in ADRB activity between the group with minimum exposure and maximum exposure and a significant increase of ADRB post-CHMI ($p \leq 0.0001$). The one volunteer who was asymptomatic and negative by microscopy had reduced PMR, but had the highest anti-schizont and MSP2 antibody (S. Hodgson et al., 2014; S. H. Hodgson et al., 2016). In the Tanzania CHMI study, Obiero measured antibody responses to CSP, liver-stage antigen 1 (LSA-1), *P. falciparum* lysate, exported protein 1 (EXP-1), and AMA-1. They stratified their participants based on *P. falciparum* lysate responses to seronegative and seropositive, here they observed a significant difference between the two groups in antibody responses to

AMA-1 ($p = 0.005$) and EXP-1 ($p = 0.04$). The seropositive Tanzanian volunteers also had a significantly lower parasite load at the time of first qPCR compared to the seronegative and pre-CHMI anti-CSP responses correlated with pre-patency period by qPCR across all Tanzanian volunteers (Pearson $r = 0.45$, $p = 0.04$) (Obiero et al., 2015). Lastly in Gabon, Lell and colleagues measured antibody responses to PfCSP, EXP-1 and MSP-1 pre- and 28 days post CHMI but found no association between antibody responses and developing parasitaemia (Lell et al., 2018).

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Table 2. 3: Summary of studies on naturally acquired immunity's effect on CHMI outcomes

Country	Exposure history	No. of participants /groups	Route	Sporozoite dose	Time to parasite positivity (TBS)	Time to parasite positivity (qPCR)	No. of participants infected by Day 21	Summary of findings	Reference
The Netherlands	Naïve	6	ID	10,000	13.0	10.0	5/6	Naïve participants had detectable parasites 3 days earlier than semi-immune participants, in low challenge dose group and 1.5 days in high dose	Obiero et al 2015 Roestenberg et al., 2013
		6		25,000	13.0	9.5	5/6		
Tanzania	Semi-immune	12	ID	10,000	15.5	13.0	11/12		
		12		25,000	13.5	11.0	10/11		
United Kingdom	Naïve	6	IM	25,000	13.0	13.0	6/6	Participants that had definite exposure were qPCR positive 3.5 days later than the naïve participants and those with minimum exposure	Hodgson et al., 2016 Sheehy et al., 2013
Kenya	Varying	2 (Minimum exposure)	IM	25,000	12.5	13.0	2/2		
		2 (Definite exposure)		25,000	12.0	16.5	2/2		
		2 (Minimum exposure)		75,000	12.0	12.0	2/2		
		2 (Definite exposure)		75,000	13.0	13.5	2/2		
		10 (Minimum exposure)		125,000	12.0	12.0	10/10		
		10 (Definite exposure)		125,000	12.0	12.0	10/10		

Table 2. 4: Summary of studies on naturally acquired immunity's effect on CHMI outcomes

Country	Exposure history	No. of participants /groups	Route	Sporozoite dose	Time to parasite positivity (TBS)	Time to parasite positivity (qPCR)	No. of participants infected by Day 21	Summary of findings	Reference
The Gambia	Semi-immune	9 (high exposure)	DVI	3,200	14.0	11.0	8/9	Low exposure group were parasite positive 2 days earlier One individual demonstrated sterile immunity (qPCR negative)	Achan et al., 2019
		10 (low exposure)			13.5	9.0	10/10		
Gabon	Naïve & Semi-immune	5 NI	DVI	3,200	12.5	8.0	5/5	Semi-immune individuals with sickle cell trait had delayed time to parasitaemia compared to those without and to naïve individuals. Four individuals achieved sterile immunity	Lell et al., 2018
		11 IA			17.0	8.0	7/11 (9/11 qPCR)		
		9 IS			19.0	10.5	5/9 (7/7 qPCR)		
Kenya	Varying	15 (High Transmission)	DVI	3,200	NA	NA	12/15	33 individuals demonstrated sterile immunity (qPCR negative)	(Kapulu et al., 2022)

Abbreviations: DVI, direct venous inoculation; IM, intramuscular; ID, intradermal; qPCR, quantitative PCR; TBS, thick blood smear; NA, not available; NI, non-immune; IA, semi-immune with haemoglobin HbAA; IS, semi-immune with sickle cell.

2.4.3 CHMI in vaccine efficacy studies

The CHMI model has played a crucial role in the systematic selection of malaria vaccine candidates, including the advanced subunit vaccines RTS,S (Sauerwein et al., 2011), R21 (Collins et al., 2017), and the Sanaria PfSPZ Vaccine (Sanaria Inc., Rockville, MD). To date, vaccine efficacy studies in Africa using CHMI have focused on Sanaria PfSPZ vaccine which has shown promise in malaria-naïve individuals (Epstein et al., 2017; Ishizuka et al., 2016; Mordmüller et al., 2022).

In Africa, I identified two countries that have tested the efficacy of the PfSPZ vaccine using CHMI, Tanzania, and Equatorial Guinea. In the Tanzania study, healthy male adult volunteers were immunized with 5 doses of the PfSPZ vaccine at 1.35×10^5 or 2.7×10^5 followed by homologous DVI CHMI to assess VE (Said A. Jongo et al., 2018). In the group receiving the lower dose, VE against homologous CHMI was 5.6% (1/18 volunteers, protected by proportional analysis), while in the higher dose group, VE was 20% (4/20 volunteers, were protected by proportional analysis). CHMI was conducted 3 weeks after the 5^{th} dose, subsequently all four protected individuals were uninfected against a second homologous CHMI 24-week post vaccination. This was the same immunization regimen used in a trial in the United States that gave 92% and 65% VE against 3- and 24-week homologous CHMI respectively (Epstein et al., 2017).

To determine whether increasing the dose of PfSPZ would increase VE, an age de-escalation study involving adults, adolescents, children, and infants was carried out in Tanzania. VE against homologous CHMI was assessed only in adults. Immunization doses were increased (to 3 doses of 9.0×10^5 *Pf*SPZ or 1.8×10^6 *Pf*SPZ by proportional analysis). In the adult group receiving 9.0×10^5 *Pf*SPZ, VE was 100% however increasing the dose to 1.8×10^6 *Pf*SPZ significantly reduced VE to 33% (Said A. Jongo et al., 2020). Recently, in Equatorial Guinea, researchers used the CHMI model to downselect the dosing regimen for the PfSPZ vaccine. Four groups of adults received multi-dose priming regimens with or without a delayed final dose at 4 or 16 weeks followed by homologous CHMI. Only the vaccine regimen of 9.0×10^5 *Pf*SPZ on study days 1, 8, and 29 provided significant protection (VE of 51.3%) (Said Abdallah Jongo et al., 2022).

Research groups are currently using infectious *P. falciparum* sporozoites in combination with concurrent antimalarial chemoprophylaxis with chloroquine as an immunization strategy (PfSPZ-CVac). Chloroquine kills parasites in the asexual stages, but allows parasite infection of the liver (the pre-erythrocytic stage) (Zhang et al., 1986). The PfSPZ-CVac model has been shown to be safe and well

tolerated in healthy naïve adults, and it prevented infection in 100% of volunteers who underwent CHMI by DVI ten weeks after the last immunization with three doses (Mordmüller et al., 2017). Two such studies have been conducted in Africa. The first study was conducted in Equatorial Guinea, which had two arms: one investigated vaccine efficacy using the PfSPZ vaccine and the other vaccine efficacy of PfSPZ-CVac assessed by DVI CHMI. Vaccine efficacy at a median of 14 weeks after the last PfSPZ-CVac dose was 55% (8 of 13, P = 0.051) and at a median of 15 weeks after last PfSPZ Vaccine dose was 27% (5 of 15, P = 0.32) (Said A. Jongo et al., 2021). The second study was carried out in Mali, where a higher dose of 2.048×10^5 infectious *P. falciparum* was used to measure vaccine efficacy against natural infections (Coulibaly et al., 2022).

Lastly, I found one study that used CHMI to assess VE from an asexual blood-stage candidate, GMZ2. It is a fusion protein of fragments of *P. falciparum* glutamate-rich protein (GLURP) and MSP-3. In this study, 50 semi-immune healthy adults were vaccinated with three doses at 4-week intervals of GMZ2 formulated in either CAF01, alhydrogel, or a control vaccine. CHMI was performed 13 weeks after the last dose to clear any *P. falciparum* infections. The researchers demonstrated that the vaccine was safe and capable of inducing immune responses however they did not observe any protection (Dejon-Agobe et al., 2019).

Table 2. 5: Summary of studies using CHMI to assess vaccine efficacy.

Reference	Year	Country	Group	No. of doses	PfSPZ dose	Timing of CHMI (weeks post last dose)	Pre-patency period (days [range])	No. of volunteers protected	VE
(Said A. Jongo et al., 2018)	2014 - 2015	Tanzania	Controls	NA	NA	-		0/18	n/a
			2	5	1.35×10^5	3	9.2	1/18	5.56%
			3	5	2.7×10^5	3	9.0	4/20	20%
			3	5	2.7×10^5	24	-	4/4	100%
			4	5	2.7×10^5	24	8.7	0/5	0%
(Said A. Jongo et al., 2020)	2015 - 2017	Tanzania	1a	3	9×10^5	3	-	3/3	100%
			1b	3	1.8×10^6	7.4	8.43 [8.0 -12.2]	2/6	33%
			1a	3	9×10^5	24	10.9 [7.8, 17.5]	3/3	100%
			1b	3	1.8×10^6	37	7.78	1/6	16.7%
			Placebo	NA	NA	n/a	7.9 [7.9–10.7]	0/6	n/a
			CHMI control	NA	NA	n/a	7.9 [7.9–10.7]	0/12	n/a

Table 2. 6: Summary of studies using CHMI to assess vaccine efficacy.

Reference	Year	Country	Group	No. of doses	PfSPZ dose	Timing of CHMI (weeks post last dose)	Pre-patency period (days [range])	No. of volunteers protected	VE
(Said A. Jongo et al., 2021; Urbano et al., 2018)	2016 - 2018	Equatorial Guinea	1a Vaccination weeks: 0,8 & 16	3	2.7×10^6	14	11 [8 -18]	7/15	27%
			1b PfSPZ-CVac Vaccination weeks: 0,4 & 8	3	1.0×10^5	15	8 [8 – 16]	8/13	55%
			Control	NA	Saline	n/a	8.5 [8 – 15]	3/7	
(Said Abdallah Jongo et al., 2022)	2018 - 2019	Equatorial Guinea	1 Vaccination days: 1,3,5,7 & 113	5	9.0×10^5	8	-	10/17	39.8%
			2 Vaccination days: 1,3,5,7	4	9.0×10^5	8	-	11/21	30.4%
			3 Vaccination days: 1,3,5,7 & 29	5	9.0×10^5	8	-	7/18	10.7%
			4 Vaccination days: 1,8 & 29	3	9.0×10^5	8	--	14/21	51.3%
			Controls	NA	Saline	NA		6/19	
(Sissoko et al., 2017)	2014	Mali	Vaccinees	5	2.7 x 105	Natural infection	-	14/41	29%
			Controls	NA	Saline		-	3/40	
(Coulibaly et al., 2022)	2017	Mali	Vaccinees Vaccination weeks: 0,4 & 8	3	2.048×10^5 (PfSPZ-CVac)	Natural infection	-		25%
			Controls	3			-		

2.5 Discussion.

The work of Sanaria in developing PfSPZ-based products has enabled research centres in endemic areas to use the CHMI model to evaluate the impact of NAI on host-parasite interactions, as well as to assess immunization strategies and vaccine efficacy in populations that closely resemble the target population for malaria vaccines. Studies have demonstrated that CHMI can be conducted safely and effectively in endemic settings in adults with pre-existing immunity.

These studies have highlighted that individuals with higher levels of pre-existing immunity are more likely to have (i) a delayed time to diagnosis by qPCR compared to naïve adults, (ii) fewer clinical symptoms, and (iii) low levels of parasitaemia detectable by qPCR but not high enough to warrant treatment. Furthermore, some individuals exhibited sterile protection. Sterile immunity can be influenced by several factors including age, previous exposure, and naturally acquired immunity. Lell et al. hypothesized that sterile immunity might involve a combination of adaptive immune mechanisms. These include antibodies that prevent hepatocyte infection, T-cell-mediated cytotoxicity that eliminates infected liver cells, and antibodies that target blood-stage infection, ensuring the efficient removal of the initial wave of merozoites that exit infected hepatocytes (Lell et al., 2018).

Additionally, some studies have also investigated the effects of CHMI on immune responses, mainly antibody responses, in malaria-exposed volunteers. The results indicated that semi-immune adults had higher antibody levels against a range of blood- and pre-erythrocytic stage antigens (S. H. Hodgson et al., 2016; Lell et al., 2018; Obiero et al., 2015). Notably, in all the studies, the researchers were able to classify the participants into "high/definite exposure" and "low/minimum exposure" groups based on previous exposure serology data measurements to pre-defined antigens. In the "high exposure groups," antibody responses to measured malarial antigens were generally higher, the time to diagnosis was delayed, and they were better able to control the infection as demonstrated by lower parasite densities compared to the "low exposure groups." This difference may be attributed to varying exposure quantities and/or exposure to multiple parasite strains which, in turn, may lead to a stronger memory B cell recall response following CHMI (Achan et al., 2019; S. H. Hodgson et al., 2014; Lell et al., 2018; Obiero et al., 2015). However, no consistent association was observed between antibody levels and PMR or parasite density across the studies, nor was there a clear association with any functional assays. This suggests that, while pre-existing immunity may provide some level of protection against CHMI, the specific immune responses involved are not yet fully understood.

A challenge in interpreting the impact of NAI using the CHMI model is the uncertainty surrounding the natural *P. falciparum* strains encountered by semi-immune individuals. In the field, *P. falciparum displays* wide genetic diversity (Neafsey et al., 2008), which is presently not represented by the available laboratory strains for CHMI. Currently, a limited number of defined *P. falciparum strains are* used in CHMI: NF54 (an isolate of West African origin) (Delemarre-Van de Waal & De Waal, 1981), 3D7 (a clone of NF54), 7G8 (a cloned line of the Brazilian IMTM22 isolate) (Burkot et al., 1984), NF135.C10 (a clone derived from a Cambodian isolate) (Teirlinck et al., 2013), and HMP02 (an isolate from Ghana), with the latter available only for blood-stage challenge (Moser et al., 2020; Stanisic et al., 2018). All the studies I reviewed used the *P. falciparum* NF54 strain (Sanaria), and this does not represent all antigenically diverse strains naturally circulating in Africa. While this may complicate assessments of the associations between exposure and clinical immunity in CHMI, it simultaneously affords an opportunity to evaluate heterologous protection within the context of CHMI, which is a pivotal concern in vaccine development. Also the Sanaria PfSPZ strain is a aseptic purified product unlike what is seen in nature, mosquito saliva contains a variety of proteins that can either enhance or inhibit the infectivity of *Plasmodium* sporozoites through for example immunomodulatory properties (Arora et al., 2023)

The use of PfSPZ, a whole organism malaria vaccine, has shown great promise in malaria-naïve individuals, showing up to 100% protection against homologous and short-term protection of 80% VE against CHMI with heterologous CHMI (Epstein et al., 2017; Lyke et al., 2017; Mordmüller et al., 2017; Seder et al., 2013). However, VE is much lower in the field. This difference may be explained by the cumulative history of *P. falciparum* exposure in African studies. People living in malaria-endemic areas are often exposed to *P. falciparum* infections multiple times throughout their lives. This could result in an altered immune system, which may lead to a downregulation/immunosuppression of immune responses to the PfSPZ vaccine when compared to the malaria-naïve population (Calle et al., 2021). This can result in a diminished ability to respond effectively to vaccination, as the immune system may be less capable of mounting a strong response due to prior infections and/or ongoing exposure, leading to a lower VE. Another explanation for the lower VE observed in the heterologous challenge could be that the challenge strain did not represent the genetic diversity of the parasite in the field, as mentioned earlier. Moser and colleagues conducted whole-genome sequencing of the Sanaria PfSPZ vaccine strain NF54 and compared this against the strains used in heterologous challenge (7G8, NF166.C, and NF135.C10), and a collection of clinical isolates relative to the reference strain 3D7 (a clone of NF54) (Moser et al., 2020). The authors confirmed that NF54 and 3D7 are genetically similar and, as expected, considering their respective

geographical regions 7G8 and NF166.C8, and NF135.C10 was genetically distinct from NF54 and 3D7, with tens of thousands of variants being detected between them. These variations may affect the ability of the immune system primed with NF54 to recognise other *P. falciparum* field strains, impairing VE against heterologous CHMI (Moser et al., 2020). Studies have also demonstrated that the efficacy of PfSPZ vaccine is dose- and regimen-dependent, with VE efficacy improving when either or both are changed in both homologous/heterologous CHMI. Caution should be taken when interpreting VE outcomes when using challenge models, as field VE may be overestimated when measured using homologous CHMI (Sissoko et al., 2021). The same applies when comparing VE results between studies conducted at different study sites, target populations, and different follow-up time points. The latter is particularly important when time-to-infection analysis is used in field studies. Further studies are underway to overcome the low VE and immune responses seen in endemic areas by optimizing doses and varying the timing.

Although the majority of the studies focused on VE towards the PfSPZ vaccine, I did find a single study that examined the effectiveness of a potential blood-stage malaria vaccine. Although this vaccination did not result in protection for the population in question, the study demonstrates that CHMI with sporozoites is a potential method to downselect blood-stage vaccine candidates in individuals living in malaria-endemic areas.

It is important to note that most of the CHMI studies described here used direct venous injection (DVI) which circumvents the skin completely. The dermis is the first stage of infection and is critical for the establishment of infection and induction of the host immune response. Various studies have discussed and described the importance of skin immunity in *P. falciparum* infection (Daily, 2018; Flores-Garcia et al., 2018; Ménard et al., 2013; Mitchell et al., 2022). The skin provides the first barrier for protection against pathogens and can recognise and respond to sporozoites, activating both innate and adaptive immune responses. Haeberlein et al. demonstrated that the route of administration of attenuated malaria parasites significantly affects the quality and mechanism of malaria immunity in a mouse model. DVI administration elicited robust systemic CD8+ T cell responses, whereas ID and subcutaneous (SC) administration induced more localised immune responses, with a stronger reliance on CD4+ T cells (Haeberlein et al., 2017). Therefore, CHMI studies using DVI as the inoculation route may have implications for assessing both NAI and VE. In this literature review, I did not find any study that looked at the different routes of challenge and vaccine efficacy. The VAC074 clinical trial will hopefully address this issue, as a challenge will be carried out by both ID and DVI.

2.6 Conclusion

In this review, I have shown that CHMI studies are being conducted successfully in several institutions in Africa. I have discussed the introduction, development, and challenges of modern-day *P. falciparum* CHMI studies conducted in Africa and the impact of naturally acquired immunity on infectivity and vaccine efficacy. CHMIs have been shown to be an invaluable tool, particularly in accelerating malaria vaccine research by downselecting potential vaccine candidates as well as offering critical insights into the mechanisms of immunity against malaria, particularly in populations with varying degrees of prior exposure Although there are limitations of CHMI studies, their strength lies in proof-of-concept efficacy data at an early stage of development, providing a faster way to select vaccines for further development and providing valuable insights into the mechanisms of immunity to malarial infection.

3 CHAPTER THREE – qPCR validation for measuring *P.falciparum* parasitaemia

3.1 Introduction

CHMI studies are established methods for evaluating the efficacy of antimalarial drugs and early-stage vaccines, as well as for defining and identifying the host/parasite immune mechanisms involved in protection. Quantification of parasite density is a crucial part of CHMI studies; the gold standard monitoring and quantification of malaria infection post-CHMI has traditionally been conducted by microscopic examination of the peripheral blood. This method is inexpensive, extremely sensitive, and specific when used by expert microscopists. The sensitivity of blood smear detection is about ~4-50 parasites/µl of blood depending on the number of fields examined (Mpina et al., 2022; Seilie et al., 2019). However, this method has limitations: the accuracy and interpretation of the method are highly dependent on the expertise of the microscopist, it is laborious and time-consuming, and it has poor specificity and sensitivity in cases with low parasitaemia, especially in areas of low malaria transmission and usually detects parasites in blood only after 10-11 days post-CHMI (Mpina et al., 2022; Okell et al., 2009).

There are alternatives to microscopic blood smear examinations, such as rapid diagnostic tests (RDTs), which have the advantage of being simple to use and provide results in 20 min, and thus are particularly useful in remote rural areas, as they do not require laboratory or specialised equipment (Kattenberg et al., 2011; Odaga et al., 2014). RDTs are based on the detection of parasite antigens by specific monoclonal antibodies, the most important of which are the histidine-rich protein 2 of *P. falciparum* (HRP2), which is specific for *P. falciparum*, and *Plasmodium spp.* lactose dehydrogenase (pLDH), which detects all species of human malaria (Wilson, 2013). Similar to microscopy, RDTs have the following limitations: firstly, the limit of detection is very high (approximately 100-200 parasites/µl) (Picot et al., 2020), Secondly, their ability to accurately identify and distinguish non-P. falciparum malaria species is poor. Thirdly, HRP2 can remain in the bloodstream post-treatment, potentially leading to misinterpretation of results as it may indicate a previous rather than a current infection. Lastly, as most RDTs in use target HRP2, any antigenic variations in this protein could result in false negative outcomes (N. Lee et al., 2006).

To overcome the challenges presented by microscopy and RDTs, various nucleic acid tests (NATs) have been developed to detect and quantify pre-microscopic patent blood-stage parasitaemia at lower parasite densities and to provide definite species identification. The most common *Plasmodium* NAT target is the conserved 18S (small subunit) ribosomal RNA coding gene (Kimura et al., 1997) or the asexual type 18S rRNA (Murphy et al., 2012) which can be detected by quantitative PCR (qPCR), nested PCR, nucleic acid sequence-based amplification (NASBA) and quantitative reverse transcription PCR (qRT-PCR) (S. H.

Hodgson et al., 2015). The 18S rRNA is a fundamental component of the ribosome, essential for protein synthesis. In *P. falciparum*, there are two distinct types of 18S rRNA genes: one is primarily expressed in the mammalian host (A-type) and the other in the mosquito vector (S-type) (Seilie et al., 2019). Each *Plasmodium* parasite expresses thousands of 18S rRNAs from a few coding genes which enhances the sensitivity of the detection methods (Murphy et al., 2012; Seilie et al., 2019). Based on extensive analytical and clinical data, the US Food and Drug Administration (FDA) has qualified the Plasmodium 18S rRNA biomarker for the diagnosis of P.falciparum infections in CHMI trial participants (Seilie et al., 2019). NATs have been shown to be more effective in detecting low levels of parasitaemia than microscopy, with higher sensitivity, specificity, and reliability. They also performed well with larger sample sizes and blood volumes, as demonstrated in studies by Hodgson in 2015 (S. H. Hodgson et al., 2015) and Imwong in 2014 (Imwong et al., 2014).

In this chapter, I focus on validating the accuracy of a qPCR method that targets the 18S RNA gene. The use of qPCR not only enables the absolute quantification of infections but also lowers the limit of detection to less than 1 parasite per microlitre (Kamau et al., 2011, 2014). In CHMI models (post-challenge), qPCR detection occurs 2-5 days earlier than blood smear, with all naive volunteers showing parasites on day 7 after CHMI (Walk et al., 2016). The use of qPCR has enhanced the reproducibility and statistical power of CHMI studies (Sauerwein et al., 2011).

3.2 Aim

To validate a previously developed quantitative PCR assay for the quantification of malarial parasite densities in whole blood following the ICH Harmonised Tripartite Guideline Part II: Validation of analytical procedures (ICH, 2014).

3.3 Rationale

Ensuring the integrity and rigor of the qPCR assay through comprehensive validation is imperative for reliable quantification of vaccine efficacy and safety, thereby facilitating informed decisions regarding potential regulatory actions. Validation of the qPCR assay is necessary to establish a heightened level of confidence in the data produced via the qPCR assay.

3.4 Materials and Methods

3.4.1 *P. falciparum* culture and maintenance

P. falciparum 3D7 culture vials with a parasitaemia of 1-5% at the ring stage were thawed rapidly in a water bath at 37°C and then moved to a 50 ml falcon tube. While gently shaking the tube, 200 µl of solution 1 (12% NaCl) was slowly added followed by five-minute incubation period at room temperature (RT) before adding an additional 10 ml dropwise amount of thawing solution 2 (1.8% NaCl), while gently mixing thoroughly and was left to stand for 5 min. Finally, another 10 ml of thawing solution 3 (0.9 % NaCl and 0.2 % glucose) was added dropwise. The cells were then washed twice using R0 (RPMI 1640 medium (Gibco/Invitrogen) supplemented with 2 mM L-glutamine, 37.5 mM HEPES (Gibco/Invitrogen), 25-µg/ml Gentamicin (Invitrogen), 0.2% glucose (Sigma) and 50.4-µg/ml Sodium Hypoxanthine (Sigma)) by centrifugation at 440 g for 5 min. After the last wash, the supernatant was discarded, and the pellet was resuspended in 5 ml R10 (R0 + 10% Fetal calf serum). The cultures were gassed for 30 seconds with mixed gas consisting of 3% (v/v) O2 , 5% CO2 (v/v) and 92% N2 and incubated at 37°C in an incubator (Trager & Jensen, 1976). Fresh culture media and fresh O^+ cells were added every 2 days by centrifugation at 440 g for 5 min, the old medium was discarded, and fresh culture medium was added. Parasite growth was followed by examination of thin blood smears stained with Giemsa stain. Parasite cultures were maintained at approximately 2-5% parasitaemia.

3.4.2 Sorbitol synchronization

Cultures were synchronised at the ring stage using the D-sorbitol lysis technique (Lambros & Vanderberg, 1979). The D-sorbitol treatment approach exploits the susceptibility of mature parasite forms, such as trophozoites and schizonts, to osmotic stress, compared to younger ring forms. This results in a predominantly synchronised population of parasites at a similar life stage. Parasites were harvested by centrifugation (440 g for 5 min), and 5% of freshly prepared, pre-warmed D-sorbitol (Sigma–Aldrich) was added to the pellet (10 times the volume of the culture pellet). The mixture was then incubated in a water bath at 37 °C for 20 min and gently mixed three times during this period. After incubation, the cultures were washed twice with R0 and the supernatant containing the mature stages was carefully removed. The remaining pellet containing synchronised ring-stage parasites was resuspended in 10 ml R10, gassed, returned to the incubator, and maintained at > 10% parasitaemia.

3.4.3 Flow Cytometry

A flow cytometry-based method adapted from Bei et al. (2010) was used to determine parasitaemia(Bei et al., 2010). The method utilised SYBR Green (Molecular Probes), which specifically adheres to double-stranded DNA, with any detected fluorescence attributable to parasite DNA. SYBR Green emits at a similar wavelength to fluorescein isothiocyanate (FITC). To confirm that the cultures were at the ring stage, a thin Giemsa slide of the culture was prepared and examined under a light microscope. This examination revealed the presence of ring-stage parasites, confirming that the cultures were at the desired stage following the sorbitol synchronization. Subsequently, the cultures were pelleted by centrifugation (1800 rpm for 5 min) and washed twice with R0. Ten microlitres of the pellet were removed and stained with SYBR Green. The remaining culture pellet was resuspended in R10 and left at RT to maintain them at the ring stage until flow cytometry analysis.

The 10 µl parasite culture was incubated with 1 ml of SYBR green (diluted to 1 in 1000) for 30 min at RT in the dark, after which the cells were washed twice in 1x PBS by centrifugation (5 min at 1800 rpm) and resuspended again in 1 ml of PBS. Once ready, 200 µl of this sample was analysed using flow cytometry data collection on a BD FACS Canto (BD Biosciences), acquiring data from approximately 100,000 events per sample. Initial gating was performed with unstained infected erythrocytes to account for autofluorescence.

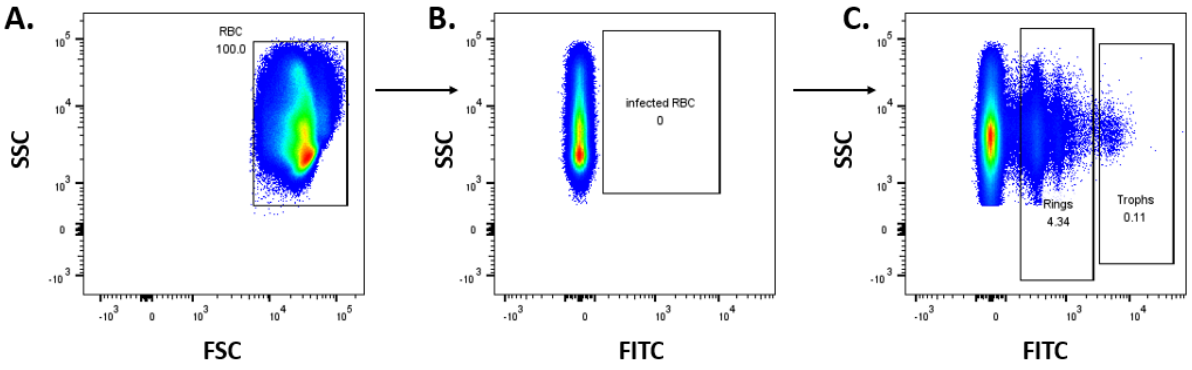


Figure 3. 1 Gating strategy used to determine iRBCs in FlowJo.

Synchronized cell cultures at ring stage, were stained with SYBR green and read on the flow cytometer. (A) Gating of red blood cells, (B) unstained control sample and (C) gating showing ring stage (early and late) and trophozoite stage of parasites. As the culture transitions from rings to early trophozoites the parasite begins to replicate its DNA.

Hence, ring-stage parasites have lower fluorescence intensity compared to trophozoites due to their smaller size and lower DNA content (Bei et al., 2010).

The percentage of rings as determined by the flow cytometry was then multiplied by $1x10^7$ packed red cells to determine the parasitaemia per microliter. Using the above example, experimentally we observed the percentage of rings to be 4.34% which would give approximately 4.34×10^5 parasites/µl.

3.4.4 Spiked *P.falciparum* blood sample preparation

To ascertain the reliability, precision, and sensitivity of the qPCR assay, a meticulous validation process was undertaken. This involved the preparation of control red blood cell samples spiked with varying levels of parasitaemia. Healthy adult donors confirmed negative for *P. falciparum* via rapid diagnostic tests (OptiMAL, Flow Inc., Portland, Oregon) provided uninfected blood samples. These samples were then spiked with known quantities of either freshly harvested or cryopreserved asexual *P. falciparum* parasites determined by flow cytometry above. A series of two-fold dilutions of the parasitized blood into uninfected blood generated samples with parasitaemia ranging from approximately 0.25 to 2500 parasites per microliter using cryopreserved parasites, and from around 5.5 to 55,000 parasites per microliter with fresh parasites.

3.4.5 DNA extraction

DNA was extracted from whole blood spiked with *P. falciparum* or cultured ring-stage parasites using an automated DNA extraction and purification QIAsymphony machine (Qiagen, Hilden, Germany) according to the manufacturer's instructions. QIAsymphony SP performs fully automated purification of nucleic acids using magnetic particle technology. Sample preparation using QIAsymphony SP typically involves four main steps: lysis, binding, washing, and elution. During the lysis step, samples were lysed at the lysis station. In the binding step, nucleic acids bind to the surface of magnetic particles. Contaminants were removed during the washing step, and purified nucleic acid was eluted.

DNA extraction was performed on four different sample volumes (200, 400, 500, and 1000 µl) to determine whether the sensitivity of the assay would be increased when using larger blood extraction volumes. Each extraction was performed alongside the known positive and negative control samples. DNA was eluted in 50 µL (for extraction volumes of 200 µL) and 100 µL of elution buffer (for 400-, 500, and 1000 µL extraction volumes).

3.4.6 Primers and probes

Primers and probes were designed as previously described by Hermsen et al. (Hermsen et al., 2001). These were based on the 18S ribosomal RNA genes of the *P. falciparum* malarial parasite (accession number: M19173).

- 18S Pf Forward - 5’ GTAATTGGAATGATAGGAATTTACAAGGT 3’
- 18S Pf reverse - 5’ TCAACTACGAACGTTTTAACTGCAAC 3’
- 18S Pf MGB -5’ FAM- AACAATTGGAGGGCAAG-NFQ-MGB 3’

3.4.7 qPCR

The Applied Biosystems 7500 Real-Time PCR system was used for amplification and qPCR. The reaction mixture, which contained 10 µl of purified DNA, was used to amplify the 18S ribosomal RNA gene using a TaqMan assay with primers and probes. The mixture included 1× of universal PCR Master Mix (Thermo Fisher), 10 pmol/µl of each primer, and a 10 µmol Non-Fluorescent Quencher with Minor Groove Binder moiety (NFQ-MGB) probe in a final volume of 50 µl per well. The thermal cycling conditions included one step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, and a final extension at 60 °C for 1 min. The qPCR runs included known cultured parasite standards with eight serial dilutions of extracted DNA run in triplicate, unknown samples in triplicate, positive controls (cultured parasites), and three replicates of Non-Template Control (NTC, (water)).

3.4.8 Generation of the standard curve

The standard curve used for all experiments was prepared from a previous clinical trial conducted at the KEMRI-Wellcome Trust Research Programme in 2019 and was stored at -80^{o}C. Briefly, calibration curve generation commenced with the cultivation of highly synchronised 3D7 ring-stage *P. falciparum* parasites, as previously described, with the percentage of parasitaemia determined via flow cytometry. An approximately 2-fold dilution series was established by serial dilution of the parasites within non-infected whole blood, spanning an eight-step gradient ranging from 15,000 to 2.5 parasites per microliter. The resultant standard curve was plotted by correlating the mean threshold cycle values with the logarithm of parasite DNA concentration. This calibration curve serves as a predictive model for estimating analyte concentrations in test samples based on the observed quantification cycle values.

3.4.9 Validation study and criteria acceptance

The assay was validated in accordance with the ICH Harmonised Tripartite Guideline Part II: Validation of Analytical Procedures (ICH, 2014). The guidelines for assay validation define the parameters that must be investigated to validate a bioanalytical assay: (1) specificity, (2) accuracy, (3) precision (repeatability, intermediate precision, and reproducibility), (4) sensitivity (lower limit of detection), (5) linearity, and (6) range.

Specificity: The specificity of the qPCR assay was determined using WHO external quality assurance (EQA). The scheme was commissioned by the WHO Global Malaria Program and UK National External Quality Assessment Service (UK NEQAS) in 2017 (Cunningham et al., 2020). The EQA scheme offers an independent means for research laboratories to verify the quality of their NAT diagnostic assay. This was achieved by providing well-characterised blinded controls consisting of mixed positive and negative *Plasmodium* samples with varying parasite densities, provided as lyophilised blood (WHO | WHO External Quality Assurance Scheme for Malaria Nucleic Acid Amplification Testing (NAAT EQA), 2018). I extracted from 500 µL of EQA samples using QIAsymphony, followed by qPCR, as described above. The results were submitted to the WHO, which then issued an EQA report.

Accuracy is defined as the degree of closeness of the measured value to the true value.

Precision was determined by the recovery percentage ($(\frac{measured\ conc.}{spiked\ conc.})$x100).

Precision (inter-assay repeatability, intra-assay reproducibility) was used to determine the repeatability and reproducibility of the assay, and DNA was extracted from two to four replicates of a high-, medium-, and low-culture quantified 3D7 parasite, followed by qPCR. Coefficient of variation (CV) was used as a measure of reproducibility.

The lower Limit of quantification (LLOQ) was defined as the ability of the assay to detect very low parasite concentrations and corresponds to the lowest parasite concentration in a sample that can be consistently detected at a 95% confidence level in all replicate wells but not necessarily quantified as an exact value (Almeida-de-oliveira et al., 2019). To establish the LLOQ experimentally, spiked parasite-positive samples were serially diluted 10-fold, covering a range of 2500–0.25 p/µl and qPCR was performed. To ensure robust and reliable results, each concentration was assayed in quadruplicate for three days. The experiment was conducted on four separate occasions with each plate containing three replicates per sample (n = 12). The LLOQ was defined as the lowest concentration that showed at least one positive replicate on each of the three days tested.

The limit of detection (LOD)) was defined as the lowest concentration or amount of analyte that can be reliably detected, but not necessarily quantified. The LOD was used to establish the point at which the target could be reliably distinguished from background noise, and the same experiment used to identify the LLOQ was used to determine the LOD.

Linearity: The linearity of the assay indicates that the relationship between the DNA concentration and cycle threshold (Ct) values is proportional, allowing for accurate quantification. Linearity was evaluated by calculating the coefficient of determination (R^2) from standard curves. The R^2 value indicates how well the experimentally defined cycle threshold (CT) values correlate with the dilution series. For quality assays, the value should be ≥ 0.980. A standard curve was created by plotting the Ct values against the logarithm of the DNA concentration, which was previously determined by flow cytometry. The standard curve is typically a linear relationship (y = mx + b), where y is the Ct value, and x is the logarithmic transformation of the DNA concentration. The slope of the standard curve was also used to calculate the efficiency of PCR using the following equation (the qPCR machine automatically computes the slope of the standard curve based on the threshold cycle (Ct) values and the logarithmic concentrations of the template DNA):

$$E = 10^{\frac{-1}{slope}} - 1$$

The slope of the standard curve should ideally fall between -3.1 and -3.6, corresponding to an amplification efficiency (E) of 90%–110%. This indicates that each PCR cycle effectively doubled the amount of the target DNA.

Reproducibility (inter-lab comparison): An inter-lab comparison was used to assess the performance of our assay against that of an independent laboratory. I obtained a standard curve of purified DNA from *P. falciparum* samples at Jenner Institute, University of Oxford, and conducted the qPCR assay. The Oxford standard curve consisted of a 10-fold dilution, with parasite quantities ranging from 100,000 parasites/ml to 10 parasites/ml. Subsequently, I quantified both the Oxford standard curve samples and unknown spiked samples prepared in-house using our standards, as described in the Methods section. Additionally, I utilised the Oxford standard curve to quantify both our standard curve and the unknown spiked in-house samples.

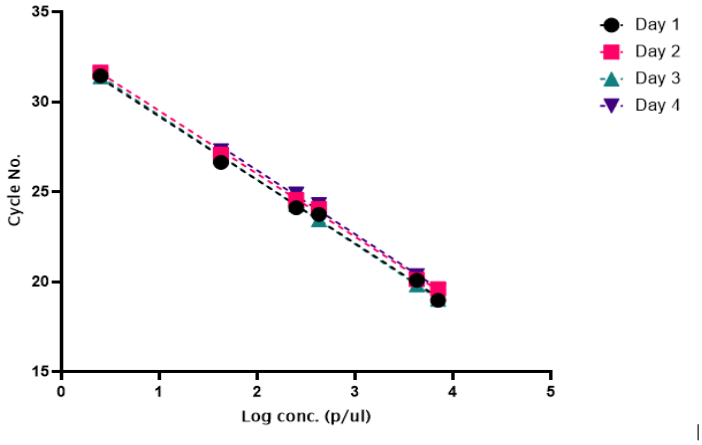
3.5 Statistical Analysis

Data were compiled using Microsoft Excel Version 2410 before being exported into GraphPad Prism version 10.1.2 (GraphPad Software Inc., USA) for Windows. Spearman's correlation coefficient was used to assess correlations between assays. To analyse the effect of sample volume on DNA extraction, the *observed* values (as determined by flow cytometry) were compared to the *expected* values (as determined by qPCR).

3.6 Results

3.6.1 Linearity

The linearity of this qPCR method was assessed by calculating the coefficients of determination (R^2), gradient of the curve, and efficiency. Standard dilutions with known parasite concentrations were prepared and qPCR assays were performed for each dilution to obtain their Ct values. Four separate standard curves were generated by plotting the Ct values against the logarithm of DNA concentration from independent assays on different days. The resulting graph (Figure 3.2) demonstrates a distinct and consistent linear relationship, as evidenced by the decreasing Ct values with increasing logarithm of the concentration. The gradient of the standard curve was calculated and ranged from -3.8 to -3.1. The R^2 values for all four days were ≥ 0.98, while the efficiency values fell within a range of 90% to 100%, indicating strong linearity and effectiveness in the qPCR assay.



	Day 1	Day 2	Day 3	Day 4
Slope	-3.526	-3.499	-3.583	-3.550
R^2	0.997	0.999	0.999	0.995
Efficiency (%)	92.1	93.1	90.4	91.2

Figure 3. 2: Construction of the standard curve for quantifying parasitaemia.

Serial six-fold dilution of cultured 3D7 parasites (n=3 for each point), ranging from 7000 to 2.5 p/µl showed a linearity (R^2 > 0.99). Parasites were extracted from 500 µl volumes. Black, red, green, and purple represent the standard curves of day 1, day 2, day 3 and day 4 respectively.

3.6.2 Concordance between qPCR and flow cytometry determined parasitaemia

To compare the accuracy of the SYBR Green I flow cytometry method with that of this qPCR assay for the determination of parasitaemia, I conducted a correlation analysis. P. falciparum 3D7 parasites were serially diluted and resulting parasitaemia was measured by the flow cytometry-based method and qPCR. A strong linear correlation was observed ($R^2 = 1$), indicating a strong positive correlation between the two methods.

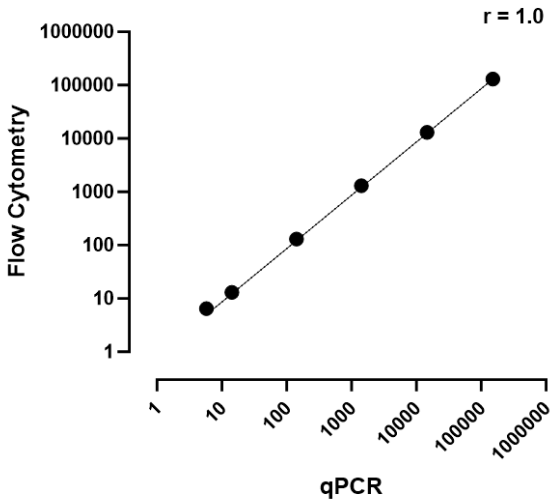


Figure 3. 3: Correlation between flow cytometry and qPCR for identification of unknown samples.

Six samples were spiked with varying parasite concentrations and measured using the two methods. Y-axis represents parasite quantities as determined by FACS (in parasites/µl), and X-axis represent parasite quantities as determined by qPCR (in parasites/µl)

3.6.3 Specificity

To determine specificity, I ran the qPCR using our primers and probes on well-characterized blinded controls. No amplification occurred in any of the non-target *Plasmodium* species tested. Additionally, no Ct value was observed in the non-template control or internal negative controls (uninfected whole blood), indicating the absence of non-specific amplification or contamination. Furthermore, only samples containing *P. falciparum* were successfully amplified, resulting in a 100% specificity (n = 9). An internal positive control (3D7 cultured parasites) was also included in the run, which showed positive amplification results.

Table 3. 1: Specificity of the qPCR method using blinded *Plasmodium* samples from UK NEQAS.

***Plasmodium* sp.**	**No. of samples**	**No. of positive results**
P. falciparum	9	9
P. vivax	5	0
P. malariae	5	0
P. ovale	6	0
P. knowlesi	1	0

Samples tested between 2021 and 2022

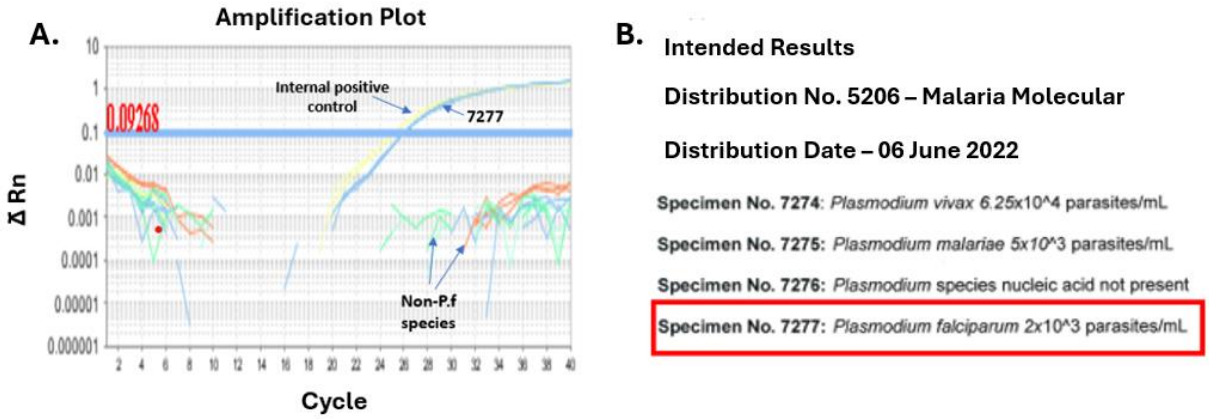


Figure 3. 4: Specificity test.

The qPCR assay was run on blinded UK NEQAS samples. (A) Only samples containing *P. falciparum* showed an amplification curve (WHO Sample 7277). (B) Extract from the WHO NAAT distribution 5206 results sheet. The positive control was an in-house known positive control (cultured iRBCs).

3.6.4 LOD and LLOQ

LLOQ: The LLOQ is the lowest concentration or amount of analyte that can be reliably quantified with acceptable accuracy and precision. To quantify the LLOQ for the qPCR assay, titrated samples with known quantities of P. falciparum 3D7 ring-stage parasites were used. These samples underwent a 10-fold serial dilution process, resulting in concentrations ranging from 2500 to 0.25 parasites/µl. Subsequent qPCR analyses established the LLOQ at 2.6 parasites/µl evidenced by a CV of 14.3% and an average cycle threshold of 34.52 (n = 12 for each parasite concentration tested). These findings suggested a 95% probability of detecting the 3D7 strain of the parasite at or above this concentration when the assay was conducted. The LOD is the lowest concentration or amount of an analyte that can be reliably detected, but not necessarily quantified, with a defined level of confidence. This threshold was experimentally determined to be 0.3 parasites/µl (n = 12 for each parasite concentration tested).

3.6.5 Accuracy

Accuracy was validated by creating four different concentrations (1300, 130, 13, and 6.5 parasites/µl) by spiking frozen culture parasites into fresh whole blood. DNA was extracted using QIAsymphony, followed by qPCR. Accuracy was assessed by determining the recovery rate, which indicated an average percentage recovery of 89.8-110.2% with a coefficient of variation below 20%. Additionally, precision was ensured by conducting replicate measurements over three days at each concentration to thoroughly validate the findings.

Table 3. 2: Measurement of accuracy of qPCR assay.

Spiked concentration	Average Ct values	CV (%) of Ct values	Average measured concentration (p/µl)	CV (%) of measured concentration (p/µl)	Average Recovery (%)
1300	21.68	0.96	1419	2.6	109.2
130	25.22	1.06	142	6.8	109.3
13	28.74	0.82	14.3	6.6	110.2
6.5	30.12	0.85	5.8	8.6	89.8

Tested by measuring the recovery of spiked red blood cells. CV acceptance criteria: <15%; average recovery acceptance criteria >80%. Three independent experiments were performed over 3 days. Abbreviations: p/µl- parasites per µl, CV- coefficient of variation

3.6.6 Precision

I measured precision by calculating the coefficient of variation of spiked blood samples and examining triplicates within a single run (inter-assay repeatability) at various parasite concentrations. Additionally, reproducibility across multiple days (n = 3) and different runs (intra-assay repeatability) was assessed through three repeated day assays conducted by different operators for four parasite quantities (high, medium, low, and LLOD). The observed variation in both inter-assay and intra-assay precision remained minimal with a coefficient of variation below 10% (Table 3 and 4).

Table 3. 3: Intra-assay repeatability based on qPCR CT values.

Control Quantity (p/µl))	No. of replicates	C_T mean	Standard Deviation	CV (%)
High	3	20.0	0.53	2.7
Medium	3	23.7	0.26	1.1
Low	3	26.6	0.09	0.3
LLOD	3	31.4	0.49	1.6
Negative	3	-	-	-

Table 3. 4: Inter-assay reproducibility based on qPCR quantity values.

Control	Samples per run	Mean Quantity (p/µl) (n = 3).			Mean quantity (n = 3)	CV (%)
		Day 1	Day 2	Day 3		
High	3	14330.8	15028.9	14525	14628.4	2.5
Medium	3	139.4	134.1	152.3	142.0	6.8
Low	3	5.83	5.34	6.34	5.8	8.6
Negative	3	-	-	-	-	-

Abbreviations: p/µl- parasites per µl, CV- coefficient of variation, – no amplification

3.6.7 Reproducibility (inter-lab comparison)

To assess the performance of this qPCR in comparison to another laboratory using the same method, an extracted and purified P. falciparum 3D7 DNA standard curve, generated from counted parasite culture, was obtained from the Jenner Institute, Oxford. The Jenner qPCR assay is essentially identical to the current qPCR assay undergoing validation both adapted from (Hermsen et al., 2001). The concentration of their standard curve ranged from 100,000, 10,000, 1000, 100, 50, 25 and 12.5 parasites per ml. The linearity of the Kilifi qPCR assay was evaluated using Jenner's established standard curve for an accurate comparison. The standard curve showed good agreement in quantitation, specificity, and linearity between sites. The R^2 of the Oxford standards was 0.988, and the efficiency was >94%, well within the acceptable range.

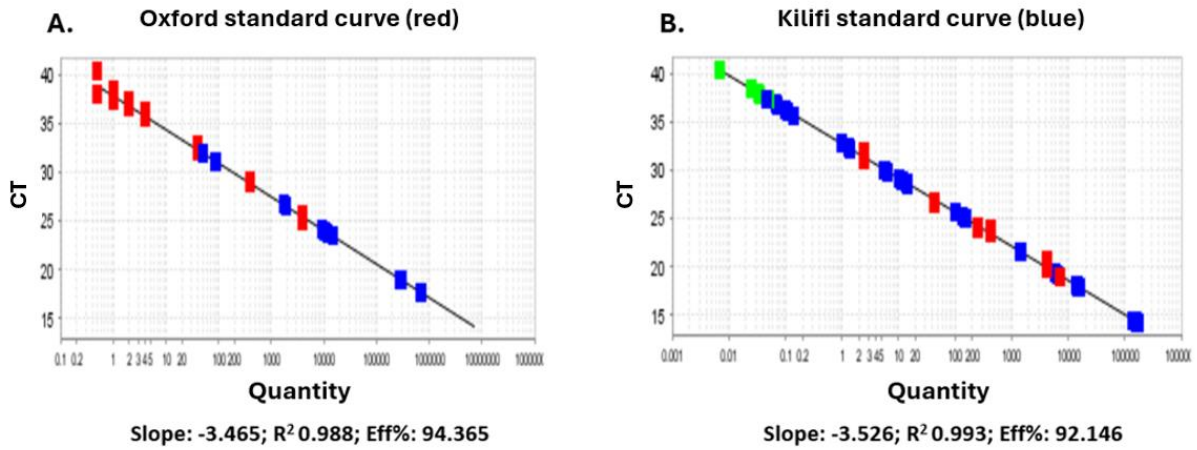


Figure 3. 5: Linearity of the standard curves

(A) Standard curve obtained from the Jenner Institute, Oxford, performed at the KEMRI-Wellcome Trust, Kilifi.
(B) Standard curve used in the Kilifi qPCR assay. Log10 parasite/µl vs Ct.

Additionally, I quantified the spiked blood samples using Jenner's standard curve alongside our Kilifi standard curve and compared them to the results obtained from flow cytometry to validate the accuracy and reliability of our qPCR assay. The results also showed a high level of agreement, with CVs <15% between the three quantities.

A.

	Estimated quantity by FACS	Observed quantity using Oxford standards	Observed quantity using Kilifi standards	CV (%)
Spiked 1	130000	121463.1	151512.4	11.5
Spiked 2	13000	11278.6	14628.4	12.9
Spiked 3	1300	1102.8	1419.4	12.6
Spiked 4	130	113.7	140	11.0
Spiked 5	13	11.8	14.3	9.7
Spiked 6	6.5	4.9	5.8	14.3

B.

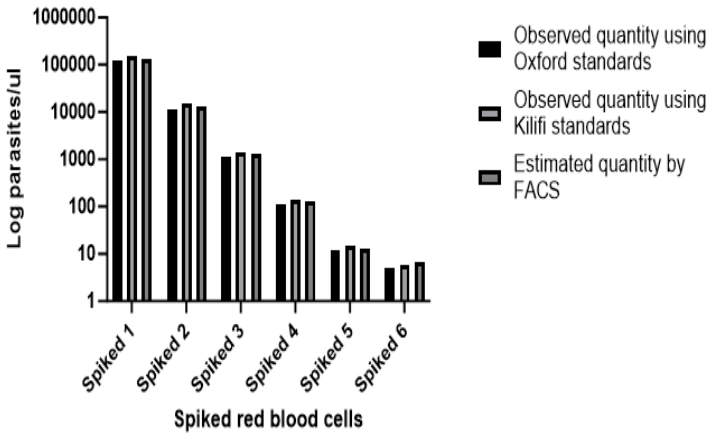


Figure 3. 6: Quantification of spiked red blood cells in parasites per microliter.

(A) Absolute quantities determined using FACS and qPCR using two independently generated standard curves (Kilifi curve and Oxford curve). (B) Graphical representation of the data showing no difference between the three different parasitaemia measured. Showing the average of 3 experiments

3.6.8 Sensitivity in relation to high blood extraction volume

I investigated the impact of extraction volume on qPCR performance by analysing DNA extracted from four different sample volumes (200, 400, 500, and 1000µl) at varying parasite concentrations (ranging from 2500 to 2.5 parasite/µl). By testing various volumes, I aimed to evaluate how the quantity of DNA in a sample affects the sensitivity, efficiency, and accuracy of qPCR. qPCR analysis showed a significant effect of the extraction volume on the assay results. A comparison between the observed (experimental) values and the expected quantities obtained from flow cytometry indicated that smaller extraction volumes resulted in values closer to the expected quantity, whereas larger extraction volumes led to higher variation and deviation from the expected values. The findings of this experiment are shown in Figure 3.7.

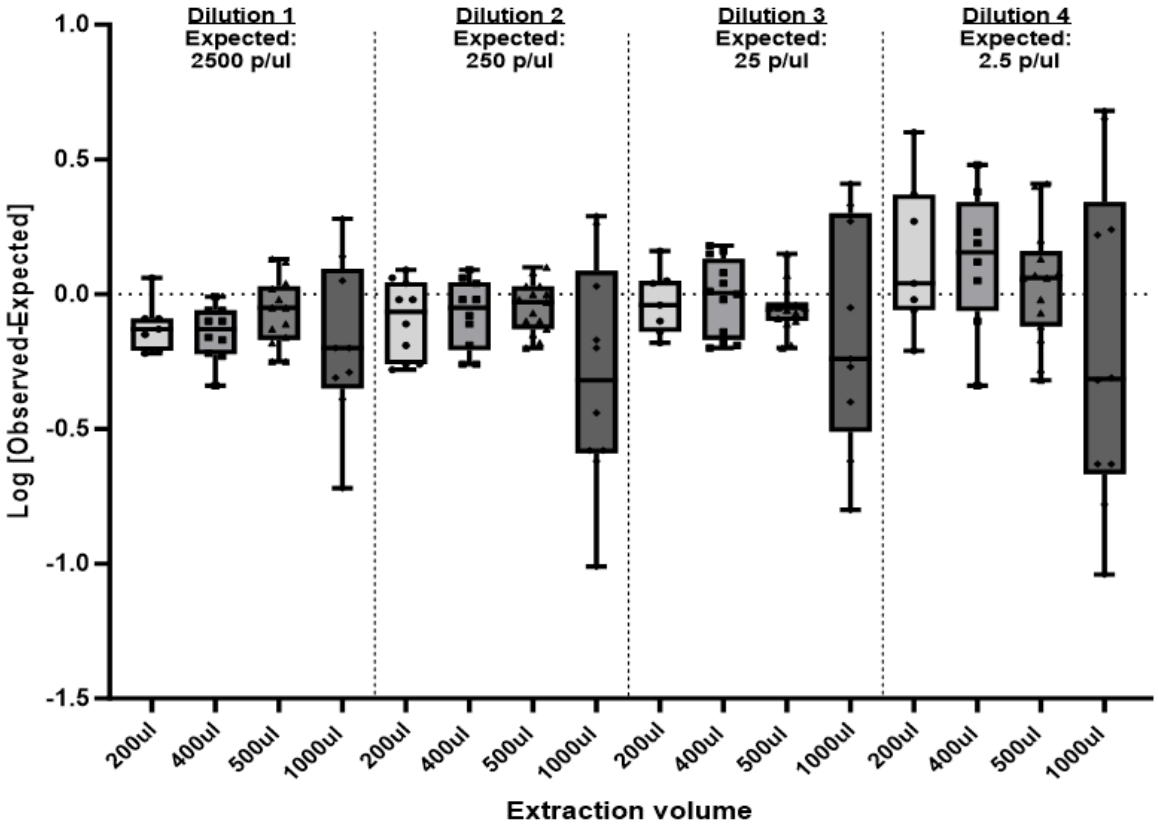


Figure 3. 7: Effects of different extraction volumes on the qPCR assay.

DNA was extracted from different sample volumes using the QIAsymphony to determine the effect of sample volume on the outcome of the qPCR assay. DNA was extracted from 200 µl, 400 µl, 500 µl and 1000 µl. Data showing [Log Observed-Log Expected] mean with ranges. Abbreviations: p/µl- parasites per µl

3.7 Discussion

Assay validation is essential for monitoring clinical trials and a high degree of confidence is required in the data obtained. Assays must be fully validated to provide this assurance, particularly when used to determine vaccine or drug efficacy. In CHMI vaccine studies, volunteers generally develop sub-microscopic parasitaemia for several days before they become thick smear-positive (Roestenberg et al., 2012; Walk et al., 2016). The pre-patency period is crucial for estimating vaccine efficacy, and early treatment mitigates the clinical risks posed to participants. Here, I describe the validation of a qPCR method for the detection and quantification of malaria parasites from whole blood of human volunteers who underwent CHMI at the KEMRI-Wellcome Trust Research Program.

These results indicated that the assay achieved a high level of precision, linearity, and specificity. The method was optimised using primers targeting the *P. falciparum* 18S RNA gene across a wide range of parasitaemia. The primers used for validation were specific to the target *Plasmodium* species, ensuring that only the desired parasite DNA was amplified and detected. This specificity was further confirmed by the absence of amplification in the non-target *Plasmodium* species and in the non-template control, confirming the reliability of the qPCR assay for accurately identifying and quantifying *P. falciparum*. Assessment of specificity was limited (n = 9). However, in routine use at the KEMRI-Wellcome Trust, the specificity is high, with 1 of 40 EQA samples being positive when in fact it was negative.

I additionally examined qPCR and flow cytometry findings to evaluate the relationship between the two techniques. The results revealed a high level of agreement between the two methods, with a correlation coefficient of 1 (Pearson's correlation, $p<0.001$). This strong correlation indicates that the qPCR assay provides reliable and accurate quantification of parasite densities, further validating its effectiveness as a tool for monitoring clinical trials and assessing the efficacy of malaria interventions. Determining the LLOQ is crucial for establishing treatment endpoints. I found that the lowest number of parasites/µl detected in the qPCR assay was 2.6, which demonstrated good sensitivity. This assay easily captured parasitaemia above the threshold with high confidence. In spiked recovery experiments, the results of the assay showed that the precision ranged from 89.8% to 100%. These findings suggest a high level of accuracy and precision of the qPCR assay, confirming the reliability of the data obtained. These results illustrate that the DNA extraction method exhibits good extraction efficiency.

The validation process of the qPCR assay included an independent comparison with standard curve samples provided by Jenner Institute, Oxford. Their qPCR assay also underwent a similar validation process for their CHMI studies. Upon receiving the standard *P. falciparum* DNA samples from Jenner

Institute, we conducted a qPCR assay to compare the linearity of our qPCR method with their standard curve. Based on experimental evidence, the comparison showed a high level of agreement between the two standard curves, with an R^2 value greater than 0.98, and a PCR efficiency of over 92% for both curves. The results showed a consistent and parallel relationship between the Ct values and logarithm of DNA concentration, demonstrating the reliability and accuracy of this qPCR technique. Additionally, I utilised both their standard curve and our own curve to quantify spiked control samples. The results indicated a high level of concordance between quantification using the two different standard curves, with a Pearson correlation coefficient (r) value of 1.0, further validating the robustness and consistency of our qPCR assay. This comparative analysis with an independent laboratory provided further assurance of the accuracy and reliability of our qPCR method, reinforcing its suitability for use in clinical studies and broader applications involving quantification of *P. falciparum* parasites.

The impact of using a larger DNA extraction volume on the sensitivity of the assay was also investigated. It has been demonstrated that increasing the extraction sample volume accelerates early diagnosis and/or increases the sensitivity when using qRT-PCR (S. H. Hodgson et al., 2015; Imwong et al., 2014). However, these studies used different methods for blood processing and DNA extraction. The data generated here showed that increasing the DNA extraction volume to 1 ml did not increase the sensitivity of this assay. In theory, a larger volume would increase the sensitivity of the assay by extracting more parasite-infected blood; however, in practice, the assay becomes less reproducible and therefore less robust at 1 ml volumes. A similar deterioration in reproducibility was not observed when 200, 400, or 500 µL extraction volumes were used. As expected at lower parasite dilutions of 0.25 parasite/µl, there is substantial variability at all extraction volumes, since 0.25 parasite/µl is lower than the established LLOQ of 2.6 parasite/µl. When the sample volume was increased to 1 ml, this qPCR assay was no longer reliable, as demonstrated by the higher variability (wider confidence intervals) and efficiency of qPCR which decreased to ~80%. Efficiency refers to the extent to which PCR amplifies the target DNA during each cycle. Efficiencies less than 90% suggest that the reaction does not perform optimally at this higher extraction volume. I speculate that this is due to PCR inhibitors, as the volume of the sample increases, as do any PCR inhibitory substances. Studies have shown that heme, IgG, leukocyte DNA, and anticoagulants (EDTA and/or heparin) can play a role in PCR inhibition by affecting either DNA polymerase activity, binding to genomic DNA, or binding to fluorescent dyes, thereby causing fluorescence quenching (Al-soud & Rad, 2001; Sidstedt et al., 2018). As a result, the reliability of the assay was compromised at the 1 ml extraction volume, leading to significant variability and inconsistency

in the data. The assay acceptance criteria were as follows: linearity of the standard curve of R2 0.98, efficiency 90-100%, accuracy 90-100%, intra-assay precision CV <5% (between replicates of the same sample), and inter-assay precision CV <10% (between plates, i.e. if the same sample was run across different plates on different days).

3.8 Conclusion

In summary, the comprehensive validation process presented here demonstrates the suitability of the malaria qPCR assay for use in clinical studies and other investigations involving the quantification of *P. falciparum* parasites in human whole blood in the field and clinical CHMI studies. The high specificity, linearity, precision, and sensitivity of the assay at volumes < 500µl make it a valuable tool for accurately detecting and quantifying malarial parasites, providing essential data for the monitoring of clinical trials and the assessment of vaccine or drug efficacy.

4 CHAPTER FOUR – Humoral responses to vaccination

4.1 Introduction

Vaccines play a crucial role in preventing the spread of infectious diseases and safeguarding public health (Rodrigues & Plotkin, 2020). Infections such as smallpox have been eradicated using vaccines (McGough & Bodine, 2024), whereas polio is on the verge of eradication (S. E. Lee et al., 2023). Although the development of an effective malaria vaccine has been challenging, the WHO has recommended two malaria vaccines for the prevention of malaria in children in the past two years: RTS, S/AS01, and R21/MM (WHO, 2023). R21/MM is the second malaria vaccine to be recommended for use in 2023, following RTS S/AS01, which was recommended in July 2022. R21 builds on the design of RTS, S which achieves only moderate efficacy in the field (Kaslow & Biernaux, 2015; Laurens, 2019; The RTS S Clinical Trials Partnership, 2011).

Both vaccines are based on a large segment of *P. falciparum* CSP, which is present on the surface of sporozoites (R. S. Nussenzweig et al., 1972). *P. falciparum* CSP is a 397 amino acid protein composed of a N-terminal region, highly repetitive central region, presenting with amino acids repetitions (NANP and NVDP) and a C-terminus region. Epitopes within the NANP central repeat region are the major protective B-cell epitopes (Seder et al., 2013; Zavala et al., 1983). Phase 3 trials with R21/MM have shown vaccine efficacy of approximately 75% in areas with seasonal malaria compared to 68% in sites with perennial malaria transmission over a 12 month period (Datoo et al., 2024). Despite impressive efficacy data from R21/MM, challenges remain. Three doses of the vaccine are needed for the primary vaccination course, followed by a booster dose one year later. Additionally, NANP antibodies wane after the third and booster doses (Datoo et al., 2024; Sang et al., 2023).

In preclinical models using pre-erythrocytic candidates, antibody responses to CSP are associated with clinical protection (Abuga et al., 2021; Goh et al., 2019; Kurtovic, Atre, et al., 2021; Moita et al., 2022; Stanisic & McCall, 2021). During the pre-erythrocytic stages, antibodies function by (i) inhibiting sporozoite motility in the dermis and/or liver (Flores-Garcia et al., 2018) or (ii) impeding sporozoite invasion into the liver ((Triller et al., 2017; Wang et al., 2020). Furthermore, antibodies may interact with different immune cells by inducing processes such as antibody-dependent phagocytosis (Leitner et al., 2020; Musasia et al., 2022), antibody-dependent complement deposition (Behet et al., 2018; Kurtovic, Agius, et al., 2019; Kurtovic, Drew, et al., 2021), and antibody-dependent cytotoxicity (Goaqian Feng & Beeson, 2024). There is clear evidence that antibodies play a significant role in providing protection against clinical malaria, particularly during the blood stage of the disease (Cohen et al., 1974; Fowkes et

al., 2010; Marsh & Kinyanjui, 2006). Vaccine-linked protection against malaria using candidates based on CSP has yet to be conclusively attributed to any specific immune mechanism (Stanisic & McCall, 2021).

Vaccine-induced immune responses are often multifaceted, making it challenging to identify immune mechanisms that confer protection against infection and/or disease. The identification of correlates of protection and the development of *in vitro* assays that accurately predict protection are crucial for assessing vaccine efficacy and informing future vaccine designs. R21/MM induces strong and sustained anti-NANP IgG responses in both children and adults, which is associated with vaccine efficacy. Studies on R21-induced immune responses have primarily focused on measuring total NANP-specific IgG levels. (Datoo et al., 2024; Sang et al., 2023; Venkatraman et al., 2019). However, the specific antibody features (affinity, avidity, and half-life) that are required for protection are currently unknown. CHMI models provide an optimal platform for investigating the correlates of protection in humans, assessing vaccine immunogenicity, and generating proof-of-concept efficacy data following Phase I trials.

In this chapter, my objective was to improve our understanding of the antibody response to the R21/MM vaccination. To accomplish this, I have focused on identifying and characterising vaccine-induced antibodies in adults. This involved investigating the specificity, affinity, and functional properties of antibodies following R21/MM vaccination. The kinetics and duration of antibody production were also examined to assess the ability of the vaccine to confer durable immunity. In addition, I explored the different isotypes induced by vaccination as well as the factors that may influence vaccine responses, specifically previous parasite exposure. Furthermore, I investigated the role of antibody interactions with the innate immune system, specifically in complement activation. A well-characterised antibody response to a vaccine is essential for evaluating its effectiveness and will provide valuable information on the mechanisms by which the R21/MM vaccine confers protection and aids in the identification of potential correlates of immunity.

4.2 Aim

In this study, I aimed to characterise and identify the dynamics of antibody responses in Kenyan adults following R21/MM vaccination. By investigating the specific immune pathways and the duration of antibody production, I aimed to shed light on the vaccine's ability to confer durable immunity and potentially correlate this with protection following CHMI.

4.3 Rationale

Vaccination is a promising strategy for combating malaria, with several candidate vaccines developed at various stages (WHO, 2021). The R21/MM vaccine, which has recently been licenced, is expected to have a significant and lasting effect on malaria control (Aderinto et al., 2024; Schmit et al., 2024). The target population for any malaria vaccine is children, as they bear the majority of the disease burden (WHO, 2023); however, due to safety and ethical reasons, CHMIs are performed in adults. This study offers a unique opportunity to study immune responses to R21/MM in an endemic area and to relate these responses to challenge infection. This chapter describes the humoral immune response elicited by R21/MM by identifying and characterising the antibodies generated and subsequently correlating these antibody features with protective outcomes following CHMI. Identifying the correlates of protection, including specific antibody features, helps to establish immunological markers that can predict vaccine efficacy. These correlates can guide vaccine development, inform clinical trial endpoints, and facilitate assessment of the effectiveness of new vaccine candidates.

4.4 Materials and Methods

4.4.1 Study Design

This section describes the study design and methods used to collect and analyse the data relevant to this chapter. My PhD project was nested within the clinical trial titled 'Safety, immunogenicity, and efficacy of R21/Matrix-M and ChAd63/MVA-ME-TRAP in the context of CHMI' (Clinicaltrials.gov ID NCT03947190). This was a Phase IIb sporozoite infection study evaluating the safety, immunogenicity, and protective efficacy of two malaria vaccine candidate antigens: R21 adjuvanted with Matrix-M and ChAd63-MVA encoding ME-TRAP. This was an open label, randomised, controlled human malaria vaccine efficacy study. The study was conducted at the KEMRI-Wellcome Research Centre in Kilifi, Kenya and was allocated the study code "VAC 074". This study recruited 80 healthy adults from the Ngerenya sub-location of Kilifi County, an area with low malaria transmission (Mwangi et al., 2005). The inclusion criteria were healthy adults aged 18–45 years, written consent, non-pregnant, non-lactating adult female or adult male, able and willing to comply with all study requirements, agreement to refrain from blood donation during the study, and use of an effective method of contraception for the duration of study for female participants. The participants were randomised into four groups: group 1 received R21/MM (N=24); group 2 received ChAd63/MVA-ME-TRAP (N=24); group 3 received R21/Matrix M (N=14); and group 4 received no vaccine (control group, N=18). Here, I focus on examining responses to R21/MM and will not discuss the ChAd63/MVA-ME-TRAP responses in the following sections.

The R21/MM vaccine was administered as a three-dose regimen on days 0, 28, and 56, and each participant received 10 µg of R21/MM mixed with 50 µg of Matrix M. Group 4 was the control group and did not receive any vaccine. For coronavirus disease 19 transmission risk mitigation, each group was equally split into two cohorts (i.e. groups 1A and group 1B respectively), and the cohorts were enrolled and received vaccinations sequentially over time. Vaccination, sampling, and challenge occurred in a staggered manner. Venous blood samples were drawn periodically for immunological assays before and after vaccination and immediately processed for plasma and PBMCS which were stored at -80 ˚C and liquid nitrogen, respectively. Four weeks after the last vaccination, three groups received ID inoculation with 22,500 aseptic, purified, cryopreserved *PfSPZ* (Sanaria ® PfSPZ Challenge), and one group received DVI inoculation with 3,200 PfSPZ. The participants were housed at an inpatient facility and monitored twice daily to assess *P. falciparum* parasitaemia from days 7 to 21 after the challenge using a quantitative PCR assay. Antimalarial treatment was administered either when i) parasite levels exceeded the threshold

of 500 parasites per µl, ii) the participants experienced signs of illness, or iii) the participant reached day 21 of monitoring on which the CHMI was completed.

4.4.2 Ethical and regulatory approvals

The study was conducted in accordance with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidelines, Good Clinical Practice (GCP), and the Declaration of Helsinki. This clinical trial was registered with ClinicalTrials.gov (NCT03947190). Ethical approval was obtained from the KEMRI Scientific and Ethics Review Unit and the University of Oxford Tropical Research Ethics Committee (approval reference number: OXTREC 38-19). All the participants provided written informed consent.

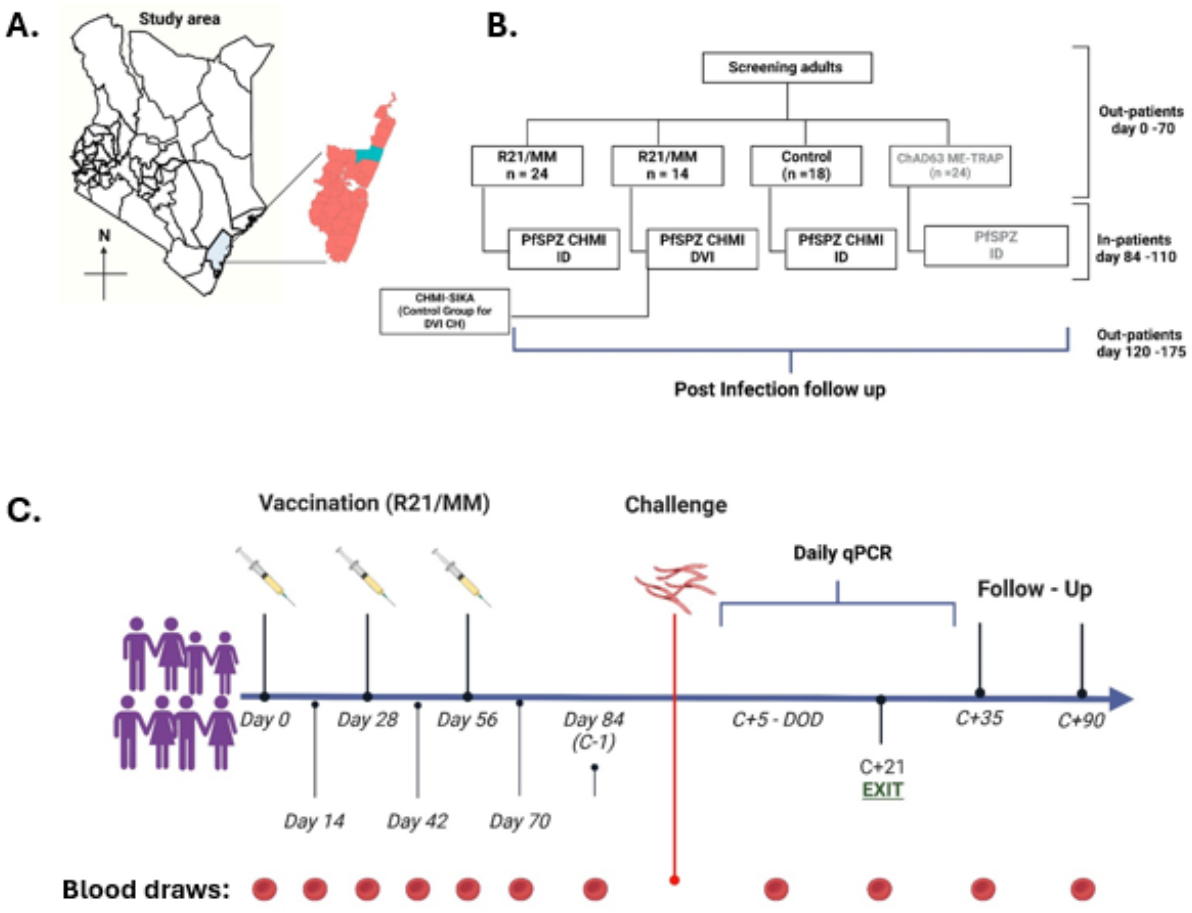


Figure 4. 1: Schematics of VAC 074 Trial Design.

(A) study participants were adults recruited from Ngerenya in Kilifi county, on the East coast of Kenya. (B) schematic of recrutiment and randomization process. (C) vaccination schedule, blood draws and challenge timeline. Syringe represents vaccination, blood cells represent blood draws for peripheral blood mononuclear cells (PBMCs) and plasma, sporozoites represent challenge timepoint. Bloods for monitoring parasite growth was collected twice a day from 7 days post challenge upto day of diagnosis. Images were created by E. Kibwana in biorender

4.5 Laboratory Methods

4.5.1 *P. falciparum* culture and maintenance

P. falciparum NF54 culture vials with a parasitaemia of 1-5% at the ring stage were thawed rapidly in a water bath at 37°C and then moved to a 50 ml falcon tube. While gently shaking the tube, 200 µl of solution 1 (12% NaCl) was slowly added followed by five-minute incubation period at RT before adding an additional 10 ml dropwise amount of thawing solution 2 (1.8% NaCl), while gently mixing thoroughly and was left to stand for 5 minutes. Lastly, another 10 ml of thawing solution 3 (0.9 % NaCl and 0.2 % glucose) was added in a dropwise manner. The cells were then washed twice using R0 (RPMI 1640 medium (Gibco/Invitrogen) supplemented with 2-mM L-glutamine, 37.5-mM HEPES (Gibco/Invitrogen), 25-µg/ml Gentamicin (Invitrogen), 0.2% glucose (Sigma) and 50.4-µg/ml Sodium Hypoxanthine (Sigma)) by centrifugation at 1800 rpm for 5 minutes. After the last wash, the supernatant was discarded, and the pellet was resuspended in 5 ml R10 (R0 + 10% Fetal calf serum). The cultures were gassed for 30 seconds with mixed gas consisting of 3% (v/v) O2, 5% (v/v) and 92% N2 and incubated at 37°C in an incubator (Trager & Jensen, 1976). Fresh culture media and fresh O^+ cells were added every 2 days by centrifugation at 440g for 5 minutes, discarding the old medium and adding fresh culture media. The growth of the parasite was followed by examination of thin blood smears stained with Giemsa. The parasite cultures were maintained at approximately 2-5% parasitaemia.

4.5.2 Sorbitol synchronization

Cultures were synchronized at the ring stage utilizing the D-sorbitol lysis technique (Lambros & Vanderberg, 1979) as described in the qPCR chapter 3.4.2.

4.5.3 Isolation of mature trophozoites

Late-stage trophozoites were isolated using magnet-assisted cell sorting (MACS) as recommended by the manufacturer. The parasite culture was centrifuged at 1800rpm for 5 minutes, and the resulting pellet was suspended in fresh media. As per the manufacturer's instructions, the MACs column, magnet, syringe, and adaptor were assembled and washed with warmed R0 to remove any air bubbles before adding the culture. The parasite culture was loaded onto the column where mature trophozoites containing paramagnetic hemozoin fragments were captured in a magnetic field. Meanwhile, eluted ring stage parasites and uninfected red blood cells from the flow through were collected and returned to culture. The column was washed with R0 until the eluted liquid was clear. The column was then removed from the magnet and

placed on a clean 50 ml falcon tube. The bound material (mature trophozoites) was eluted by adding 50 ml of R0 to the top of the column and allowed to run through. The eluted material was centrifuged at 1800 rpm for 5 minutes and washed once with R0. The culture media containing the mature trophozoites was returned to media to obtain schizonts.

4.5.4 Isolation of schizont extract

Following the isolation of mature trophozoites a thin blood smear was made to determine the parasite if confirmed to be mainly schizonts the cultures are harvested by centrifugation, and the pellet resuspended in 500ul of RPMI-HEPES (incomplete medium). Cells were lysed by three rounds of freeze-thaw followed by sonication for 30 minutes. Aliquots of the lysed schizont extract were stored at -80^{o}C, for future experiments.

4.5.5 Schizont ELISA

The ELISA assays were conducted to measure the serum antibody endpoint titres. I adapted an in-house standardized ELISA to include a standard curve from a pool of hyperimmune Kenyan adult sera. The standard curve was used to determine an arbitrary unit (AU) for test samples. Briefly, Nunc-Immuno Maxisorp 96 well plates (Fisher) were coated overnight with 100 µl of *P. falciparum* NF54 schizont lysate in carbonate-bicarbonate coating buffer at 4^{o}C. The plate was washed five time with phosphate-buffered saline/0.05 Tween 20 (PBST) and blocked with 200 µl per well of casein (Thermo Scientific) for 2 hours at RT. The plates were washed to remove excess casein and, 100 µl of plasma sera diluted at 1:1000 was added in triplicate and incubated at RT for 2 hours then washed as before. The plates were incubated with 100 µl of Goat anti-human IgG (γ-chain) conjugated to alkaline phosphatase ((AP) (Sigma)) diluted at 1:1000 for 1 hr at RT. Following a last wash, plates were developed by adding 100 µl of p-nitrophenyl phosphate (pNPP (Sigma)) at 1 mg/mL in diethanolamine buffer ((DEA), (Pierce)) and OD was read at 405 nm using Bio-Tek microplate reader using the Gen5 software. For all the ELISAs the x-axis intercept of the dilution curve was used to determine serum antibody endpoint titres reported as AU. Samples were considered seropositive if they had an AU higher than the mean plus three standard deviations of the naive U.K controls. Each assay included a standard positive serum sample as a reference control and a four-malaria negative naïve serum samples.

4.5.6 MSP-1 ELISA

I adapted a standardized internal ELISA protocol previously described by Biswas et al (Biswas et al., 2014). MSP-1 was expressed and purified in-house (donated by Dr Jame Tuju at the KEMRI-Wellcome Trust Research Programme). Plasmids encoding the full ectodomain of MSP1 antigen from 3D7 *P. falciparum,* were codon-optimized for expression in human cells and transfected into Expi293F cells for protein expression. For the ELISA, 2 µg/ml of purified MSP-1 was coated overnight at 4°C on 96-well plates. The plates were then washed four times with PBST and blocked with 1% casein in PBS for 2 hours at RT. After four additional washes, plasma samples diluted to a ratio of 1:500 in casein were added to the plates and incubated for 2 hours at RT, followed by another set of four washes. Subsequently, the plates were incubated for an hour at RT using secondary anti-human IgG antibody AP (Sigma, diluted to a ratio of 1:1000) to evaluate total antibody responses. Following further washing rounds, the plates were developed with a pNPP/DEA substrate buffer and OD at 405nm were read on Bio-Tek ELx800 Microplate Reader with Gen5 software.

4.5.7 Validated NANP ELISA and Kilifi NANP ELISA

The antigens for CSP-based ELISAs were provided by the Jenner Institute, University of Oxford. NANP ELISA was conducted using a synthetically produced peptide consisting of six NANP repeats and a cysteine residue $(NANP)_6C$ (Think Peptides, ProImmune, Oxford, UK). To assess the immune response to NANP, a validated NANP ELISA developed at the Jenner Institute in Oxford and the same assay developed in Kilifi were used. In Kilifi, I modified the Oxford NANP ELISA by establishing our own positive control pool to generate the standard curve and incorporate negative control samples. The same samples were then tested using both assays to compare the reproducibility of the assays in different laboratories. For both assays, 96-well NUNC Immuno plates (Fisher) were coated with 50 µl of 0.2 ug/ml of $NANP_6C$ in carbonate bicarbonate coating buffer (Sigma) and left to incubate overnight at 4°C. The following day, the plates were washed five times with 200 µL of PBST (Sigma) and blocked with 100 µl of casein (Thermo Scientific) for one hour at RT. After blocking, the plates underwent another wash before plasma samples diluted in casein were added in triplicate at a dilution ratio of 1:500 (pre-vaccination (d0)) or 1:1000 (post-vaccination). Plates were then incubated for two hours at RT. Subsequently, the plates were washed again and incubated with 50 µl of anti-human IgG AP (Sigma) at a concentration of 1:1000 for one hour. Following another round of washing, the plates were developed using a substrate solution containing pNPP/DEA and OD at 405nm read on Bio-Tek ELx800 Microplate Reader with Gen5

software. Each plate included a standard curve using hyperimmune sera pool alongside positive control plasma sample as well as four naïve control samples all tested in triplicate to ensure accuracy and reliability.

4.5.8 R21 and C-terminus ELISA

I modified the NANP ELISA above to test for responses to synthetically produced R21 (Serum Institute of India) and C-terminus (thinkpeptides, ProImmune, Oxford, UK) antigens. In brief, 96-well plates (Fisher) were coated overnight at 4°C with 50 µl of 1.5 µg/ml of R21 or C-terminus in carbonate bicarbonate coating buffer. The following day, the plates were washed five times with PBST and then blocked with 100 µl of casein for one hour at RT. Plasma samples, diluted in casein at a ratio of 1:500 (pre-vaccination (d0)) or 1:1000 (post-vaccination), were then added in triplicate and allowed to incubate for two hours at RT. Subsequently, after washing the plates again, 50 µl of anti-human IgG AP secondary antibody was added and incubated for 1 hr at RT. Following another round of washing, substrate solution was applied for development and absorbance was measured at 405nm to quantify levels of antibodies against R21 and C-terminus antigens. Each plate contained a standard curve utilizing hyperimmune sera pool as well as positive and negative control plasma samples tested in triplicate.

Table 4. 1: Sequence information on CSP proteins

Antigen		**Sequence**	**Manufacturer**
R21	Protein	MDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANP NANPNANPNANPNANPNANPNA NPNANPNKNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSD KHIKEYLNKIQNSLSTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYANDIEKKICKMEKCSSVPV TNMENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGSPVCLGQNSQSPTSNHSP TSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVC PLIPGSTTTNTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSW AFAKYLWEWASVRFSWLSLLVPFVQWFVGLSPTVWLSA IWMMWYWGPSLYSIVSPFIPLLPIFFC LWVYI	Serum Institute of India
$NANP_6$	Peptide	NANPNANPNANPNANPNANPNANP	Thinkpeptides, ProImmune, Oxford, UK
C-terminus	Peptide	epsdkhikeylnkiqnslstewspcsvtcgngiqvrikpgsankpkdeldyandiekkickmekcs	Thinkpeptides, ProImmune, Oxford, UK

4.5.9 Isotype ELISAs (IgA and IgM)

To assess the immune responses to R21/MM vaccination, ELISAs were performed to measure IgA and IgM levels against NANP, R21 and C-terminus using the same procedure as those for IgG above. However, instead of using anti-human IgG-AP, we utilized anti-human IgA alkaline phosphate (Sigma, 1:1000) or anti-human IgM alkaline phosphate (Sigma, 1:1000), both diluted in casein. The method for conducting the IgA and IgM ELISAs remained consistent with the procedures used for the IgG ELISAs regarding plate coating, sample dilution, incubation period, washing steps as well as standard curves and control samples.

4.5.10 Experimental controls and standardization on ELISAs

To standardize experiments across multiple ELISA plates, I used a pool of hyperimmune sera from adults vaccinated with R21/MM from a previous study (Sang et al., 2023) as a positive control for the CSP based ELISAs. The reading time range between (12- 30 minutes) and was determined by the point at which the internal control reached an OD405nm of 1.2 (1.0- 1.4) for NANP, 1.0 (0.8-1.2) for R21 and 1.2 (1.0 -1.4) for C-terminus. For the blood stage antigens (Schizont extract and MSP-1 ELISAs) I used a pool of hyperimmune adults who had previously undergone challenge with PfSPZ (Kapulu et al., 2018, 2020). Arbitrary units (AUs) for all ELISAs were determined using a four-parameter logistic (4PL) curve derived from a two-fold serial dilution of pooled hyperimmune sera. A smooth sigmoidal curve was generated by plotting absorbance values against dilution factors and analysing them with a 4PL regression model. The OD values of test samples were measured and interpolated from this curve, and the AUs were calculated relative to the reference. The 4PL standard curve was generated using a Gen5 v3.00 ELISA reader (Bio-Tek). The conversion of ODs to AUs using a standard curve from pooled hyperimmune sera normalised the results, enabling consistent comparisons across various experiments, plates, or time points.

Samples with triplicate CV >20% were re-run, except for samples with an OD of less than 0.2 (lower limit of detection of the plate reader). For these samples I averaged out what the AU values were at OD of 0.2 and assigned all samples a single fixed value. This value was antigen dependent. Samples with absorbance readings surpassing the Abs405 threshold of 3.4 OD or showing a "high OD" message underwent retesting using a more diluted sample, to obtain accurate results. Also included on each plate were negative controls consisting of malaria naïve sera from U.K donors. Samples were considered seropositive if they had an AU higher than the mean plus three standard deviations of the naive U.K controls.

4.5.11 Meso Scale Discovery (MSD)

The MSD is a multiplex assay (Custom Malaria 4-Plex Assay) for quantification of human serum Ig and plasma Ig antibodies against the following 4 malaria vaccine antigens: six repeats of CSP NANP peptide (NANP6) with a cysteine residue added to the C-terminus (NANP6C) (ProImmune, Oxford, UK), CSP C-terminus peptide (ProImmune, Oxford, UK), full length R21 protein and Hepatitis B surface antigen (HBsAg) (both produced by Serum Institute of India Ltd). This assay is a validated assay performed at the Jenner Institute, where it is routinely used in clinical trials of malaria vaccines (Stockdale et al., 2024). The procedure employed by MSD differs from that of ELISA in that it utilises electrochemiluminescence for detection rather than the colorimetric reaction used in ELISA. This assay applies sandwich immunoassay principles to detect the concentration of each analyte. Secondary antibodies are conjugated with specific electrochemiluminescent labels, and the emitted signal was measured using a microplate reader equipped with an electrochemiluminescence detection system.

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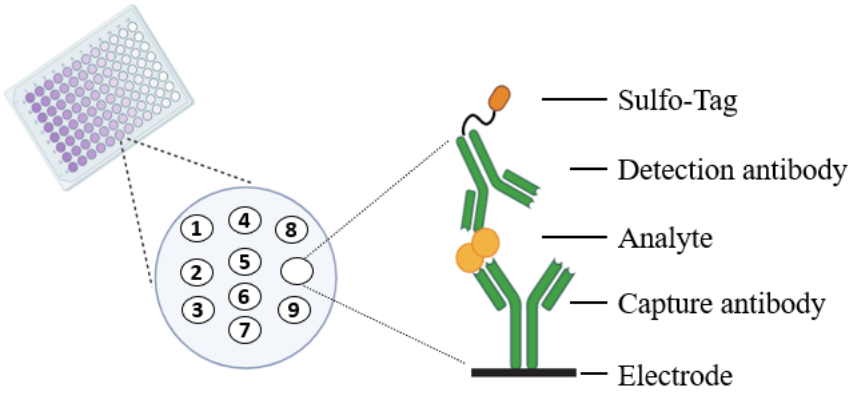

Figure 4. 2: A multiplex plate spot diagram.

Displaying the placement of capture antigens on the left. The software visualization tool maintains a numbering convention for the different antigen spots. On the right illustrates the sandwich immunoassay principle used to measure analyte concentrations (created by E. Kibwana in Biorender).

Each assay plate was pre-coated with the four malaria vaccine antigens arranged in independent spots on the base of each well and stored at 4 °C until required (mesoscale diagnostics). To begin, the plates were brought to RT and then blocked with casein for 30 min at RT while being gently shaken. The plates were washed thrice with PBST and dried by blotting on paper towels. For quality assurance purposes, a reference standard control was prepared to establish a standard curve and positive control samples were prepared. Thereafter, 50 µl of the reference standard, positive controls (high, medium, and low), and diluted samples were added to the plates and incubated at RT while being shaken for 2 h. The plates were washed again before 50 µl/well of SULFO-TAG detection antibody was added at a concentration of 100 µg/ml. The plates were then incubated at RT while being shaken for another hour before being washed one final time. Finally, 150 µl/well of MSD GOLD buffer was added to the plates, which activated the electrochemiluminescence reaction. Plates were read immediately using an MSD multiplex instrument and analysed using the MSD Methodical Mind Software.

4.5.12 Total IgG Avidity ELISA

To assess the overall strength of the interaction between vaccine-induced antibodies and antigen/s, the sodium thiocyanate (NaSCN)-displacement ELISA method previously outlined by Biswas et al. was used. (Biswas et al., 2014). The avidity assay followed similar steps to the total IgG ELISAs mentioned above but included some adjustments. Plasma samples were normalised individually in casein to achieve an OD_{405}= 1.0 (based on the ELISA units and the OD value of the standardised total IgG ELISA for each antigen), ensuring that they contained equivalent titres of antigen-specific IgG and developed similarly in wells where no NaSCN was added. Samples were added at 50 µl/well in 16 wells and incubated for 2 h at RT. During this period, an NaSCN gradient was created by diluting an 8 M stock solution in PBS to produce a range of concentrations from 0M to 7M. After incubation and washing, duplicate wells down the plate received increasing levels of NaSCN (ranging from 0M to 7M) before being left for an additional 15 min at RT. Afterwards, the plates were washed, secondary antibody was added, and the plates were developed according to the standard procedure for total IgG ELISA. The plates were left to develop until the OD of the wells in the first row (without NaSCN) reached approximately 1.0 (± 20%). The avidity index was then determined based on the concentration of NaSCN needed to decrease the OD405 levels by 50% compared to those without added NaSCN.

4.5.13 Complement (C1q) ELISA

ELISA plates were coated with 50µl of either purified $NANP_6C$ (at a concentration of 0.2 µg/ml) or C-terminus (at a concentration of 1.5 µg/ml) and incubated overnight at 4°C. The plates were then washed four times with PBS, followed by blocking with 200 µl of casein for two hours at 37°C. Subsequently, plasma samples (50µl), diluted to a ratio of (1:100) in casein, were added to the plate and allowed to incubate for two hours at 37°C. Plates were washed and 40µl of recombinant human complement C1q (Calbiochem) diluted to 10 µg/ml in casein was added to the wells and incubated for 30 min at 37 °C. The plates were washed again, followed by the addition of anti-C1q antibody conjugated to horseradish peroxidase (HRP) (50 µl; diluted at a ratio of 1:100 in casein (Abcam)) for 1 h at 37 °C, followed by a final wash. The development was performed using 50 µL of the OPD substrate for 30 min at RT. Finally, the reaction was stopped by adding 2 M $H_2SO_4$and ODs was read at 492 nm using the ELISA reader Gen5 v3.00 (Bio-Tek).

4.5.14 Inhibition of Sporozoite assay

To quantify the functional capacity of antibodies against sporozoite surface proteins, that is, to prevent sporozoites from infecting hepatocytes *in vitro,* I used an inhibition assays which uses chimeric *P. berghe*i parasites that express *P. falciparum* CSP and green fluorescent protein (GFP) that can be detected by flow cytometry.

4.5.15 Hepatocyte cell line culture

Cryo-preserved Huh7 cell lines were kindly provided by Dr Duncan Bellamy of the Jenner Institute at the University of Oxford. Cells were thawed in a 37 °C water bath and transferred into a falcon containing 10 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine ((D10) (all reagents were obtained from Sigma Aldrich)). The cells were washed twice with 10 ml of sterile PBS by centrifugation at 1800 rpm for 5 min, and the supernatant was discarded after each wash. After the final wash, the pellet was resuspended in 10 ml of D10, transferred to a T175 flask, and cultured at 37 $^{\circ}$C and 5% CO_2. Cell cultures were split every two days until required for the inhibition assay by first examining the cells under light microscopy to confirm confluence. The cell medium was aspirated from the flask and washed twice with sterile PBS by centrifugation (1800 × g for 5 min). 7.5 ml of 0.05% Trypsin-EDTA was then added to the flask, which was incubated for 5 min at 37 $^{\circ}$C to detach the cells. After 5 min, trypsin-EDTA was neutralised by adding D10 at 4 × the volume of trypsin-EDTA. The cells were transferred into a 50 ml

falcon tube and centrifuged at 1800 rpm for 5 min before resuspending in 10 mL of D10. Cells were split into three T175 flasks to ensure that there was always a backup flask (e.g. by adding 1 ml of cells to 59 ml D10, 2 mL of cells to 58 mL D10, and 3 mL of 57 mL D10) and returned to culture at 37 ℃ and 5% CO_2.

4.5.16 Huh 7 Cell Plating

Cells that were in culture for several days were plated overnight before assay set up in a similar manner to cell splitting above. Following the removal of trypsin-EDTA step, I removed 50 µl of the cell suspension and added 50 µl of Trypan blue to visualize and count the cells by microscopy. To calculate the number of cells I used the equation below:

Average live count x 10^4 (haemocytometer chamber volume) x 2 (dilution factor) x number of mls

The number of cells required for the assay is 30,000 cells in 100 µl/well. The cell suspension was centrifuged and resuspended in the required volume of D10. 100 µl of cell suspension was added to each well to a labelled 96-well flat-bottomed sterile tissue culture plate. Cells were then rested in a 37°C and 5% CO_2 incubator for overnight before infection.

4.5.17 Sample preparation.

Plasma samples were normalized individually in D10 to achieve an OD_{405}= 1.0 (based on the ELISA units and the OD value of the standardized total IgG ELISA), ensuring that they contain equivalent titres of antigen-specific IgG

4.5.18 Mosquito Dissection

Mosquito infection was carried out by Adam Truby, briefly transgenic chimeric *P. berghei* sporozoites expressing *P. falciparum* CSP and GFP were isolated from female *Anopheles stephensi* mosquitoes around 19-24 days after feeding on *P. berghei* blood stage infected donor mice at the Jenner Institute. Mosquitoes were dissected by placing the petri dish containing the mosquitoes on ice to anaesthetize them. The salivary glands were carefully removed using a dissecting microscope and placed in a homogenized kept on ice. The salivary glands were then homogenized using a mini pestle to release the sporozoites, transferred to an Eppendorf and counted under a phase contrast microscope. To calculate the number of sporozoites obtained I used the below formulae:

Average count in 16 squares x 10^4 x 1 (dilution factor) x volume in Eppendorf (mls)

Ideally, 15,000 sporozoites/well are required for infection.

4.5.19 Hepatocyte infection

The media was aspirated from the 96-well plate containing the cells using a multi-channel pipette. I then added 100 µl of sera which was normalized to ensure each sample contained approximately the same anti-R21 IgG titres followed by 100 µl of sporozoites. I included assay controls: 4 positive and 4 negative controls:

- Positive controls – Cells + sporozoites, no serum/ plasma
- Negative controls – Cells only, no sporozoites and no serum/ plasma

Plates were centrifuged at 1600rpm for 5min at 4°C to increase infectivity and placed in a 37°C and 5% CO_2 incubator for 20-26 hrs.

4.5.20 Assessment of infectivity by flow cytometry

Following the incubation period, I aspirated the media from the cells using a multichannel pipette, washed the wells twice with 100 µl of PBS. 50 µl trypsin was then added to each well and the plate was incubated at 37^{o}C for 8 minutes. The trypsin was neutralized by the addition of 100ul of 10% FCS in PBS and the cells were transferred to a V-bottom plate. Plates were washed twice with 100µl of 10% FCS in PBS by centrifugation (1800 rpm for 5 mins). The supernatant was then flicked off and the cells were resuspended in 80 µl of 1% FCS in PBS and kept at 4^{o}C until acquisition. 10 min prior to running the cells on the flow cytometer, 5 µl of 4′,6-diamidino-2-phenylindole dihydrochloride ((DAPI) (diluted to 1 in 1000 in 1% FCS in PBS) was added to every sample to exclude dead cells. Cells were acquired BD high throughput sampler (HTS) with the LSR II^{TM} flow cytometer (BD Biosciences) using FACSDIVA^{TM} software V 6.2 (BD Biosciences).

Upon analysing the data, I observed no infection in any of the assay conditions, therefore I shall not present data on this assay in this thesis. A potential reason for this could be the mosquito's infectivity was lower than expected or that the sporozoites were not viable. Due to time constraints, I was unable to repeat this experiment.

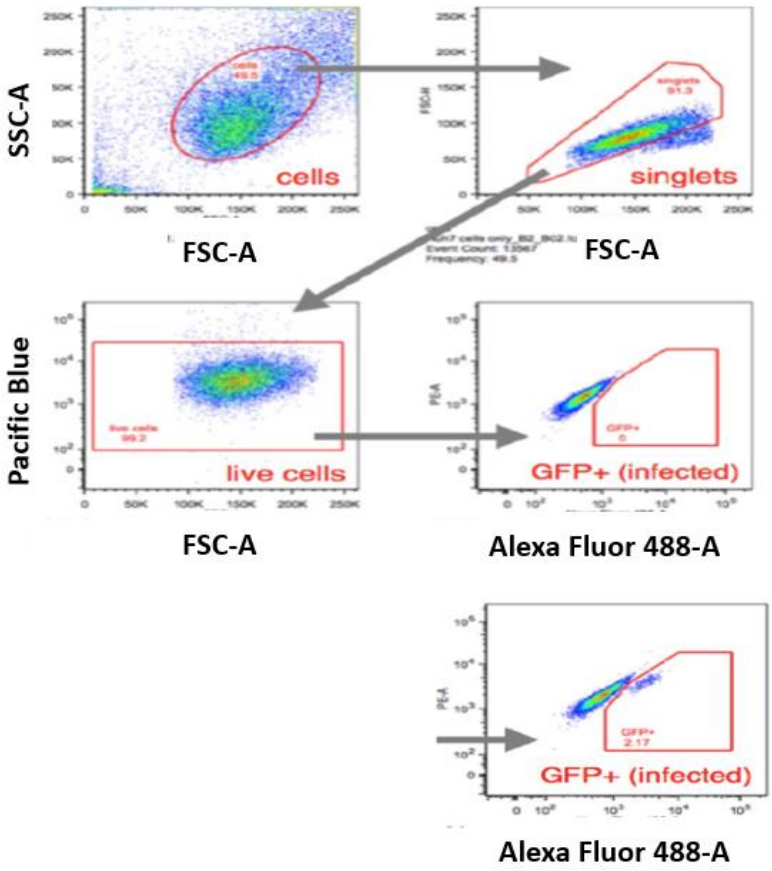

Figure 4. 3: Gating strategy for detection of *P. berghei* infected liver cells.

Image adapted from Dr Duncan Bellamy, Jenner Institute.

4.6 Statistical Analysis

The data obtained from the ELISA assays were analysed using GraphPad Prism version 10.1.2 for Windows. The antibody endpoint titres were determined for each sample by interpolating the absorbance values against the standard curve. The data are presented as medium (± 95% confidence intervals (CI)) or geometric mean titre (± 95% CI). Mann Whitney test was used when comparing the median of two distinct group. Participant dropout and experimental challenges led to varying sample sizes and inconsistencies in paired data availability. Therefore, the Friedman test was replaced with the Kruskal–Walli's test, which is appropriate for an unpaired dataset and different sample sizes. When I analysed a subset of complete datasets using the Friedman test, the results were consistent with the Kruskal–Walli's test (Dunn's multiple comparisons), confirming the robustness of the findings. Experimental and participant dropout rates ranged from 17.2% to 25.1%, depending on the time point and assay. Statistical significance was set at $P < 0.05$. For avidity index analysis, only samples with positive antibody titres (as measured by ELISA) were used. All correlations were determined using Spearman's rank correlation to determine whether there was a statistically significant correlation between two variables, and the significance level was set at <0.05.

4.7 Results

4.7.1 Baseline demographic characteristics

Between June and August 2022, 80 healthy adults aged between 18 and 45 years were enrolled and randomised into four groups. Two groups received 3 doses of 10µg R21/ 50 µg Matrix-M, and the last group was the control group who were not vaccinated. The mean age of the participants was 28 years (standard deviation [SD] = 6), and the majority were male (71%). One participant was *P.falciparum* positive at baseline but was treated prior to vaccination; no participant was positive for HIV or COVID-19, and all were negative for hepatitis B surface antigen (HBsAg) and/or hepatitis C (HCV IgG).

Table 4. 2: Baseline demographic characteristics.

Table provided by Prof M Kapulu.

Characteristic	**R21 (Intradermal) n=24**	**R21 (Intravenous) n=14**	**Control (Intradermal) n=18**	**Overall N=80**
Age in years, mean (SD)	28 (6)	26 (5)	29 (6)	28(6)
Sex, female, n (%)	4 (17%)	4 (29%)	6 (33%)	23 (29%)
Sex, male, n (%)	20 (83%)	10 (71%)	12 (67%)	57 (71%)
BMI, kg/m^2, mean (SD)	21.7(2.0)	20.2 (1.8)	20.2 (1.7)	21.7 (3.1)
PCR (parasites/) Median (min-max)	0 [0,0]	0 [0, 0.0167]	0 [0,0]	0 [0,0.0167]
PCR positive, n (%)	0 (0%)	1 (7.1%)	0 (0%)	1 (7.1%)
Diastolic, mean (SD)	75 (7)	74 (7)	72 (10)	74 (8)
Systolic, mean (SD)	122 (9)	124 (11)	118 (10)	121 (10)

n = number of healthy volunteers enrolled to each vaccination group. BMI, Body mass index, PCR (parasites/) was recorded at pre-vaccination (pre-vac). Data are presented as either mean and SD (standard deviation), medium (min-max) for continuous variables and n (%) for categorical variables. Percentages are presented with denominator as number of healthy volunteers per vaccination groups.

4.7.2 Comparison of NANP Titres: validated Oxford ELISA vs. Kilifi ELISA

To determine whether the NANP ELISA set up in Kilifi was fit for purpose and the degree of agreement between the two assays, I measured the total IgG responses to NANP on days 14, 42, 70, and 84 in the KEMRI-Kilifi and Jenner laboratories (Figure 4.4). The assays were comparable between the two laboratories, and no significant differences were observed on any day. Peak antibody responses were observed on day 70 in both the ELISAs. The GMT in the Kilifi assay was 1886 (95% CI: 1459 – 2438, n = 38), whereas that in the validated Oxford assay was 2009 (95% CI: 1584 – 2548, n = 37). Spearman's correlation test further confirmed the correlation between the two assays with all timepoints demonstrating a strong significant positive association (figure 4.5).

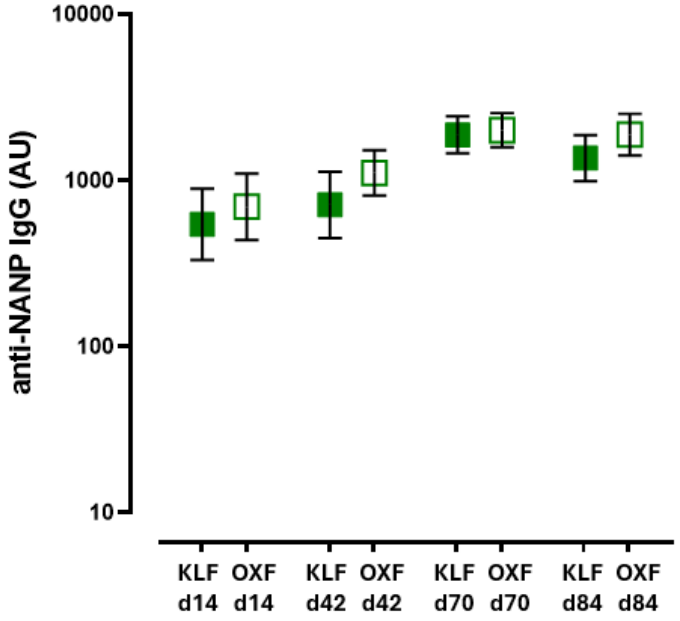

Figure 4. 4: Assessment of anti-NANP IgG antibody responses by the two assays

Antibody titres of the vaccinees were compared using the validated NANP ELISA (Jenner Institute) and the Kilifi NANP ELISA on days 14, 42, 70, and 84. Antibody titres are specified as AU, shown are geometric mean titres (GMT) ± 95% CI. Solid green squares represent results from the Kilifi ELISA, whereas open squares represent results from the Oxford ELISA. The limit of detection of the ELISA was OD 0.2, and values with an OD <0.2 were assigned a value of 1 (as per the assay validation).

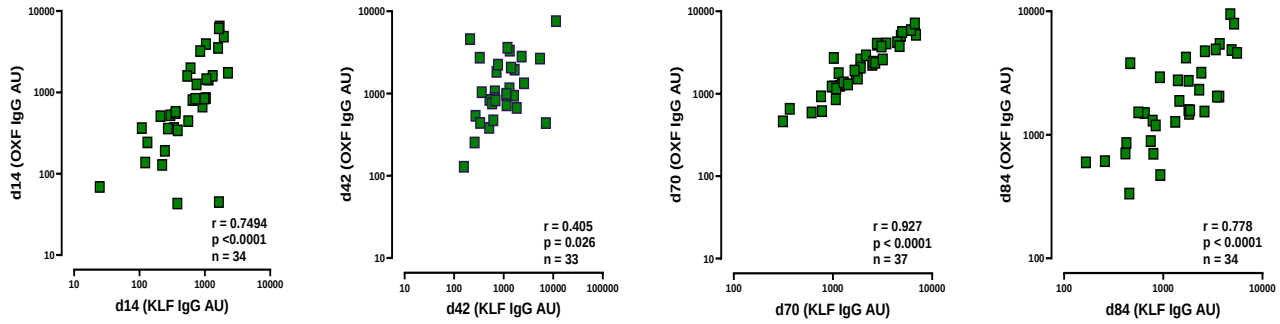

Figure 4. 5: Association between validated Oxford ELISA and the Kilifi NANP ELISA

Spearman's correlation analysis was performed to assess the relationship between the two assays measuring the total anti-NANP IgG titres Samples were tested at multiple time points (d14, d42, d70 and d84) using both assays, and the resulting data were compared to evaluate the level of agreement between the methods.

4.7.3 R21/MM vaccination induces NANP, R21 and C-terminus specific IgG antibodies.

R21 comprises HBsAg, the C-terminus, and the NANP repeat region (of CSP), serving as the main B-cell epitope; anti-NANP responses are the primary endpoint in R21/MM studies. Antibodies against the C-terminus region were also detected. Studies show responses to both antigens correlate with vaccine efficacy (Chaudhury et al., 2021; Datoo et al., 2024; Dobaño, Sanz, et al., 2019). My goal was to assess responses to R21 protein and specific epitopes, including the NANP repeat and C-terminus, in vaccinated adults. Standardised ELISA tests were conducted for each vaccine antigen using a hyperimmune serum pool to establish standard curves on ELISA plates. Plasma samples from malaria-naïve individuals in the U.K served as the negative control. Data are presented as arbitrary units (AU).

4.7.3.1 R21 IgG responses

First, I assessed the kinetics and durability of antibody responses to, R21/MM vaccination from day 0 (day before vaccination) up to day 84 (day before sporozoite challenge). I observed an increase in anti-R21 IgG titres in the vaccinees. GMT at day 0 was 22.06 (95% CI: 16.45 – 29.58, n = 38), day 14 1479 (95% CI: 1040 – 2103, n = 37), day 42 10353 (95% CI: 8037 – 13335, n = 38), day 70 12229 (95% CI: 9737 – 15360, n = 38), and day 84 9324 (95% CI: 7591 – 11453, n = 34). The peak antibody response occurred on day 70. There was a significant difference in antibody titres two weeks following the first

and second dose: (Kruskal-Wallis test with Dunn's multiple comparison test: (day 0 vs day 14; p = 0.003) and (day 14 vs day 42; p <0.0001)). However, there was no significant difference in antibody titres following the third and final dose (Kruskal-Wallis test with Dunn's multiple comparison test (day 42 (GMT 10353) vs day 70 (GMT 12229); p > 0.99). In the non-vaccinees (control group), antibody titres remained low throughout the trial and were well below the positivity threshold. GMT was 20.54 (95% CI: 7.69 – 54.81, n = 9) at day 0, and 25.82 (95% CI: 9.17 – 72.69, n = 9) at day 84.

4.7.3.2 NANP IgG responses

Next, I evaluated total IgG antibody responses to NANP. Similar to R21 responses, the levels of NANP antibodies increased with each additional dose of R21/MM. GMT at day 0 was 17.4 (95% CI: 14 – 21.4, n = 38), day 14, 545.8 (95% CI: 332.9 – 894.9, n = 38), day 42, 714.8 (95% CI: 451.2 – 1131, n = 38), day 70, 1886 (95% CI: 1459 – 2438, n = 38), and day 84 1366 (95% CI: 992.6 – 1880, n = 34). Peak antibody responses for NANP also occurred on day 70. Significant differences were observed 2 weeks following the first dose, day 0 vs day 14 (p < 0.0001). However, there was no significant difference two weeks after the second (day 14 vs day 42 p > 0.99) and third immunization (day 42 and day 70 p = 0.327). Analysis was carried out using Kruskal-Wallis test with Dunn's multiple comparison test. The non-vaccinees (control group) did not show any positive response and remained below positivity threshold, GMT 17.34 (95% CI: 14.04 – 21.42) at day 0 and 14 (95% CI: 14 – 14) at day 84.

4.7.3.3 C-terminus IgG responses

Lastly, I investigated antibody responses to the C-terminus antigen. After the first R21/MM dose, no detectable IgG responses were detected against the C-terminus, with responses falling below the positivity threshold (day 0, GMT 77.8 (95% CI: 61.28 – 98.77, n = 38) and day 14, GMT 58.58 (95% CI: 46.38 – 73.99, n = 38). Total IgG antibodies were observed following the second dose on day 42, GMT 2543 (95% CI: 1792 – 3610, n = 38). A statistically significant difference was noted when comparing doses 1 and 2 (Kruskal-Wallis test with Dunn's multiple comparison test p < 0.0001, comparing days 0 and 14 to days 42 and 70). Antibody responses peaked on day 70, with a GMT of 3921 (95% CI: 2955 – 5201). The non-vaccinated group remained negative on day 0, GMT 54.25 (95% CI: 34.52 – 85.24) and day 84 GMT 53.2 (95% CI: 40.25 – 70.31).

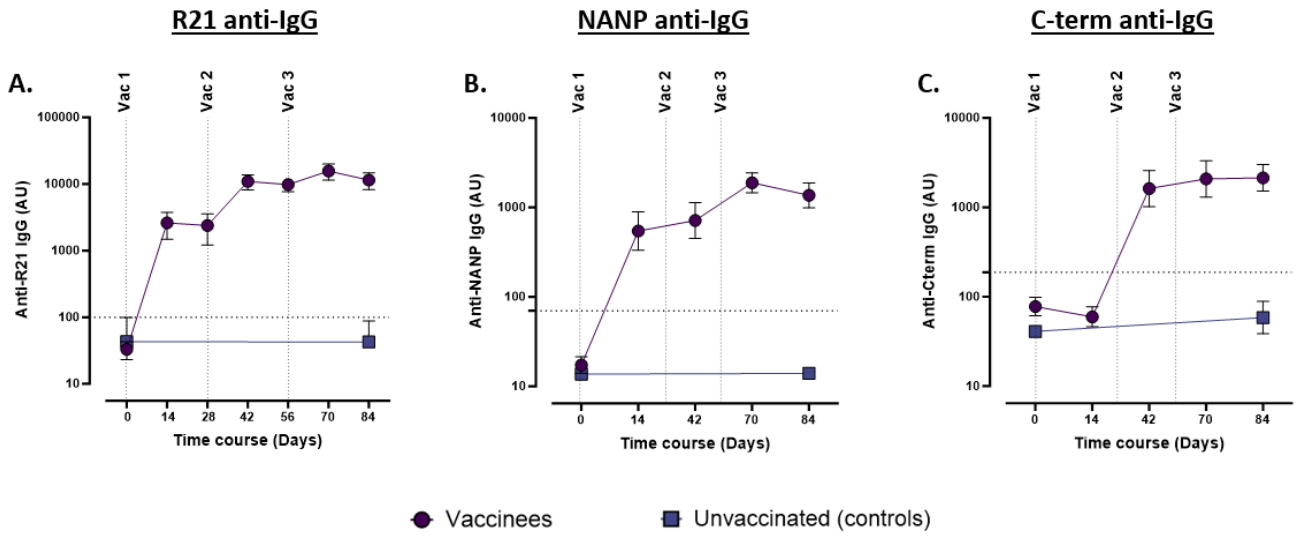

Figure 4. 6: Assessment of anti-IgG CSP responses.

Total IgG responses to (A) anti-R21, (B) anti-NANP, and (C) anti-C-terminus were analyzed over time from pre-vaccination (day 0) to day 84 using standardized ELISA. Geometric mean antibody titers (±95% CI) are presented as log10 AU. The positivity threshold, indicated by a horizontal dashed line, represents the mean + 3x standard deviations of malaria-naïve negative controls. Samples exceeding this threshold were classified as positive. Vaccination time points are marked by vertical dashed lines. The ELISA detection limit was OD 0.2; values below this limit were assigned AU values of 11 for R21, 14 for NANP, and 41 for C-terminus. Purple circles represent vaccinees (n = 38), and blue squares represent unvaccinated controls (n = 9). Total IgG antibody response data were available for both cohorts.

4.7.4 R21/MM vaccination induces NANP, R21 and C-terminus specific anti-IgA and -IgM antibodies.

Recent research has indicated that RTS,S vaccinations produce IgA and IgM antibodies against CSP (Kurtovic et al., 2020; Mugo et al., 2021; Suau et al., 2021). Here, I examined whether R21/MM vaccination induced similar or stronger isotype responses in adults, using a standardised ELISA method. Antibody levels were assessed at baseline, two weeks after each dose, and before challenge (days 14, 42, 70, and 84), except for IgA C-terminus, which was measured on days 0, 42, 70, and 84. An additional IgA response to R21 was observed on day 56. The results showed that R21/MM vaccination induced strong IgM and IgA responses against all three CSP antigens, persisting until day 84 (28 days after the third booster).

4.7.4.1 Total IgA responses to CSP antigens

Following administration of a single R21/MM dose, anti-IgA responses increased for all three antigens: NANP, R21, and C-terminus. Anti-R21 responses were GMT 30 (95% C: 30 – 30, n = 28) at day 0, 843.9 (95% CI: 527 – 1351, n = 19) at day 14, 1455 (95% CI: 827.1 – 2559, n = 19) at day 42, 1908 (95% CI: 1174 – 3103, n = 19) at day 70 and 1845 (95% CI: 1172 – 2904, n = 17) at day 84. A significant increase in R21 IgA response was observed after the initial dose (between days 0 and 14, p = 0.0005). However, no significant increase in antibody response was noted following the second and third doses (i.e. between days 14 and 42 and days 42 and 70), as determined by the Kruskal-Wallis test with Dunn's multiple comparison test.

Responses to NANP also rose in a stepwise manner, the GMT at day 0 was 17.34 (95% CI: 14.04 – 21.42, n = 28), 545.8 (95% CI: 332.9 – 894.9, n = 19) at day 14, 1145 (95% CI: 451.2 – 1131, n = 19) at day 42, 1886 (95% CI: 1459 – 2438, n = 19) and 1366 (95% CI: 992.6 – 1880, n = 17) at day 84. A significant increase was observed after the first dose (Kruskal–Walli's test with Dunn's multiple comparison test: days 0 and 14, p = 0.02). No significant increase in antibody titres was observed following subsequent doses (i.e. doses 2 and 3). Peak antibody responses to R21 and NANP were observed on day 70.

I also observed IgA responses to C-terminus, albeit at lower quantities; 13 out of 19 participants (68%.4) had detectable antibody responses by day 70 (post 3 doses). Antibodies rose from GMT of 19 on day 0 (n = 19) to peak on day 70, where GMTs were 137.0 (95% CI: 64.94 – 289, n = 17). Non-vaccinated controls (n = 9) remained negative throughout the follow-up period (days 0–84).

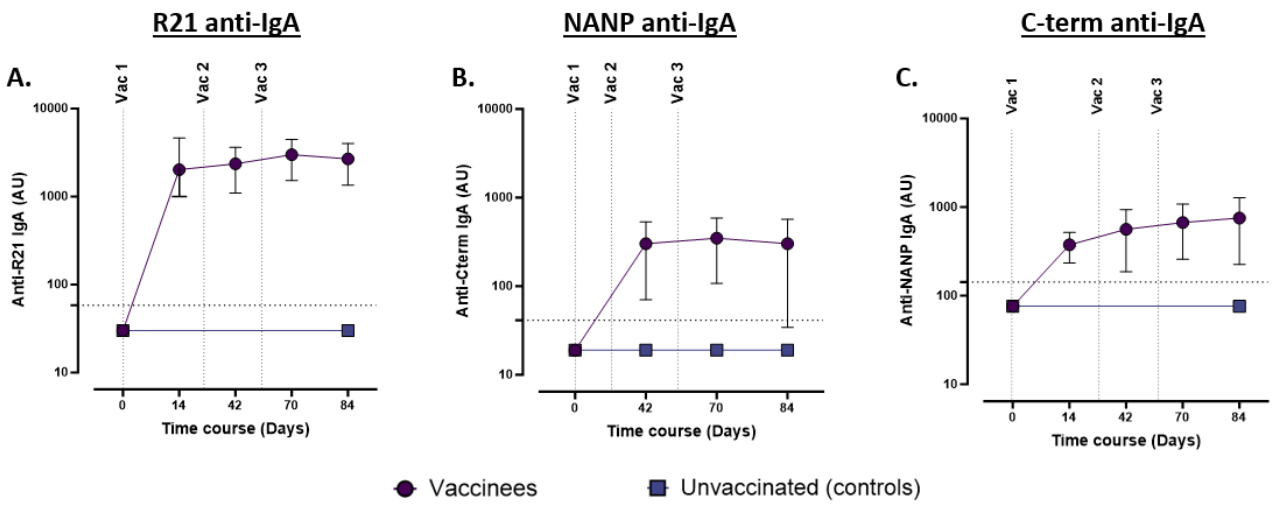

Figure 4. 7: Assessment of anti-IgA CSP responses.

Anti-IgA responses to vaccination were measured over time from baseline (day 0) to day 84 post-vaccination using standardized ELISA. (A) Anti-IgA responses to R21, (B) anti-IgA responses to NANP, and (C) anti-IgA responses to C-terminus are shown as geometric mean antibody titers (+95% CI) in arbitrary units (AU). The seropositivity cutoff, indicated by the horizontal dashed line, corresponds to the mean + 3x standard deviation of malaria-naïve controls, with samples above this threshold considered positive. Vaccination time points are marked by vertical dashed lines. The ELISA detection limit (OD 0.2) was assigned AU values of 30 for R21, 76 for NANP, and 19 for C-terminus for samples below this limit. Purple circles represent vaccinees (n = 19), and blue squares represent unvaccinated controls (n = 9). IgA antibody response data were available for cohort 1 only.

4.7.4.2 Total IgM responses to CSP antigens

Anti-IgM CSP responses increased with additional vaccine doses from day 0 to day 70, peaking on day 70 with a GMT of 1953 (95% CI: 1354–2815, n = 36). Significant increases in R21 IgM responses occurred after the first and second doses (days 0 - 14, p = 0.0001; days 14 - 42, p = 0.0032), but not after the third dose (days 42 - 70, p > 0.99), according to the Kruskal-Wallis test with Dunn's multiple comparison test. At baseline, 21 of the 38 (55%) participants had detectable anti-NANP IgM responses. Post-vaccination, NANP responses peaked on day 70, with a GMT of 885.2 (95% CI: 612–1280, n = 38). Non-vaccinees remained below the positivity threshold, although there was a slight increase in NANP antibody titres from day 0 (GMT 110, 95% CI: 85.4–142.6, n = 38) to day 84 (GMT 132.5, 95% CI: 505–1079.9, n = 34). IgM responses to C-terminus were not presented due to the substandard optimisation of the ELISA assay, as the standard curve did not meet the required criteria.

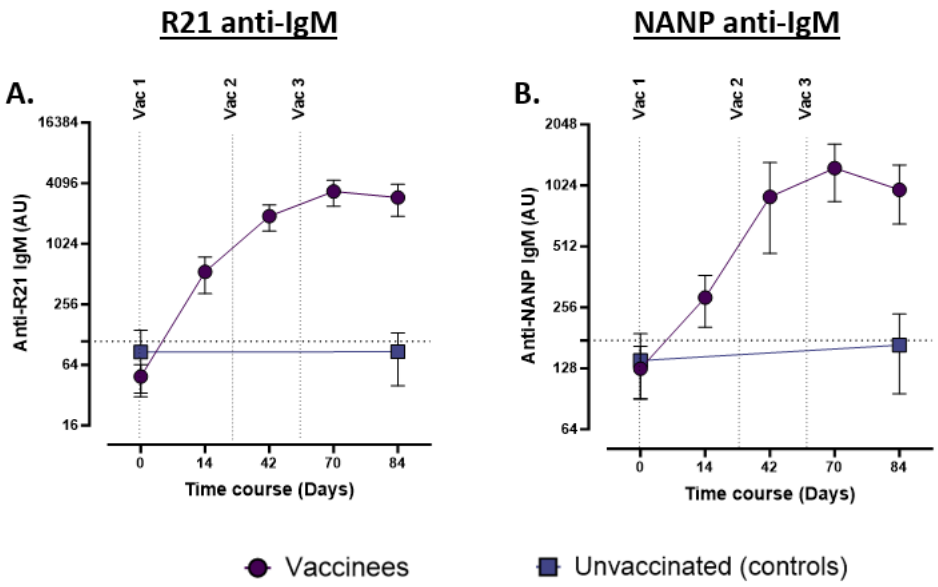

Figure 4. 8: Assessment of anti-IgM CSP responses.

Anti-IgM responses to vaccination were measured over time from baseline (day 0) to day 84 post-vaccination using standardized ELISA. (A) Anti-IgM responses to R21 and (B) anti-IgM responses to NANP are shown as geometric mean antibody titers (+95% CI) in arbitrary units (AU). The seropositivity threshold, represented by the horizontal dashed line, was defined as the mean + 3x standard deviation of malaria-naïve sera. For samples with an OD < 0.2, AU values were assigned as 38 for R21 and 50 for NANP. Purple circles represent vaccinees, and blue squares represent unvaccinated controls. Data were available for both cohorts.

4.7.5 Total IgG responses to R21 vaccine measured by the MSD assay.

To assess the collective IgG responses to all vaccine components, I conducted a multiplex assay using the validated mesoscale discovery platform at the Jenner Institute (Stockdale et al., 2024). This method enables simultaneous detection of four vaccine antigens (R21, NANP, C-terminus, and hepatitis B surface antigen (HBsAg). Responses were evaluated at baseline (day 0) and on days 42, 70, and 84 post-vaccination. I observed a similar increase in antibody responses among all vaccinated individuals for the three malaria antigens (R21, NANP, and C-terminus), with the highest responses occurring on day 84, following three doses of R21/MM. Antibody responses to R21 increased from a median of 14.53 [IQR 30.95, n = 38] on day 0, and peaked at day 84, median 124,459.4 [IQR 605,566.6, n = 34]. Responses to NANP rose from median of 4.82 [IQR 13.5, n = 38] and peaked at day 84 with median values of 69,411.2 [IQR 404,297.8, n = 34], similarly, antibody responses to C-terminus peaked at day 84, median 54,294.4

[IQR 300,447.1, n = 34], from a median of 14.16 [IQR 6., n = 38] at baseline. The responses at day 84 to all antigens were significantly different from their baseline levels (day 0, p <0.0001).

Responses to HBsAg showed a modest increase following each vaccination dose, with median values of 0.49, [IQR 0.79], 3.54 [IQR 4.05], 5.16 [IQR 10.89], and 43.67 [IQR 190.2] on days 0, 42, 70, and 84, respectively. The responses were not significantly different between the vaccinated and unvaccinated groups on days 0 or 84. This suggests that HBsAg may not be adequately present on the surface of R21 particles, limiting the immune system's ability to recognise and respond to it effectively. Antibody responses in the unvaccinated control group remained relatively low, with no significant difference between baseline responses and day 84.

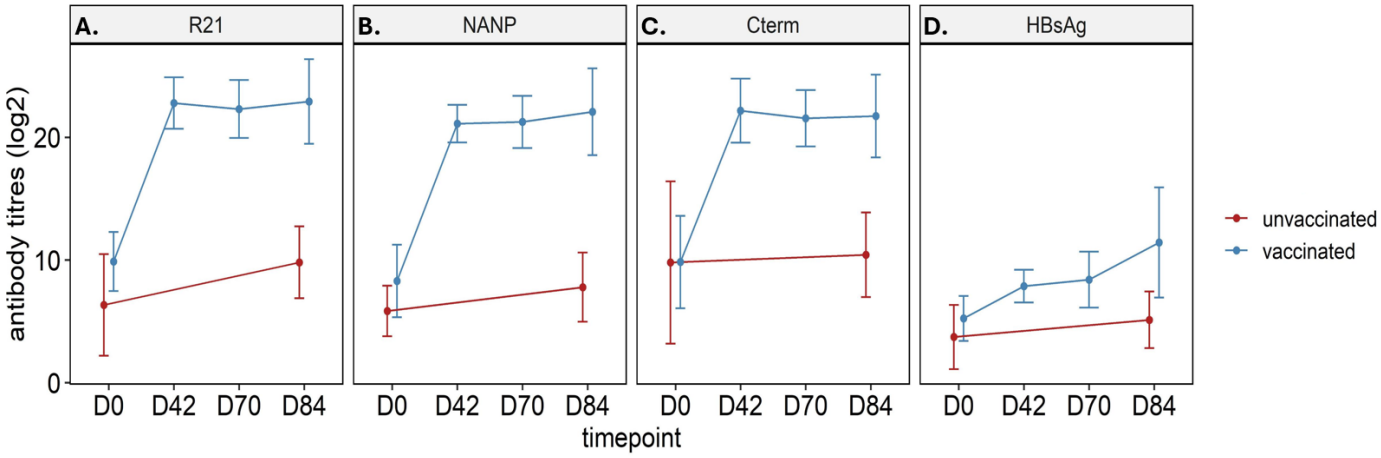

Figure 4. 9: IgG responses measured by a multiplex assay MSD.

IgG antibody responses to the individual components of the R21/MM vaccine were assessed using the MSD assay. (A) Responses to R21, (B) responses to NANP, (C) responses to C-terminus, and (D) responses to HBsAg are shown. Antibody levels are presented as grouped medians at each time point, with interquartile ranges (IQR) indicating variability within the group.

4.7.6 Antibody avidity to R21/MM vaccination.

Evaluating the overall IgG binding strength against vaccine antigens provides insight into vaccine-induced antibody responses. An NaSCN-displacement ELISA was used to measure total IgG avidity to assess whether R21/MM vaccination increases avidity and if additional doses further enhance it. The avidity index was calculated on days 14, 42, and 70 (2 weeks after each dose) and monitored up to day 84. Avidity was not reported on day 0 because of difficulties in measuring low or negative IgG responses.

R21, NANP, and C-terminus showed minimal changes in avidity post-vaccination. The median avidity index for R21 was 2.22 (n=11), 1.8 (n=19), 2.1 (n=19), and 1.6 (n=17) for days 14, 42, 70, and 84 respectively (Kruskal-Wallis test with Dunn's multiple comparison test, $p > 0.9$). For NANP, the median avidity indices were 1.29, 1.0, 0.90, and 1.1 for the same days ($p > 0.9$). The median avidity indices for the C-terminus were 0.84, 0.79, and 0.77 at days 42, 70, and 84, respectively. Avidity at day 14 for the C-terminus was not measured because of the low IgG antibody levels.

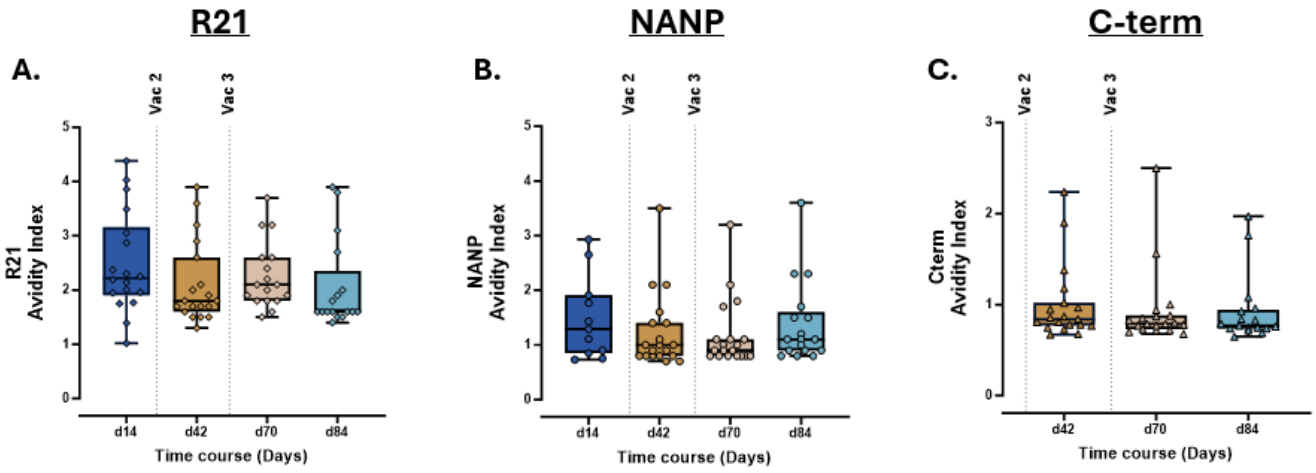

Figure 4. 10: Assessment of IgG avidity post R21/MM vaccination.

Avidity index was determined as the molar concentration of NaSCN required to reduce the OD405 to 50% of the value observed in the absence of NaSCN. (A) Avidity to R21, (B) avidity to NANP, and (C) avidity to C-terminus are shown. Samples with negative antigen-specific total IgG responses by ELISA or insufficient antibody levels for avidity testing at a given time point were excluded from the analysis. Individual avidity responses are displayed alongside the group median and 95% confidence intervals (CI) to illustrate the variability and overall trends in avidity over time

4.7.7 Associations of total IgG and antibody avidity to NANP, R21 and C-terminus

I explored whether there was any relationship between antigen-specific total IgG and total IgG avidity at different time points after vaccination. Interestingly, there was a moderately negative association between antibody titres and avidity towards R21 and NANP at earlier time points and little or no association at later time points (days 70 and 84). This association was strongest at days 14 (n = 11, r = -0.855, p = 0.0015, [95% CI -0.9630 to -0.5075]) and 42 (n = 19, r = -0.519, p = 0.0195, [95% CI -0.7987 to -0.0858]) for NANP, and at day 42 for R21 (n = 19, r = 0.530, p = 0.022, [95% CI: -0.7934 to -0.0714]). Correlations with C-terminus antigen were consistently negative, and a statistically significant negative association was observed on day 84 (r = -0.511, p = 0.038, n = 17). However, the statistical significance of the association appears to be driven by a single outlier, suggesting that the result may not be representative of the broader dataset (Figure 4.11). These results underline the importance of measuring antibody levels and assessing the quality of the immune responses generated by the vaccine.

Table 4. 3: Association of total IgG and antibody avidity following vaccination with R21/MM

Time point	**R21 responses**			**NANP responses**			**C-terminus responses**		
	r	p	n	r	P	n	r	p	n
Day 14	0.300	0.226	18	-0.855	0.001	11	*n.d*	*n.d*	*n.d*
Day 42	-0.519	0.022	19	-0.530	0.019	19	-0.273	0.258	19
Day 70	-0.372	0.116	19	-0.036	0.889	19	-0.142	0.566	19
Day 84	-0.385	0.127	17	-0.075	0.777	19	**-0.511**	**0.038**	**17**

Association between total IgG to NANP, R21 and C-terminus are reported with their corresponding avidity index. Spearman's rank correlation and p values are shown. *n.d* = not done (too few data points to carrying out an association analysis)

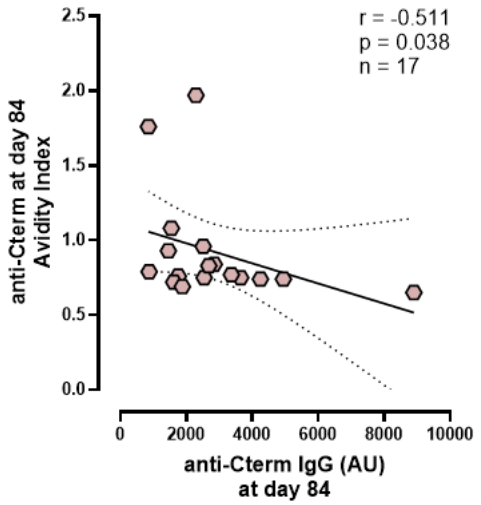

Figure 4. 11: Relationship between total anti-C-terminus IgG antibodies and C-terminus avidity at day 84.

Spearman's correlation was performed at day 84 to assess the relationship between total anti-C-terminus IgG antibody levels and C-terminus avidity.

4.7.8 Anti-*Plasmodium* IgG responses in Ngerenya adults vary greatly.

One challenge we face is understanding how pre-existing immune responses acquired through prior exposure to malarial parasites might interfere with our ability to mount vaccine-induced responses. Therefore, the impact of previous exposure to malaria on the immunogenicity of the R21/MM vaccine was explored. First, I wanted to establish the level of exposure of the participants. The assay included two antigens: MSP-1, a key antigen involved in red blood cell invasion, and NF54 schizont extract, which contains a broad array of *P. falciparum* antigens derived from the NF54 strain. These measurements provide a baseline assessment of pre-existing immunity to *P. falciparum*. I observed a wide range of responses to both the antigens. Total IgG ranged from a minimum AU of 43 to a maximum AU of 3637 for anti-schizont responses, and a minimum AU of 23 to a maximum AU of 3267 for MSP-1 responses. Among those tested for schizont responses 61.7% (29/47) were positive at baseline with a GMT of 155.6 AU (95% CI: 108.1 – 224.2, n = 47). Participants responding to MSP-1 were slightly higher at baseline with 70% (33/47) of individuals having a positive response with a GMT of 103.2 AU (95% CI: 73.5 – 144.8).

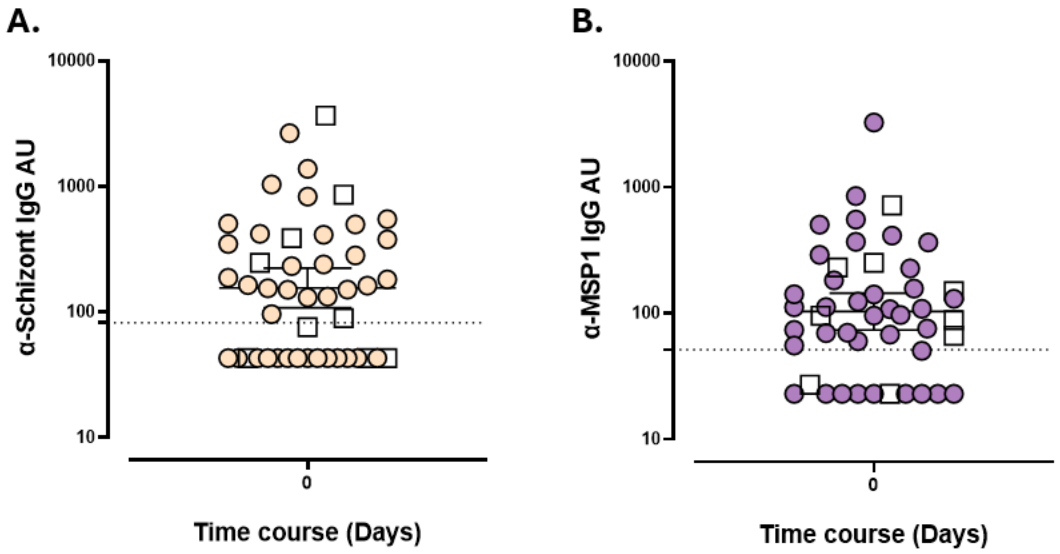

Figure 4. 12: Total anti-*Plasmodium* IgG antibody responses.

Anti-IgG measured by standardized ELISA at baseline (day 0) to determine the previous malaria exposure in the participants. (A) Schizont ELISA, d0 n =47 (B) MSP-1 ELISA, d0 n =47. Results were expressed as arbitrary units (AU). Data shown is median ±95% CI. The limit of detection of the ELISA was OD 0.2, values with an OD <0.2 were assigned an AU value of 43 in the schizont ELISA and AU 23 in the MSP-1 ELISA. The y-axis is in logarithm 10 scale. Horizontal dash line represents the positivity threshold (mean plus 3x standard deviation of naïve individuals). Circles in both graphs represent participants who were later vaccinated, while open squares denote control participants.

4.7.9 Positive Correlation Between MSP-1 and Schizont IgG Responses at baseline.

Spearman's correlation test was used for data analysis because of its ability to function without assuming a normal distribution, allowing me to determine whether there is a relationship between MSP-1 antibodies and anti-schizont IgG responses among the study participants. A strong positive association was observed between schizont IgG responses and MSP-1 responses on day 0 ($r = 0.574$, $p = 0.0003$, $n = 36$), indicating that participants with higher MSP-1 antibody levels also exhibited elevated anti-schizont IgG responses.

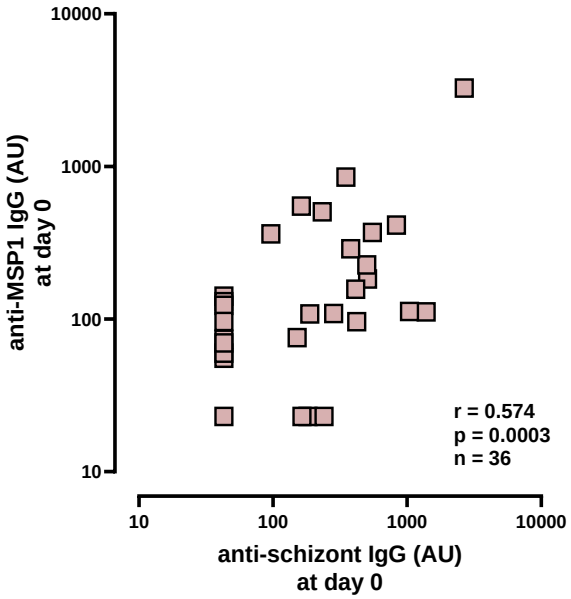

Figure 4. 13: Relationship between total anti-schizont extract antibodies and MSP-1 antibodies.

Antibody responses at baseline (Day 0) were quantified using a standardized ELISA, with results expressed in arbitrary units (AU). Data shown for both cohorts (n = 36). Statistical analysis was performed using Spearman's correlation test.

4.7.10 Previous malaria exposure does not impact vaccine induced antibody responses.

To evaluate whether previous exposure to malaria had an effect on vaccine-induced antibodies, I carried out a correlation analysis and compared the total IgG responses to NF54 schizont extract and MSP1 on day 0 (before vaccination) against NANP, R21, and C-terminus responses on day 84 (1 month after the third dose). The analysis revealed no significant correlation with any of the three vaccine antigens (NANP, R21, and C-terminus), suggesting that malaria exposure, as determined by responses to schizont extract and MSP-1, does not influence antibody responses to the vaccine.

Table 4. 4: Relationship between total IgG responses to schizont extract and MSP-1 with vaccine induced CSP responses post three doses.

	Anti-Schizont IgG Day 0			**Anti-MSP-1 IgG Day 0**		
	r	p	n	r	p	n
R21 IgG	-0.287	0.09	34	0.08	0.63	32
NANP IgG	-0.09	0.60	34	0.21	0.25	32
C-terminus IgG	-0.008	0.96	34	0.17	0.35	32

4.7.11 Association between naturally acquired anti-schizont antibodies and vaccine-induced antibody avidity

I investigated the influence of previous exposure on the avidity of vaccine-induced antibodies by comparing the total anti-schizont IgG antibodies at day 0 against the antibody avidity index of NANP, R21, and C-terminus following the third dose of R21/MM (day 84). I observed a moderately positive association between total schizont responses with NANP (Spearman's correlation test r = 0.483, p = 0.052, n = 17) and R21 (Spearman's correlation test r = 0.459, p = 0.06, n = 17) avidity. Interestingly, there was a significant positive correlation between schizont responses and C-terminus avidity on day 84 (Spearman's correlation test, r = **0.736, p = 0.0012**, **n = 17**). Similarly, a strong positive association was observed between total IgG responses to MSP-1 at baseline and C-terminus avidity on day 84 (Spearman's correlation test, r = **0.612, p = 0.009, n = 17** (graph not shown). This finding suggests that pre-existing immunity to malaria antigens, MSP-1, and schizont-specific IgG responses may play a role in shaping the quality and avidity of vaccine-induced antibodies targeting the C-terminus region. While a few outliers may be affecting this trend, further investigation with a larger sample size is warranted to confirm and expand upon these results.

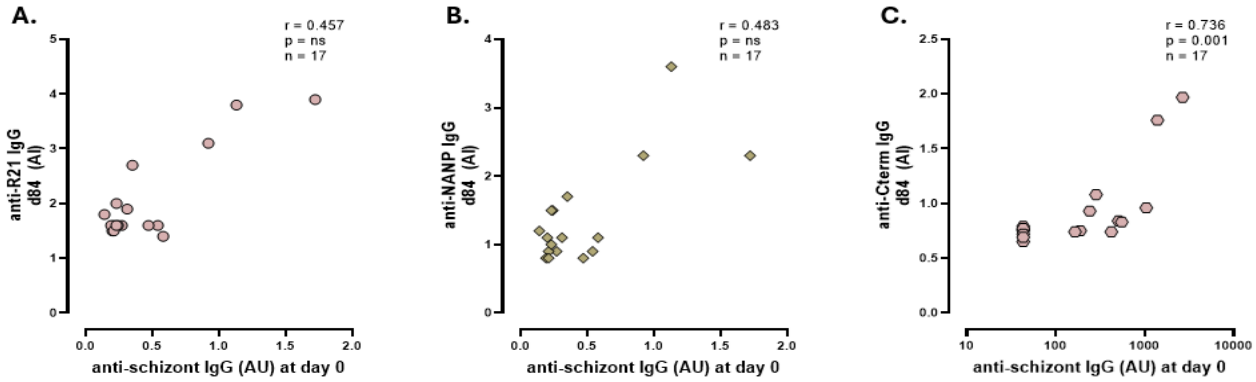

Figure 4. 14: Correlations of schizont IgG response and vaccine induced antibody avidity.

A) Associations between anti-schizont IgG responses at day 0 and anti-R21 avidity at day 84. (B) Associations between anti-schizont IgG responses at day 0 and anti-NANP avidity at day 84. (C) Associations between anti-schizont IgG responses at day 0 and anti-Cterm avidity at day were analyzed. The strength and direction of these associations were evaluated using Spearman rank correlation. Statistical significance was defined as $p < 0.05$.

4.7.12 Vaccination induces complement-fixing antibodies targeting NANP and C-terminus

I performed a complement fixation assay to assess if R21/MM vaccination produces C1q-fixing antibodies and their longevity. Immune antibodies can fix and activate complement against CSP, particularly the central-repeat or C-terminus regions, and are potential protection correlates in RTSS-vaccinated children (Kurtovic et al., 2018; Kurtovic, Drew, et al., 2021). NANP responses were measured on days 0- and two-weeks post-booster (days 14, 42, 70), and day 84 for both cohorts. Following the initial dose, there was a significant rise in NANP complement-fixing antibodies on day 14 (median OD_{405nm} 0.474, 95% CI: 0.41–0.51, n = 38) versus day 0 (median OD_{405nm} 0.232, 95% CI: 0.23–0.24, n = 38), $p < 0.0001$. By day 14, 89% (34/38) of vaccinees had developed these antibodies, which increased with subsequent doses. By day 42, all vaccinees were positive for complement-fixing antibodies, with no significant increase with doses 2 and 3 ($p > 0.9$). Peak antibody levels were on day 70 (median OD_{450nm} 0.614, 95% CI: 0.571–0.689; n = 38), followed by a decline. The control group remained negative at day 84 (low complement-fixing antibodies, median OD_{450nm} 0.256, 95% CI: 0.24 – 0.26, n = 18) and this is statistically significant when compared to the day 84 vaccinees (Mann Whitney test, $p = 0.0018$).

A similar pattern is observed in the development of complement-fixing antibodies targeting the C-terminus antigen, complementing the increase observed with NANP antibodies, although complement

fixing activity was greater towards NANP than C-terminus. However, a notable difference is that two doses of R21/MM are required to elicit complement-fixing antibodies as previously demonstrated when examining total IgG responses to the C-terminus region (Figure 4.6 C). After the second vaccination, there was a significant difference between the dose groups (d0 and d14 vs d42; $p < 0.0001$). Peak antibody levels were reached on day 42, with 73% of vaccinees exhibiting complement-fixing antibodies (median $OD_{450nm} = 0.524$, 95% CI: 0.468 - 0.61). There was no significant difference noted with the addition of the final dose (d42 vs d70; $p > 0$ .9), as determined by Kruskal-Wallis Test with Dunn's multiple comparison test. By day 70, positive rates among vaccinees had increased slightly to reached 79%; however, this percentage declined again at day 84 to settle at 73%. Unlike NANP responses not all vaccinees had C-terminus C1q responses. Suggesting the C-terminus antigen does not readily induced antibodies that activate complement. As observed earlier with the NANP responses, responses to C-terminus at day 84 by the unvaccinated group was also negative. These findings imply that vaccination with R2l/MM induces the production of complement fixing antibodies targeting NANP and the C-terminus antigen.

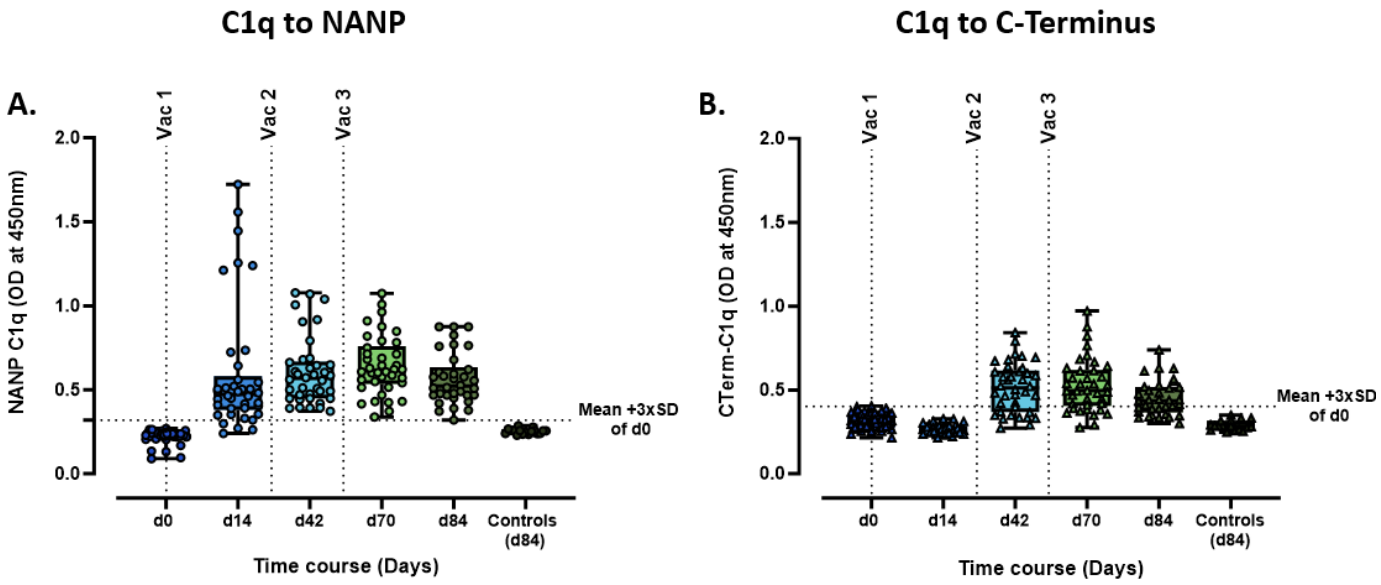

Figure 4. 15: Vaccine induced antibodies promote complement fixation to NANP and C-terminus.

Semi-immune adults vaccinated with R21/MM were tested for C1q fixation to NANP and C-terminus antigens (A) NANP and (B) C-terminus. Data shown as box plots whereby top, centre, and bottom horizontal lines represent the 75th percentile, median, and 25th percentile, respectively; upper and lower whiskers represent the highest and lowest values. The positivity cut-off was the mean plus 3 standard deviations of day 0 (dashed line).

4.7.13 Positive correlations between anti-NANP and anti-C-terminus IgG and anti-C1q responses

I evaluated the relationship between total IgG titres specific to NANP and the C-terminus region and complement-fixing antibodies two weeks post-vaccination. A strong, significant positive correlation was found between total IgG titres to both NANP and the C-terminus region, and anti-C1q complement-fixing antibodies. This indicates that higher levels of anti-CSP IgG are correlated with enhanced complement activation, suggesting increased antibody functionality.

Table 4. 5: Associations between total IgG antibody titres to anti-C1q responses to NANP and C-terminus

Time point	**NANP**			**C-terminus**		
	r	p	n	r	p	n
Day 14	0.668	0.002	19	*n.d*	*n.d*	*n.d*
Day 42	0.642	0.003	19	0.928	<0.0001	19
Day 70	0.785	<0.0001	19	0.864	<0.0001	19
Day 84	0.79	0.0003	17	0.774	0.0004	17

Association between total IgG to (i) NANP and (ii) C-terminus and complement fixing antibodies are reported. Data shown on group 1 and 3 (i.e. all those that received 3 doses of R21/MM vaccination). Spearman's rank correlation and p values are shown. *n.d* = not done (too few data points to carrying out an association analysis)

4.8 Discussion

The R21 vaccine has already demonstrated high efficacy in the field ((Datoo et al., 2024) and was recently recommended by WHO for the prevention of malaria in children. Several studies have demonstrated that vaccination with R21 induces high antibody responses to the NANP region of CSP in both adults and children (Datoo et al., 2024; Sang et al., 2023; Venkatraman et al., 2019). Building upon this previous research on antibody responses to R21/MM vaccination, my current work aims to advance the understanding of vaccine-induced immunity against malaria. In this chapter, I delved into a comprehensive analysis of the antigen-specific immune responses elicited by the vaccine in adult volunteers who were later experimentally infected with malaria. In this in-depth examination I focused on characterising and assessing the specificity and strength of the immune response against key malaria antigens.

To determine the total IgG titres and the total IgM and IgA responses to the three CSP antigens included in the vaccine, I utilized standardised ELISAs. Prior to the start of my thesis, there was no standardised ELISA for R21, NANP and C-terminus, hence I undertook various optimisation steps to establish an assay that was fit for purpose. Following successful assay set up, I compared NANP responses across three different independent assay platforms, namely the validated NANP ELISA (Jenner Institute, Oxford), Kilifi NANP ELISA, and the multiplex MSD assay, and the results were strongly correlated across the assays, which further validated my optimized NANP assay. For my analysis/experiments, I utilized the Kilifi ELISAs that I set up.

At baseline (day 0) all participants anti-CSP IgG responses, were below the threshold for positivity defined using U.K naïve control serum. In natural exposure, CSP is not as immunogenic as other antigens such as AMA-1 or MSP-1 (Ondigo et al., 2020) therefore it is not surprising that all participants were negative for CSP antibodies at this time, particularly in this low transmission setting. After vaccination with R21/MM, I observed significant increases in total IgG responses to all three vaccine antigens (NANP, R21, and the C-terminus) compared to baseline levels and the non-vaccinated control group. Antibody responses peaked day 70 for all three antigens. I did not observe any further increase in antibody titres following the second dose of R21. Further, I observed antibody responses to the C-terminus region of the vaccine require two doses of R21/MM to be elicited, unlike R21 and NANP responses that significantly increased after a single dose. This specific region includes an α-thrombospondin repeat (αTSR) domain, which is crucial for hepatocyte invasion, and also contains CD4 and CD8 T cell epitopes (Chaudhury et al., 2021; Coppi et al., 2011). T cell epitopes are mainly present in the C-terminus region rather than potent B cell stimulants; therefore, this may explain why two doses might be necessary before eliciting a humoral

response. C-terminus has been shown to be involved in sporozoite entry into the liver, therefore high functional anti-C-terminus antibodies could also contribute to preventing hepatocyte invasion. Overall, the induction of IgG responses to this region further emphasizes the multifaceted immune response induced by R21/MM.

Using the MSD assay, I was also able to investigate responses to the HBsAg part of the vaccine. One major advantage of R21 over RTS,S is that it is composed of a single CSP-HBsAg fusion unlike RTS,S meaning more of the CSP portion is displayed versus the HBsAg (Collins et al., 2017). This was evident in MSD assay results, showing lower IgG responses to HBsAg compared to CSP. R21's design minimizes HBsAg exposure, focusing on inducing strong CSP responses over hepatitis B. While the limited presentation of HBsAg may hinder the immune system's ability to fully recognize and respond to it, the observed responses remained above the WHO-defined threshold for protection (anti-HBsAg > 0.01 IU/ml) (WHO, 2011), suggesting that vaccination with R21 may also elicit or boost protective responses against hepatitis B. I also observed the same IgG kinetics to R21, NANP and C-terminus as seen in the standardized ELISAs. With IgG responses increasing after the first two doses and sustained until day 84. In these first set of experiments, I have shown the summary kinetics of IgG responses, their peak titres days and durability of vaccine induced responses. I demonstrate that the R21/MM vaccine, induces a strong humoral immune response to different regions of CSP and to a much lesser extent hepatitis core antigen in adults residing in low malaria transmission areas, potentially offering protection against malaria infection and hepatitis B.

Furthermore, I examined the IgM and IgA responses induced by R21/MM vaccination. Research has indicated that IgA and IgM responses can be induced after CSP based vaccinations, though their specific role in protection is not fully understood. Anti-parasitic IgM antibodies, generated through natural exposure, have been shown to activate the complement system, thereby inhibiting the *in vitro* growth of *Plasmodium* parasites. Furthermore, these antibodies are significantly correlated with protection against clinical malaria in human cohort studies (Boyle et al., 2019; Kurtovic et al., 2018). Studies conducted by Zenklusen and colleagues have demonstrated that vaccination with Sanaria's PfSPZ vaccine induces, in addition to IgG, IgM antibodies that are capable of blocking sporozoite invasion of liver cells *in vitro* and fixing complement to whole sporozoites (Zenklusen et al., 2018). Additionally, Tan and colleagues have shown that exposure to *P. falciparum* sporozoites through infection or immunization prompts a functional IgA response, they also isolated a monoclonal IgA antibody that was capable of inhibiting liver cell invasion *in vitro* (Tan et al., 2021). My observations revealed consistently strong responses to R21, NANP,

and C-terminus among the vaccinees; with the highest anti-IgM and -IgA responses seen for R21 followed by NANP then C-terminus. The high responses to R21 could be partly explained by IgA responses to the hepatitis core B antigen, nonetheless we still observe significant increases towards NANP and C-terminus alone suggesting the IgA may still play a role. All responses at day 84 were significantly higher than the non-vaccinated individuals, baseline (day 0) levels and those of U.K malaria-naïve adults. These robust responses were sustained until day 84, indicating a broad multi-isotype immune response but further exploration into their protective capabilities is needed. These findings underline the significance of studying different isotypes when examining vaccine-induced responses to malaria vaccines while supporting the potential of R21/MM in eliciting broad immune responses against malaria infections.

Next, I investigated the avidity of these vaccine induced antibodies. Analysis of antibody avidity following vaccination provides insights into the quality and strength of the vaccine-induced immune response. High-avidity antibodies are key indicators of an effective immune response, as they display better function and may be more indicative of protective antibodies (Bauer, 2021). The avidity of antibodies typically increases over time through affinity maturation and may serve as a useful surrogate marker for priming. In the context of other infectious diseases such as measles (Mercader et al., 2012) and *Haemophilus influenzae* type B (Breukels et al., 2002), antibody avidity has been used to identify vaccine failures; those with lower antibody avidity are more likely to have experienced vaccine failure. There is mixed evidence that antibody avidity correlates with antibody titres and/or protection against malaria. For instance, two phase IIb studies evaluating the avidity of anti-CS antibodies in infants following vaccination with RTS, S, found no association between avidity and vaccine efficacy or protection (Ajua et al., 2015; Olotu et al., 2014). Dobaño and colleagues demonstrated in infants that anti-NANP IgG concentrations and avidity to the C-terminus region correlate with increased vaccine efficacy of RTS,S/AS01E, and that C-terminus avidity emerged as a significant contributor to vaccine-induced protection (Dobaño, Sanz, et al., 2019). Furthermore, a modelling study also found that incorporating both avidity and antibody titre provided a substantially better fit to data on protection from malaria infection following RTS, S/AS01 vaccination compared to antibody titre alone (Thompson et al., 2020). Lastly, Biswas et al. noted an increase in IgG avidity for $MSP1_{19}$ after vaccination with blood-stage antigens, although the relationship between avidity and vaccination was not consistent across the antigens tested, with AMA1 showing no changes following vaccination, highlighting the antigen-specific nature of avidity (Biswas et al., 2014). These studies indicate that multiple variables affect avidity such as study population, malaria intensity of the area, ages of the population, antigen being measured and lastly the methods used to measure avidity indicating the

complexity of the relationship between vaccination, avidity, and protection against malaria. In this study, the sodium thiocyanate displacement method was used to assess the avidity of antibodies induced by the R21/MM vaccine. Antibodies were normalised to an OD of 1.0, allowing reasonable observation of comparability between assays. The results revealed only minimal changes in avidity to R21, NANP, and C-terminus after vaccination. There was a trend towards a higher avidity index for both R21 and NANP on day 14, compared to subsequent time points on days 42 and 70; this trend was not statistically significant. Overall, the results suggest that additional vaccine doses (doses two and three), do not lead to an increase in avidity, as measured by the NaSCN method. One can speculate that the immune response may reach a plateau or already form a stable response regarding antibody quality after the first dose and does not require further optimisation through additional doses. This suggests limitations in how the immune system responds to repeated vaccinations, particularly in terms of enhancing the binding strength of antibodies. When investigating the associations between total IgG titres and antibody avidity, I observed a negative association between total IgG anti-C-terminus antibodies at day 84 and C-terminus avidity on day 84. This indicates that while vaccination induces high antibody titres, it does not necessarily correlate with improved antibody functionality in terms of binding affinity. It is also possible that the NaSCN displacement ELISA may not be the best assay for assessing antigen and antibody binding strength to these antigens. A more sensitive assay, such as Biolayer Interferometry (BLI), may be more appropriate for measuring binding strength because it directly measures the kinetic properties of antigen-antibody interactions, such as association and dissociation rates. BLI offers dynamic real-time analysis of binding interactions, allowing for a detailed assessment of both the affinity (strength of binding) and kinetics (how quickly the antibody binds and dissociates from the antigen) (Dennison et al., 2018).

Individuals with previous exposure to malaria may develop some level of immunity to the disease, which can affect their immune response to vaccination and the quality of antibodies produced (Bell et al., 2023; Obiero et al., 2015; S. et al., 2014; Sissoko et al., 2021). Repeated parasite exposure can led to atypical B cells (Braddom et al., 2020), as well as B and T cell exhaustion which may then affect effector functions of these cells (Frimpong et al., 2019). Understanding the effect of exposure will offer valuable insights into the possible effect of pre-existing immunity on vaccine efficacy, immune responses to vaccines and guide strategies for optimizing vaccination effectiveness. Firstly, I determined what the level of exposure was in this population. I measured IgG responses to schizont extract and MSP-1 (a purified blood stage antigen) as a marker of exposure. The participants were from Ngerenya, where malaria prevalence began to decline in the early 2000s, reaching nearly zero by 2009, and has remained at this low level ever since

(Rono et al., 2015). Given that the average age of participants was 28 years, it was unsurprising to find a wide range of anti-*Plasmodium* responses. About 30-40% of the individuals were sero-negative to schizont extract, it is plausible to assume that these individuals have not experienced clinical malaria in several years. I also performed a Spearman's correlation between anti-schizont and anti-MSP1 and found that there was a strong positive association between the two. This is also not unexpected, as schizont extract is a mixture of all antigens derived from the schizont stage and includes several thousand proteins that are involved in the asexual stages of infection, one of which is MSP-1. Given that MSP-1 is a key antigen expressed on the surface of merozoites, which are released from the schizont following cell rupture, it is likely that antibodies targeting schizont proteins also cross-react with MSP-1. This overlap in antigenic targets can contribute to the observed correlation between anti-schizont and anti-MSP1 antibody responses. Although schizont extract and MSP-1 have been used as markers of previous exposure in this study, the kinetics and longevity of antibody responses to malaria antigen varies between antigens as well as an individual's. For example, antibody responses to MSP-1 have been suggested to be a marker of recent or concurrent infection, while AMA-1 has a longer half-life and is considered a marker of past exposure (Leonard et al., 2023; M. T. White et al., 2014; Yman et al., 2022). In future, the combination of several different antigens should be used in conjunction for more reliable results to determine previous and or recent malaria exposure.

Having established the amount of exposure the participants had; I then investigated the potential impact of previous malaria exposure on vaccine-induced responses (quality and quantity). I compared baseline levels (pre-vaccination) of both schizont and MSP1 IgG responses to post-vaccination IgG responses at day 84. A Spearman's correlation test revealed a weak negative association between anti-schizont and anti-MSP1 IgG levels with vaccine-induced total IgG antibody responses, however this was not significant. This finding suggests that the humoral response to R21/MM vaccination is not significantly influenced by prior malaria exposure. It is important to recognize that this study population originates from an area characterized by low transmission of malaria, a factor that may significantly impact the applicability of these findings to individuals residing in regions with high malaria transmission. The influence of pre-existing immunity on vaccine-induced responses could differ depending on the level of exposure to malaria in the studied population, as the dynamics of immunity may vary according to the intensity of malaria transmission in the region. Although, a previous study conducted among adults living in an area with high transmission revealed similar results, as it demonstrated increased levels of anti-CS antibodies after vaccination with RTS,S/AS02A or RTS,S/AS01B, suggesting repeated exposure did not

interfere with vaccine antibody responses (Polhemus et al., 2009). Therefore, vaccination with R21/MM may not necessarily be influenced with transmission rates, at least in responses to anti-CS antibodies in adults.

Interestingly, analysis of the impact of prior malaria exposure on vaccine-induced IgG avidity revealed a significant positive correlation with the avidity of C-terminus at day 84 for both schizont extract and MSP-1 total IgG responses at baseline. This suggests that previous exposure to malaria might enhance the development of vaccine-induced antibodies, resulting in increased avidity, particularly towards the C-terminus region of the vaccine, which could be associated with improved protection against the disease. This observation sheds light on the intricate interplay between natural immunity and vaccine-induced immune responses. However, owing to the limited sample size (n = 17), this finding may be coincidental and requires validation with a larger dataset. Nevertheless, these results emphasise the importance of considering an individual's history of malaria exposure when evaluating vaccine responses and formulating effective immunisation strategies.

Next, I analysed the ability of R21/MM to induce antibodies capable of activating complement against NANP and the C-terminus region. The ability of antibodies to fix complement is a crucial aspect of the humoral immune response, as it contributes to the effective clearance of pathogens. Complement activation against *Pf* sporozoites has been shown to associate with protection in children (Behet et al., 2018; Kurtovic et al., 2018; Kurtovic, Atre, et al., 2021). I noted that R21/MM does indeed elicit anti-C1q antibodies to NANP and to a lesser extent to C-terminus, with their concentrations increasing after vaccination in a similar pattern to the kinetics of total IgG. However, these levels are lower and decline more rapidly than those of total IgG. I also noted a consistent and positive correlation between total IgG responses and antibodies that fix complement at all timepoints. This indicates that R21 vaccination has the potential to generate an antibody response capable of fixing complement to the CSP antigens targeted by the vaccine, highlighting the potential of R21/MM to not only prevent malaria infection but also effectively combat the pathogen in the event of exposure.

This analysis of the antigen-specific immune responses, including antibody isotypes, antibody avidity and complement-fixing abilities, provides valuable insights into the mechanisms underlying the potential efficacy of R21/MM vaccination in a low malaria transmission setting. These results lay a solid foundation for further exploration and development of R21/MM as a promising tool in the fight against malaria.

4.8.1 Limitations

The primary limitation of this study is the small sample size for certain assays, which reduced statistical power and limited the generalizability of the findings. Additionally, using schizont extract as a marker of exposure poses a limitation due to its lack of standardization, necessitating bulk parasite cultures to generate the extract. Assessing the frequency and timing of exposure, alongside the relationship between antibody levels and past exposure, is also challenging. To address these issues, a combination of several antigens targeting recent and past exposures might be more effective.

Avidity ELISA has limitations in detecting low antibody levels, potentially missing high-avidity antibodies at early time points. Differentiating between avidity from natural exposure and vaccination-induced avidity is also not feasible. Although an early time point like day 7 could offer more insight into vaccine-induced immunity, it was not possible with the current study design.

Technical difficulties and time constraints prevented the inhibition of sporozoite assays, which would have provided information on antibody functionality. Venkatraman and colleagues reported a 50% increase in the inhibition of sporozoite invasion in naïve individuals post-vaccination with R21/MM compared to baseline (Venkatraman et al., 2019). Animal and *in vitro* studies have shown that vaccine-induced CSP antibodies can block sporozoite invasion (Behet et al., 2014; Rodríguez-Galán et al., 2017; Zenklusen et al., 2018). It is likely that some sporozoite inhibition would have been observed with the vaccine-induced antibodies from this study.

4.8.2 Conclusions

In conclusion, I have demonstrated strong and long-lasting antibody responses to the R21/MM malaria vaccine in adults living in low-transmission areas. These findings highlight its potential for conferring protection against malaria infection and emphasise the need for further research to evaluate the efficacy and durability of this immune response across diverse populations. It also underscores the importance of assessing the functional efficacy of these antibodies in clearing malarial parasites through *in vitro* assays or animal studies. Overall, these findings highlight the promising role of R21/MM vaccine in inducing sustained and potentially functional antibody responses. The ability of the R21/MM vaccine to elicit such a broad response suggests its potential effectiveness in providing long-term immunity and protection against parasites.

5 CHAPTER FIVE – Cellular responses to vaccination

5.1 Introduction

The success of any vaccine depends on memory induction. Memory B and T cells are vital for the development of secondary immune response. In infectious diseases, the presence and function of memory B cells appears to be crucial for long-term protection against infection (Palm & Henry, 2019). Memory B cells (MBC) are responsible for the rapid production of specific antibodies upon re-exposure to the malarial parasite, preventing reinfection or reducing the severity of the disease. They can quickly proliferate and differentiate into antibody-secreting plasma cells upon re-exposure to the same pathogen. This rapid response typically occurs within 2-4 days (Ly & Hansen, 2019; Pérez-Mazliah et al., 2020). Although MBCs have limited effectiveness in inhibiting sporozoites during the initial stages of infection, they are essential for long-term immunity and preventing subsequent infections. A previous study showed that smallpox vaccine-specific MBCs last for >50 years in individuals administered a single vaccine (Crotty et al. 2003). However, in *P. falciparum* infections, humoral immunity develops over the years and requires frequent infection. Epidemiological evidence indicates that antibodies against *Plasmodium* antigens are poorly produced and rapidly decline in the absence of ongoing parasite exposure. This suggests that malaria may hinder the development of long-term immunological B cell memory (Palm & Henry, 2019). Atypical B cells have been proposed to explain the slow and incomplete immunity observed in malaria (Portugal et al., 2015; Weiss et al., 2009). Atypical B cells are a distinct subset of B cells characterised by a lack of expression of CD21 and CD27, which are common markers found in classical MBCs and are often associated with chronic antigen stimulation (e.g. in HIV and malaria) and autoimmune diseases (e.g. systemic lupus) (Ambegaonkar et al., 2022; Pérez-Mazliah et al., 2020; Weiss et al., 2009). The development of an effective malaria vaccine that can induce and maintain robust MBC responses remains challenging (Duffy et al., 2020; El-Moamly & El-Sweify, 2023). Understanding the mechanisms that promote the generation and longevity of MBCs is imperative for the design and improvement of malarial vaccines. Additionally, identification of specific antigens or epitopes that can elicit strong MBCs is essential for the development of effective vaccines against malaria.

When a B cell encounters an antigen via either infection or vaccination, B cells become activated in the secondary lymphoid tissue and receive helper signals from CD4+ T cells at the border of the B cell follicle and T cell zones. Activated B cells proliferate and differentiate into short-lived plasma blasts, GC B cells, MBCs, and long-lived plasma cells within secondary lymphoid organs (see Figure 1.5 in chapter 1) (Ly & Hansen, 2019; Pérez-Mazliah et al., 2020). Follicular helper T-cell (Tfh) and GC B-cell responses are

essential for generating isotype-switched long-lived plasma cells and MBCs (Pérez-Mazliah et al., 2020). During subsequent infection or vaccination, MBCs transform into short-lived plasma blasts that quickly produce both switched and unswitched antibodies.

R21/MM primarily provides protection via antibodies. Tfh cells play a crucial role in guiding GC reactions, supporting the selection of high-affinity B cells, and providing essential signals for the development and sustenance of MBC populations (Soon et al., 2021). The interplay between Tfh and B cells is pivotal for establishing immunological memory and enhancing the effectiveness of the adaptive immune response.

Tfh cells are primarily found within the GC, but sampling these cells is inconvenient. However, a subset of CD4+ T cells that circulate in peripheral blood shows functional, phenotypic, and transcriptional similarities with their GC counterparts, including the expression of chemokine receptor CXCR5 and inhibitory receptor programmed cell death (PD-1). These cells can act as proxies to investigate GC responses *ex vivo* (Heit et al., 2017; Kurup et al., 2019; Morita et al., 2011). cTfh can be further divided into three cTfh subsets based on the expression of CXCR3 and CCR6: cTfh1 cells (CXCR3+CCR6−) which express T-bet and Bcl-6 and produce IFN-γ; cTfh2 cells (CXCR3−CCR6−) which express GATA3 and produce IL4, IL5, IL13 and cTfh17 cells (CXCR3−CCR6+) which express RORT and express IL17 and IL 22 (X. Ma & Nakayamada, 2021). Tfh2 and Tfh17 are particularly efficient in inducing antibody production (Moris et al., 2018; Morita et al., 2011; Wahl et al., 2022). These subsets of Tfh cells have specialised functions in promoting B cell responses and antibody production, with each subset playing a distinct role in immune system defense against various pathogens. Tfh2 facilitates B cell activation, survival, and differentiation into antibody-secreting plasma cells and antibody class switching *in vitro*, whereas Tfh1 cells have been found to be poor helpers of B cells *in vitro* (Crotty, 2019; Locci et al., 2013; Morita et al., 2011). Higher numbers of cTfh2 and cTfh17 cells are associated with increased autoantibody production (Morita et al., 2011), more severe symptoms in autoimmune diseases (Ríos-ríos et al., 2020), and the development of allergies (Kobayashi et al., 2017). Tfh2 cells also lead to the production of broadly neutralising antibodies in people with HIV (Locci et al., 2013), whereas Th1-cTfh cells are associated with antibody production following viral infections and vaccinations, such as severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (Gong et al., 2020; Verma et al., 2020). Therefore, the subset type induced by vaccination may serve as an indicator of the antibodies produced.

To date, there has been limited analysis of the impact of R21/MM vaccination on the kinetics and frequencies of Tfh and their subsets, as well as data on the types and longevity of MBCs and the role they

play in generating vaccine-induced antibodies. Long-term immunity is crucial for sustained protection against malaria infection, especially in low-transmission areas where sporadic exposure to the parasite may not be sufficient to maintain immunity. Examining the formation of immune memory cells, such as MBCs and long-lived plasma cells, as well as Tfh responses in response to R21/MM vaccination can shed light on the vaccine's ability to confer durable protection.

5.2 Aim

In this chapter, I aimed to examine the induction of MBCs specific to antigens in the R21/MM vaccine. By utilising the FluroSpot assay, I aimed to identify which epitopes within the vaccine antigens are targeted by MBCs, providing insights into the specificity and durability of the immune response. In addition, I aimed to investigate the role of Tfh cells in supporting the activation and maturation of MBCs. This includes examining the kinetics, frequency, and phenotype of Tfh cells in response to vaccination, as they play a crucial role in helping B cells to differentiate into high-affinity antibody-producing cells. Additionally, this chapter explores the impact of booster vaccine doses on these immune cell populations, assessing whether additional doses enhance or modulate MBC response and Tfh cell activity. Overall, the research aims to shed light on the mechanisms by which the R21/MM vaccine induces long-term immunity and how different immune cell interactions contribute to the development of a protective response against malaria.

5.3 Materials and Methods

5.3.1 Peripheral blood mononuclear cells Isolation (PBMC)

Venous blood samples were drawn periodically for immunological assays, as described in section 4. Processed plasma and PBMCS were stored at -80°C and liquid nitrogen, respectively. Briefly, 30 mls of blood was collected in three 10 ml sodium heparin tubes and processed within 4 hrs of collection in the immunology laboratory at the KEMRI-Wellcome Trust research program. PBMCs were isolated using lymphoprep and leucosep tubes via density gradient centrifugation. Before PBMC isolation, 15 ml of lymphoprep was added to the leucosep tubes and centrifuged for 1 min at 1800 rpm. 30ml of heparinized blood was layered into two 50ml tubes of leucosep containing lymphoprep and the tubes were centrifuged at 1200 rpm for 13 min at RT with the brake off. Plasma was then carefully collected from the top layer of each leucosep tube and stored in labelled cryovial at -80°C. PBMCs were carefully collected from the interface using a sterile wide-mouth Pasteur pipette and placed into a new well labelled 50 ml falcon tube containing 15 ml of R0 (RPMI supplemented with 100 U/mL penicillin, 10 mM HEPES, 100 μg/mL streptomycin, 2mM L-glutamine (all from Invitrogen, Life Technologies)). The tube was topped with 40 ml of R0 and spun at 1400 rpm for 7 min at RT. After the final wash, cells were washed twice with R0 by centrifugation (1800 rpm for 5 min). The supernatant was discarded, and the pellet was resuspended in 10 ml R10 for counting. Cells were counted using a Vi-Cell XR cell counter and cell viability analyser (Beckman Coulter).

5.3.2 Freezing of PBMCs

Following cell counting, the cells were centrifuged at 1800 rpm for 5 min and the supernatant was discarded. The cells were resuspended in ice-cold FCS to give between 5-10 million cells per vial. The cells were incubated on ice for 10 min before adding an equal volume of ice-cold 20% DMSO and mixed by pipetting up and down. PBMCs were then transferred into cryovials (1 ml per vial) and placed on ice before being transferred to chilled Mr. Frosty containers which were immediately stored at -80 °C overnight. The following day, the vials were transferred to a liquid-nitrogen tank.

5.3.3 Thawing of cryopreserved PBMCs.

Before retrieving PBMCs, R20 media (RPMI supplemented with 20% fetal calf serum (FCS), 100 U/mL penicillin, 10 mM HEPES, 100 µg/mL streptomycin, 2mM L-glutamine (all from Invitrogen, Life Technologies)) and 50U/ml benzonase endonuclease (Novagen) were prepared and incubated in a 37 °C water bath. PBMCs of interest were retrieved from liquid nitrogen on dry ice, and vials were rapidly thawed in a water bath before being transferred to warm thawing medium (R20) in a tissue culture hood. The cells were washed twice with 20 ml R10 solution by centrifugation (1800 rpm for 5 min). The resulting pellet was resuspended in 10 ml of R10 and left to rest in an incubator at 37 °C and 5% CO_2 for at least 2 hrs. While resting, PBMCs were counted using *a Vi-Cell XR* cell counter and cell viability analyser (Beckman Coulter) for FluroSpot assays (performed at KEMRI-Wellcome Trust Research laboratories) or CASY counter for Tfh phenotyping assays (performed at the Jenner Institute). Due to insufficient numbers of PBMCs being available at every time point, it was not possible to conduct all analyses for all individuals who had received the vaccine. Consequently, the group sizes for certain assays were smaller than optimal, as depicted in the figure legends.

5.3.4 Antigen specific memory B cells using FluroSpot

5.3.4.1 Preparation and stimulation of PBMCs

Memory B cells require polyclonal stimulation to produce detectable antibody levels. After resting the PBMCs and adjusting them to a concentration of 250,000/200 µl, the cells were stimulated with 1 µg/mL resiquimod (R848) and 10 ng/mL recombinant human IL-2 (both from Mabtech) in R20 culture media. The cells were cultured for five days at 37 °C and 5% CO_2 to allow sufficient time for activation and antibody production. After the 5-days, the cells were harvested, washed by centrifugation (1800 rpm for 5 min), and resuspended to give a final concentration of 250,000 cells per 100 µl per well.

5.3.4.2 FluroSpot assay

The FluroSpot assay is a variant of the ELISPOT assay, which uses fluorescence detection instead of chromogenic detection. The main advantage of FluroSpot is that it allows simultaneous detection of multiple Ig isotypes (and/or cytokines) in the same assay well, therefore is beneficial when working with limited samples.

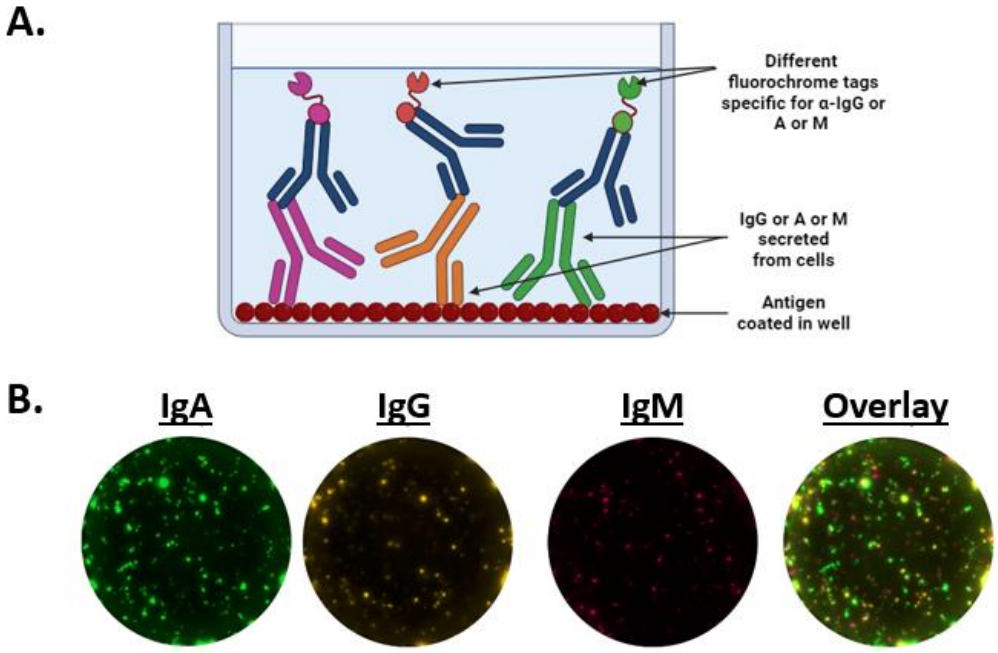

Figure 5. 1: Memory B cell FluroSpot assay.

(A) image depicted the steps involved in the assay (B) image depicted IgA (green), IgG (yellow) and IgM (red) antigen secreting memory B cells assay. Image created in Biorender.com by E. Kibwana.

The assay was conducted as previously described by (Jahnmatz, 2020). Low-fluorescent polyvinylidene difluoride membrane (PVDF) plates (Mabtech) were activated by the addition of 15 µl/well of 30% ethanol for 1 min and washed five times with 200 µl of distilled water. The plates were then coated in duplicate with 100 µl of antigen solution: (i) R21, (ii) C-terminus, (iii) capture monoclonal antibodies ((mAb), (IgG, IgA, and IgM)) and (iv) PBS (negative control to account for any non-specific binding of cells on the plate) overnight at 4 °C. The next day, plates were washed with 200 µl PBS and blocked with 100 µl of R10 per well at 37° C, 5% CO_2 for 1 hr. After removing the block buffer, 250,000 stimulated cells/100 µl/well were added to antigen and negative control coated wells, and 50,000 cells/100 µl/well to the positive control wells to measure total IgG, IgA, and IgM memory B cells. Plates were then incubated at 37 °C, 5% CO_2 for 20 hr. The next day, the plates were washed with 200 µl PBS and 100 µl of the conjugated detection mAb (anti-human IgG550, anti-human IgA-490, and anti-human IgM-640 (all from Mabtech)) diluted 1:500 in PBS containing 0.1% Bovine serum albumin (BSA) was added to all wells. After a 2-hr incubation, plates were then washed five times with 200 µl PBS before adding 50 µl of

fluorescent enhancer (Mabtech), for 5-10 min at RT. After removing the fluorescent enhancer and the plate underdrain, plates were air-dried overnight in the dark. Fluorescent spots indicating individual antibody-secreting cells were analysed using an IRIS FluroSpot Reader and counted using the Apex software version 1.1.8. provided by Mabtech. The reader is equipped with filters to detect fluorophores absorbing and emitting light at 350/470, 490/520, 550/ 570 and 640/660 nm. For each sample tested, "Total IgG or IgA or IgM" sites on wells serve as positive controls while antigen-free wells containing PBS operate as negative controls. A well was deemed positive if any antigen-specific antibody secreting cells (spot-forming cells), were detected in the assay. The magnitude of MBC responses was conveyed as a proportion of antigen-specific spots per total IgG, expressed as a percentage (% of antigen-specific MBC/total IgG or IgA, or IgM). In cases where I was unable to obtain enough cells from a participant (at least 3 million cells) following stimulation, I did not continue with the assay.

Table 5. 1: Antigen concentrations for coating wells

	R21	**C-terminus**	**Total IgG/A/M**	**PBS**
Stock (µg/ml)	568	1000	500	10x
Final concentration (µg/ml)	2.5	2.5	15	1x

5.3.5 Antibody Optimization tests

The protocol for characterizing Tfh cells was a routine protocol used at the Jenner Institute, as such a panel of antibodies had already been optimized for use (Bowyer, Grobbelaar, et al., 2018).

PBMCs were retrieved from liquid nitrogen storage and defrosted as previously outlined. The cells were then allowed to rest for 2 hrs at 37 °C. Following this rest period, the cells underwent centrifugation at 1800 rpm for 5 minutes. The resulting supernatant was removed, and the cell pellet was resuspended in 1 ml of R10 medium. To determine the cell count, 10 µl of the cell suspension was mixed with 10 ml of CasyTone solution and analysed using a CASY cell counter. Subsequently, the cells were centrifuged once more and resuspended to achieve a final concentration of 1.5 million cells per 200 µl. The antibody panel used for the experiments is shown below:

Table 5. 2: Staining antibodies for cell viability

Master mix I (MMI)

	Manufacturer	Concentration	µl/well
Aqua	Thermofisher	1:40	0.25
FACS buffer	In house		9.75
Total			10

Table 5. 3: Staining antibody panel of the identification of Tfh sub-sets

Master mix II (MMII)

	Manufacturer	Clone	Concentration	µl/well
PD1-BV650	Biolegend	EH12.2H7	1:100	1
CD45RA-eF450	Biolegend	HI100	1:50	1
CXCR5- FITC	Biolegend	J252D4	1:50	1
CCR6-PE	Biolegend	G034E3	1:25	2
CD4-APC-eF780	ebiosciences	RPA-T4	1:50	1
CD3-AF700	ebiosciences	UCHT1	1:33	1.5
CXCR3-APC	Biolegend	G025H7	1:17	3
Biolegend staining buffer	Biolegend			39.5
Total				50

Cell staining was performed in 96-well V-bottom plates. Briefly, 200 µl of cells (at 1.5 x10^6 PBMC) were added to the wells and washed twice with 200 µl of FACS buffer by centrifugation (1800 rpm for 5 min). After the last wash 10 µl of MM I was added to each well and the plate was incubated in the dark for 20 min at RT. Following this step, the plates were washed twice and 50 µl of MM II was added to each well, and the plate was incubated at 37 °C for 30 min. The plates were washed again, and the cells were fixed in 80 µl/well of 1% paraformaldehyde (PFA (43368, Alfa Aesar)). Cells were acquired immediately on the BD LSRFortessa cell analyzer (BD Biosciences). Compensation was performed using single-stained One-Comp beads for monoclonal antibodies and ARC beads for AQUA. Analyses were conducted according to the gating strategies shown in the result section. Flow cytometry data was analysed on Flow Jo version 10.10.0 software (Tree Star, San Carlos, CA, USA) and GraphPad Prism 10.2.1. FCS data cleaning was performed before analysis using the software program FLOWAI (Monaco et al., 2016) which detects and removes anomalies such as interrupted flow or signal acquisition issues. As such samples that

did not reach the set quality standards were not included in the analysis. The gating strategy used to identify cTfh is shown in Figure 5.2 as described by (Bowyer, Grobbelaar, et al., 2018).

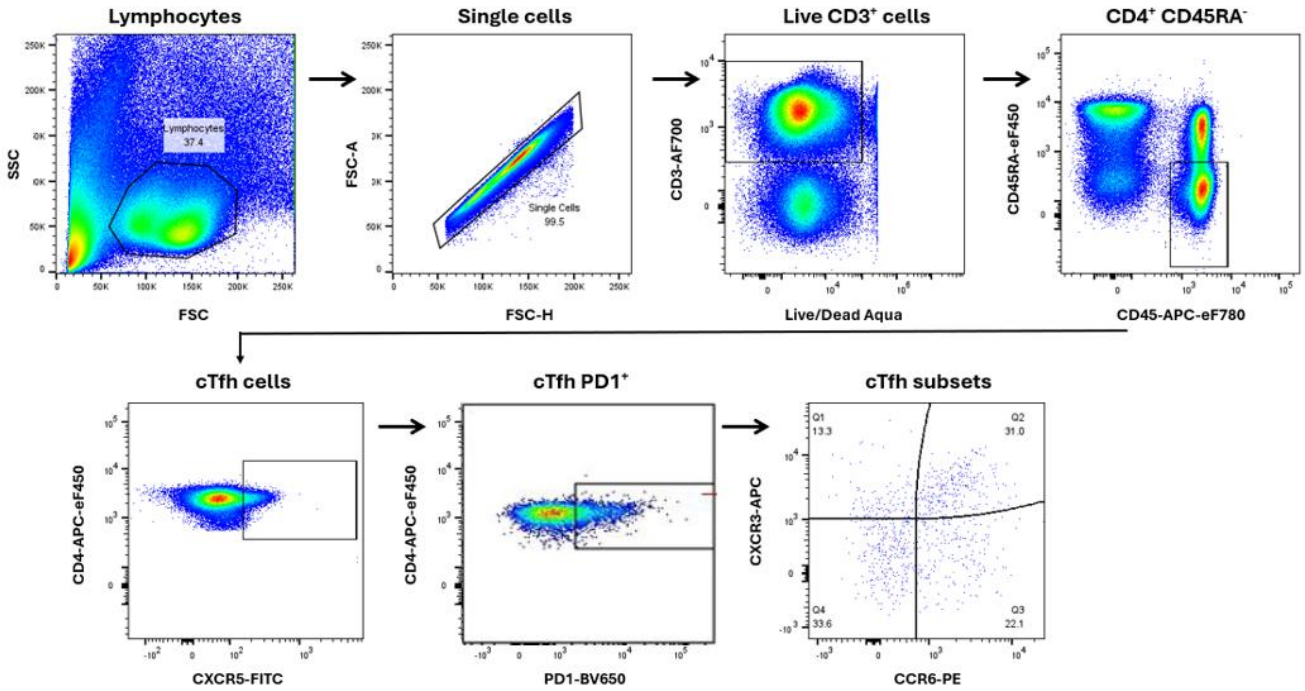

Figure 5. 2: Gating strategy for *ex vivo* cTfh phenotyping

PBMCs were thawed, stained, and analysed by flow cytometry. Lymphocytes were gated based on forward scatter area (FSC-A) and side scatter area (SSC-A), followed by selection of single cells using FSC height (FSC-H) versus FSC-A to exclude cell aggregates. T cells were identified as Live/Dead Aqua-negative (indicating viable cells) and CD3-positive. Within the CD4+ T cell population, memory CD4+ T cells were defined as CD45RA-negative, and memory T follicular helper (Tfh) cells were identified by the co-expression of PD1 and CXCR5. Further phenotyping of memory CD4+ and Tfh cells was performed using CXCR3 and CCR6 expression. Memory Tfh cells were categorized as Tfh1 (CXCR3+ CCR6+), Tfh2 (CXCR3- CCR6-), and Tfh17 (CXCR3- CCR6+).

5.4 Statistical analysis

Data analysis was performed in GraphPad Prism 10.2.1. Non-parametric tests were performed for all analysis. Due to participant dropout and occasional experimental failure, the sample size varied across different analyses, leading to inconsistencies in paired data availability. Thus, the Friedman test that needs complete paired datasets was not used. Instead, I used the Kruskal-Wallis test with additional post-hoc analysis with Dunn's correction for multiple comparisons as it accommodates unpaired datasets and allows for comparisons across groups with varying sample sizes. Sample sizes for each timepoint are included in the text. Associations between parameters were performed with Spearman correlations test. Statistical significance level was defined as < 0.05 and all P values are two tailed. Linear regression with line of best fit and 95% CI was added to correlation plots.

5.5 Results

5.5.1 Vaccination with R21/MM induces memory B cell responses to R21 and C-terminus.

The FluroSpot assay was used to assess whether R21 vaccination elicited MBC responses to various CSP antigens in the vaccine. I evaluated IgG, IgM, and IgA MBC responses to R21 and C-terminus on days 0, 42, 70, 84, and 90 after sporozoite challenge. Owing to contamination during B cell culture, I lacked a complete dataset of day 70 samples. The data are presented as the % of antigen-specific MBC per total IgG, IgA, or IgM.

5.5.1.1 Antigen specific IgG responses

As with the antibody responses, the MBC was generated in a stepwise manner. Following vaccination, there was a significant increase in the IgG MBC to the R21 protein. The frequency of R21 MBC increased from a median of 0.026% (95% CI: 0 – 0.039, n =15) at day 0 to 6.34% (95% CI: 4.12 – 12.36, n = 15) at day 42, following two doses of R21/MM. Peak responses occurred on day 70, with a median frequency of 7.09% (95% CI: 4.1 – 13.1, n = 8). On day 84, a day before challenge, MBC responses had fallen to a median of 1.63% (95% CI: 0.96 – 3.2, n = 16), but 90 days post-challenge responses had risen slightly to 2.27% (95% CI: 0.45 – 3.23, n = 11). This suggests that sporozoite challenge may boost the IgG MBC pool. There was a statistically significant difference between day 0 and subsequent time points. Kruskal-Wallis test with Dunn's multiple comparison test revealed p values of < 0.0001 for days 42, 70 and 84, and p = 0.0006 at C+90.

Significant differences in MBC frequencies were observed at days 42 (6.34%), 70 (7.09%), and 84 (1.65%) compared with the background wells (0.03%, 95% CI: 0.026–0.039; Kruskal-Wallis test with Dunn's multiple comparisons, $p < 0.0001$). Additionally, day 84 vaccinees (1.63%, 95% CI: 0.96–3.2, n = 16) showed significantly higher MBC frequencies than unvaccinated controls (0.016%, 95% CI: 0 – 0.0048, p = 0.037, n = 8), confirming that vaccination induced the observed increase. In contrast, R21 MBC frequencies in the unvaccinated group remained low and stable from baseline (0.026%, 95% CI: 0–0.039, n = 15) through day 84 (0.016%, 95% CI: 0.004–0.034, n = 8) and C+90 (0.013%, 95% CI: -0.02–0.106, n = 4). These results suggest that sporozoite challenge alone is insufficient to elicit a robust MBC response to R21 antigens without vaccination.

In addition to R21-specific MBCs, MBC frequencies were measured against the C-terminus antigen post-vaccination. Median frequencies increased from 0.036% (95% CI: 0–0.066, n = 15) at baseline to 0.21% (95% CI: 0.16–0.81, n = 14) by day 42. By Day 70, the frequency was 0.104% (95% CI: 0.045–0.21, n = 8), rising to 0.14% (95% CI: 0.12–0.32, n = 15) on day 84. Post-challenge, the frequencies further

increased to 0.319% (95% CI: 0.099–0.744, n = 11) at C+90. Kruskal-Wallis test with Dunn's multiple comparisons showed significant increases on days 42 (p = 0.0014), 84 (p = 0.0089), and C+90 (p = 0.0003) compared to baseline. Data for seven participants were lost on day 70 because of contamination, potentially explaining the lack of significance at this time point. It is likely that these participants would have shown similar or higher levels than those on day 42.

5.5.1.2 Antigen specific IgA responses

The data revealed a significant increase in IgA MBC responses to R21 post-vaccination, with 86% (12/14) of the participants generating responses after two doses. Median IgA MBC frequencies rose from 0.076% (95% CI: 0.0–0.152, n = 15) at baseline to 1.064% (95% CI: 0.34–1.70, n = 12) on day 42 (p = 0.0073). Frequencies declined to 0.354% (95% CI: 0.017–1.968, n = 8) on day 70 and 0.2% (95% CI: 0.086–0.303, n = 15) on day 84, stabilizing thereafter. Post-challenge (C+90), responses remained unchanged at 0.203% (95% CI: 0.048–0.715). In the unvaccinated group, the IgA MBC frequencies remained low and stable at 0.12%, 0.06%, and 0.033% on days 0, 84, and C+90, respectively.

IgA MBC responses to the C-terminus showed a slight, non-significant increase on day 42 (median 0.0965%, 95% CI: 0.0–0.025, n = 12) compared to day 0 (median 0.082%, 95% CI: 0.026–0.103, n = 15). The frequencies remained steady at 0.079, 0.037, and 0.054% on days 70, 84, and C+90, respectively.

5.5.1.3 Antigen specific IgM responses

The frequencies of R21 IgM MBCs showed a similar stepwise increase from day 0 (median = 2.03%, 95% CI: 1.62 – 3.41, n = 15), with the increase in mirroring IgG MBC responses peaking at day 42 (median = 5.34%, 95% CI: 4.77 – 8.34, n = 12) and declining to baseline levels by day 84 (median = 1.6%, 95% CI: 1.42 – 2.68, n = 15). I only observed a significant difference in the vaccinees on day 42 when compared to baseline (day 0), PBS, and day 84 (n = 14, Kruskal-Wallis test with Dunn's multiple comparison test p =0.042 (baseline vs. d42); p = 0.018 (PBS vs. d42); and p = 0.003 (d84 vs. d42)). IgM MBCs frequencies to the C-terminus also showed similar kinetics to MBC responses to R21 following vaccination; however, there were no significant differences at any time point.

These findings suggest that the R21/MM vaccine induces a robust and sustained MBC responses which are further boosted following sporozoite challenge, particularly for IgG MBC against R21 and the C-terminus region.

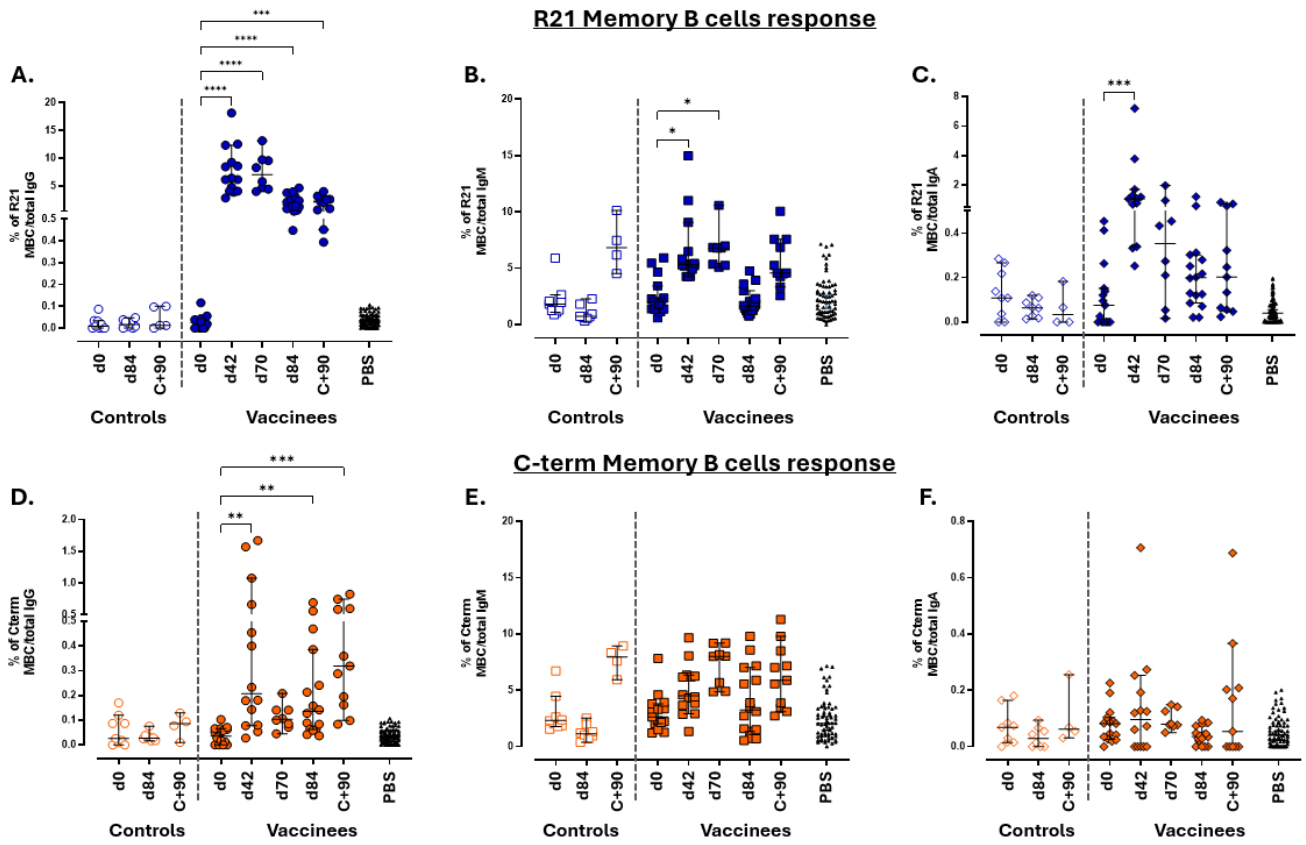

Figure 5. 3: Memory B cell responses following vaccination.

Magnitude of MBC responses to R21 and C-terminus measured by B cell FluroSpot in pre-exposed adults vaccinated with R21/MM. (A) proportion of R21 MBC per total IgG producing cells; (B) proportion of R21 MBC per total IgM producing cells; (C) proportion of R21 MBC per total IgA producing cells; (D) proportion of C-terminus MBC per total IgG producing cells; (E) proportion of C-terminus MBC per total IgM producing cells and (F) proportion of C-terminus MBC per total IgA producing cells.

5.5.2 Antigen specific memory B cell frequencies do not correlate with total antibody titres.

I performed a Spearman's correlation analysis on day 84 to assess the relationship between MBC frequencies and IgG antibody levels. No significant correlation was observed between IgG R21- or C-terminus-specific MBC frequencies and their corresponding IgG titres, despite both exhibiting similar kinetics. IgG responses to R21 ($r = -0.303$, $p = 0.253$, $n = 16$) and the C-terminus ($r = -0.024$, $p = 0.935$, $n = 16$) showed a moderately negative association with IgG titres.

MBC responses to IgA and IgM demonstrated weak positive associations between IgA antibody titres and IgA MBCs to both R21 ($r = 0.203$, $p = 0.448$, $n = 16$) and C-terminus ($r = 0.277$, $p = 0.314$, $n = 15$) however this observation is not significant. Similarly, anti-R21 IgM antibodies and IgM MBCs to R21 at day 84 showed a moderate positive correlation ($r = 0.44$, $p = 0.08$, $n = 16$) but again not significant. I did not have total IgM antibodies against C-terminus as I was unable to optimize the C-terminus IgM ELISA.

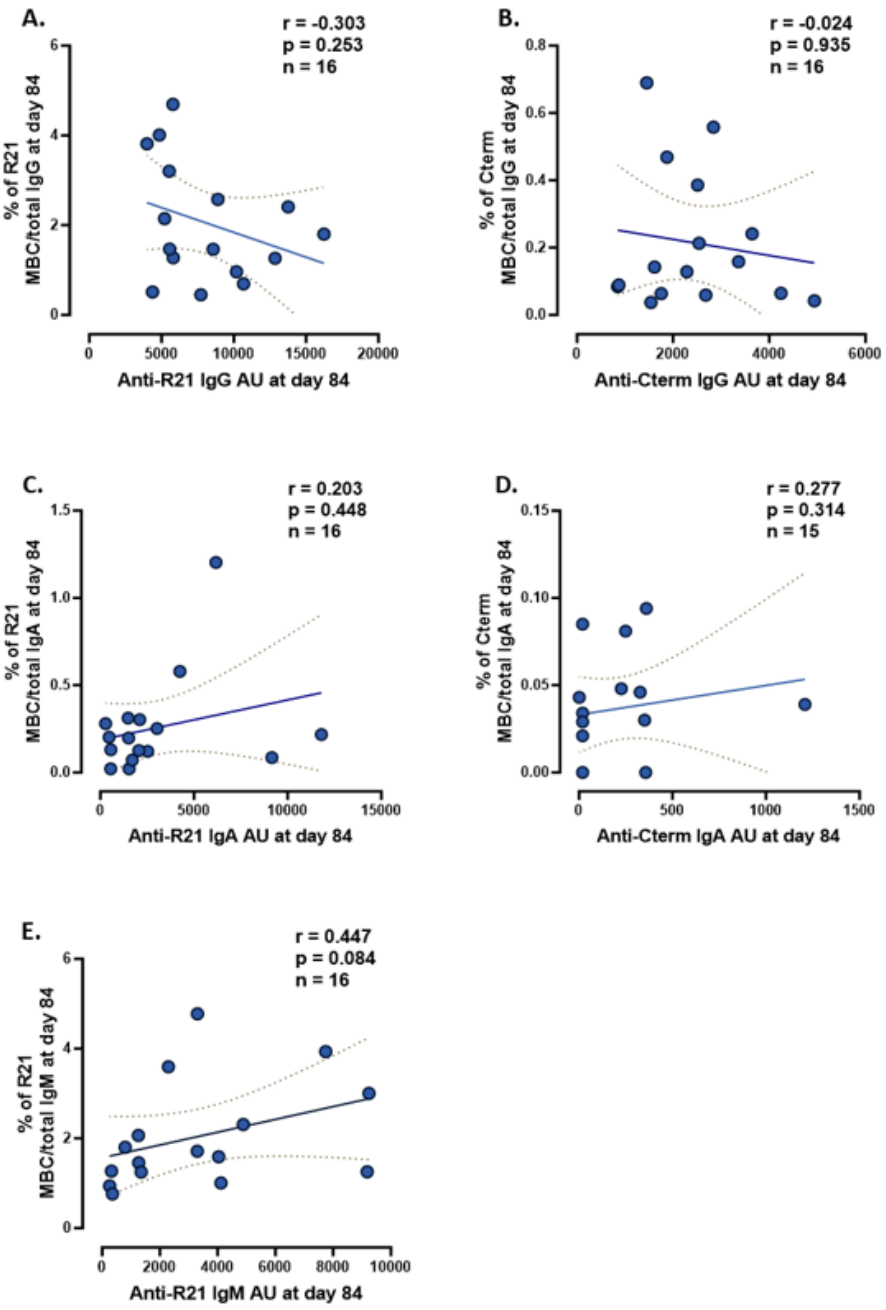

Figure 5. 4: Relationship between antibodies and antigen specific memory B cell at day 84.

A Spearman's correlation analysis was conducted to examine the relationship between total anti-IgG, IgA, and IgM antibodies and the frequencies of antigen-specific MBCs at day 84 (A) R21 IgG responses. (B) C-terminus IgG responses. (C) R21 IgA responses. (D) C-terminus IgA responses. (E) R21 IgM responses. Spearman's correlation test rank correlation with line of best fit calculated with linear regression with 95% CI.

I also carried out a correlation analysis to look at the relationship between MBCs at day 84 and total IgG antibody responses at C+90. A later timepoint may yield a stronger correlation between MBC responses and antibody titres, as the immune response evolves over time and early timepoints, may not fully capture the maturation of the MBC response. Here, I observed a weak negative correlation between MBC and IgG antibodies to R21 (r = -0.1536, p = 0.5844, n = 15) and notably there was a moderate positive association with MBC responses and antibodies towards C-terminus (r = 0.454, p = 0.092). Although, both observations were not significant. I did observe a positive correlation with total IgM antibodies and MBC frequencies, which was significant (r = 0.557, p = 0.03, n =15). However, this analysis was conducted on a relatively small group, which can affect the robustness and generalizability of the findings.

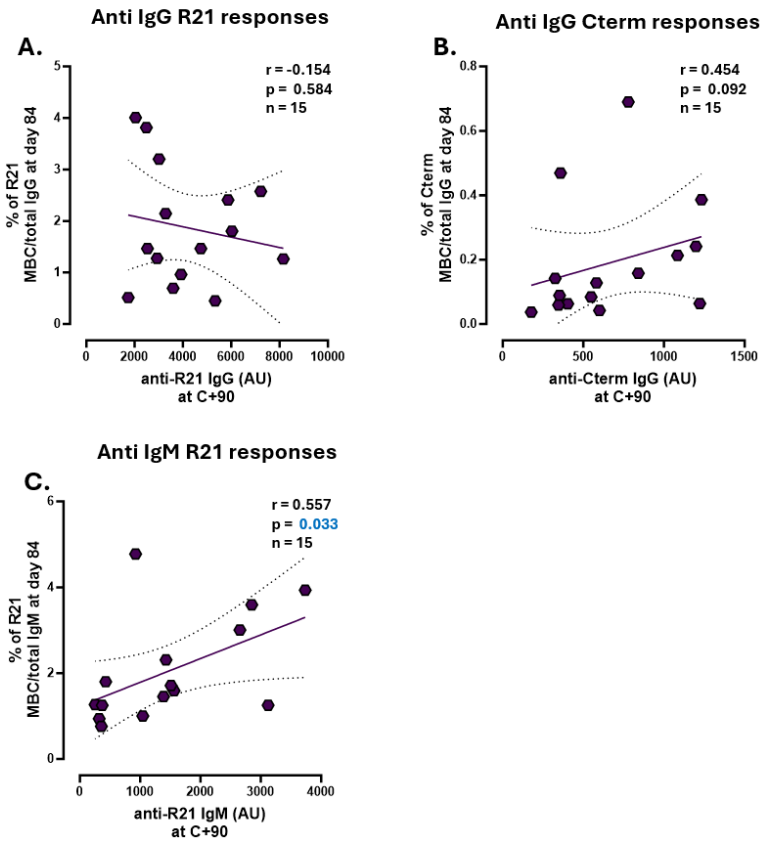

Figure 5. 5: Relationship between antibodies at C+90 and antigen specific memory B cells at day 84.

Spearman's correlation test was performed to interrogate the relationship between total anti-IgG/IgA and IgM antibodies at 90 days post challenge and the frequencies of antigen specific MBC at day 84. (A) R21 IgG responses. (B) C-terminus IgG responses. (C) R21 IgM responses. Spearman's correlation test rank correlation with line of best fit calculated with linear regression with 95% CI.

5.5.3 Phenotyping of circulating Tfh cells

I analysed cTfh cell frequencies and phenotypes using multiparameter flow cytometry to assess their role following R21/MM vaccination. PBMCs were isolated on day 0 (pre-vaccination) and 14 days after each dose (days 14, 42, and 70) to determine if additional doses increased the total cTfh pool. No significant change in total CD4 memory T cells (CD4+/CD45RA-) was observed across timepoints, with frequencies of 33.35%, 32.6%, 35.5%, and 33.8% at Days 0, 14, 42, and 70, respectively ($p > 0.9$, Kruskal-Wallis with Dunn's multiple comparison). This suggests vaccination does not affect the total CD4+ T cell pool.

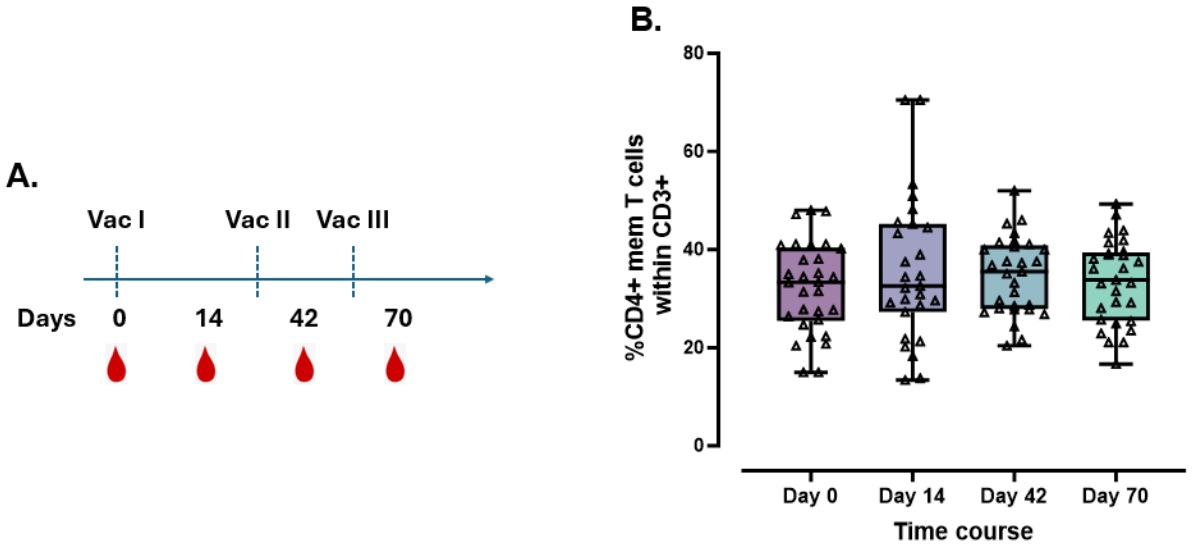

Figure 5. 6: Analysis of total CD4 memory T cells following R21 vaccination.

(A) R21/MM vaccination and PBMC sampling scheme (days represent blood draws). (B) PBMCs were stained *ex vivo* for phenotypic markers to identify memory CD4 T cells and analysed by flow cytometry. Comparisons were done by Kruskal-Wallis non-parametric with Dunn's multiple comparison test. Each point represents a vaccinated individual. Data for figure B shown as box plots whereby top, centre, and bottom horizontal lines represent the 75th percentile, median, and 25th percentile, respectively; upper and lower whiskers represent the highest and lowest values.

5.5.4 Circulating Tfh cells increase following a single dose of R21/MM

Next, I looked at the frequencies of cTfh, I defined cTfh as either $CXCR5^+$ or $CXCR5^+/PD1^+$ within $CD4^+$/CD45RA- (memory CD4 T cells). For cTfh cells defined by the expression of CXCR5+ the median at day 0 was 4.1% (95% CI: 4.0 – 7.77, n = 30) and 9.65% (n = 29, 95% CI: 18.3 - 15.1) at day 14, p = 0.0026. While the median for cTfh defined by expression of $CXCR5^+/PD1^+$ was 3.4% (n = 30, 95% CI: 2.2.8 – 8.3) at day 0 and 9.8% (n = 29, 95% CI: 7.44 – 12.8) at day 14, p = 0.0003. There was no significant difference between the two definitions of cTfh cells. These findings indicate a significant increase in cTfh frequencies following R21/MM vaccination, suggesting that the vaccine induces a Tfh-mediated immune response, potentially enhancing humoral immunity. Furthermore, the lack of further increase in cTfh cells after subsequent doses suggests that a single dose is sufficient to elicit a robust Tfh response, with additional doses providing no further enhancement of this specific immune response.

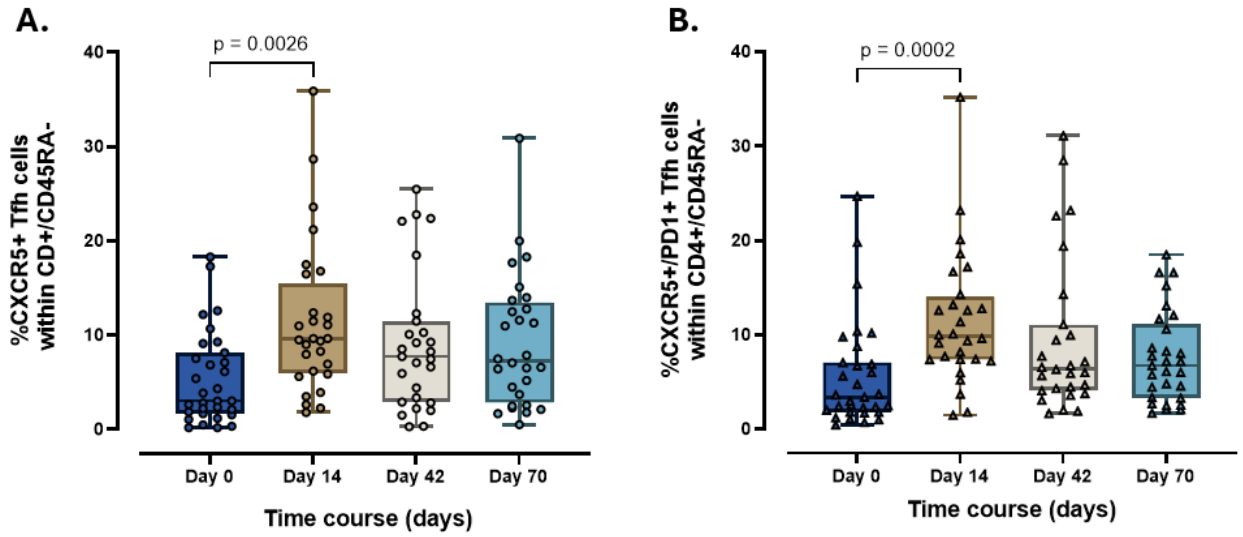

Figure 5. 7: Frequency of cTfh cells.

cTfh cells were defined by their expression of (A) CXCR5+ and, (B) CXCR5+/PD1+. Cryopreserved PBMCs from day 0, day 14, day 42 and day 70 were stained *ex vivo* and analysed by flow cytometry. Shown are box plots whereby top, centre, and bottom horizontal lines represent the 75th percentile, median, and 25th percentile, respectively; upper and lower whiskers represent the highest and lowest values. Kruskal-Wallis non-parametric with Dunn's multiple comparison test were used to compare the median between timepoints.

5.5.5 Tfh2 and Tfh17 are the predominant subsets before and after R21/MM Vaccination

Further analysis of cTfh cells focused on the CXCR5+/PD1+ subset. Additionally, Tfh subsets were characterized based on their CXCR3/CCR6 expression into (i) Th1 (CXCR3+CCR6-), (ii) Th2 (CXCR3-CCR6-) and (iii) Th17 (CXCR3-CCR6+). I observed no significant differences within the frequencies of each subset. Within the Th1 subset (median frequencies were 1.6, 4.4, 2.6, 3.0 (%)), in the Th2 cells (median frequencies 24.3, 27.6, 27.4, 25.7(%)) and Th17 (median frequencies 31.8, 26.1, 29.4 and 26.5 (%)) at day 0 (n = 30), 14 (n = 28), 42 (n = 30) and 70 (n = 30) respectively. Within the cTfh cells, Th2 and Th17 were the predominant subsets, and though not significant there was an increase of Th2 and Tfh1 cells from day 0 to day 14. There was also a subtle decrease of Th17 cells from vaccination to day 14 but this rose by day 42 and started to decrease again by day 70. The frequencies of Th1 remained unchanged throughout the duration of the follow-up and were at much lower numbers than those of Tfh2 and Tfh17.

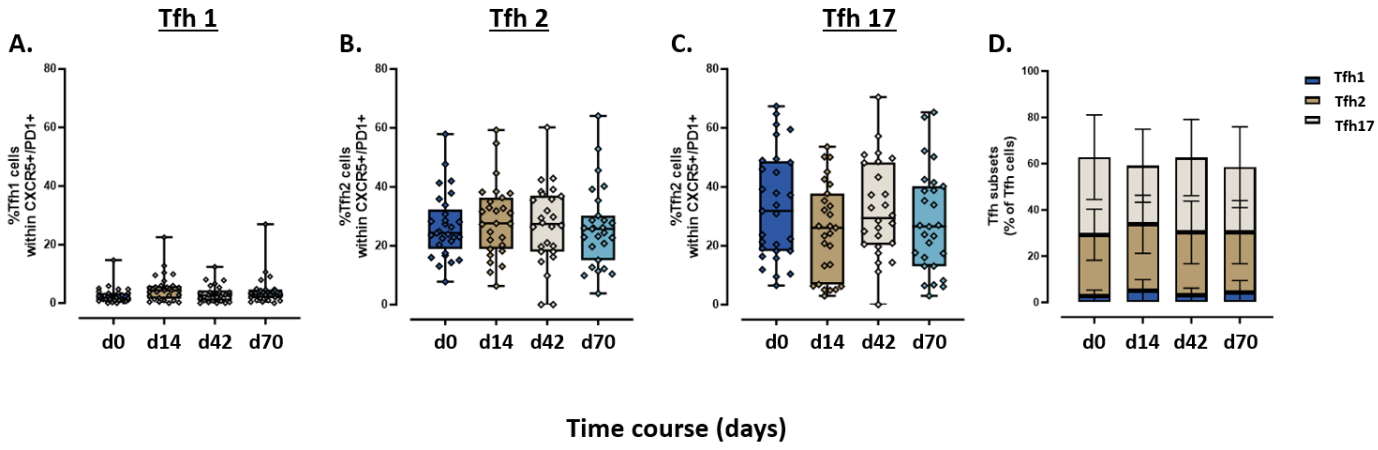

Figure 5. 8: Frequency of circulating T follicular subsets.

Defined by their expression of CXCR3 and CCR6 cTfh subsets into (A) Th1 (CXCR3+CCR6-), (B) Th2 (CXCR3-CCR6-), (C) Th17 (CXCR3-CCR6+) and (D) showing the median responses of Th1, Th2 and Th17.

5.5.6 Frequencies of cTfh correlate with R21 memory B cells but not with total IgG antibody titres.

I ran a Spearman's association test to assess the relationship between the frequencies of cTfh cells at day 14 against (i) the percentage of IgG antigen specific memory B cells to R21 and to C-terminus at day 84 and (ii) total antibody titres to R21 and C-terminus IgG at day 84. I observed a moderate positive association between total circulating Tfh cells and R21 IgG MBCs (r = 0.577, p = 0.043, n = 13). Suggesting that cTfh cells play a role in the activation or maintenance of memory B cells I also saw a similar positive correlation between total cTfh and C-terminus IgG MBC (r = 0.4011, p = 0.1759, n =13) however this was not a significant finding.

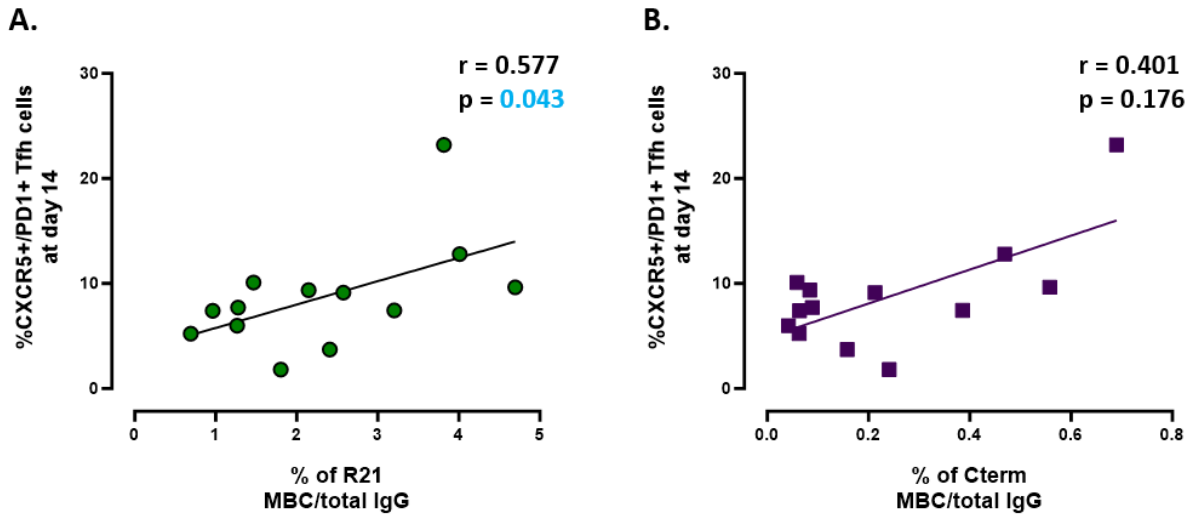

Figure 5. 9: Total cTfh at day 14 correlates with R21 IgG MBC at day 84.

Spearman's correlation was run to determine the relationship between the frequency of cTfh cells and (A) R21 specific MBC (n = 13) (B) C-terminus specific MBCs (n = 13). Each point represents a vaccinee and significant was set at p <0.05.

When I performed a correlation analysis to determine the relationship between total cTfh at day 14 and total IgG antibody titres at day 84, I observed a weak positive association between NANP and total Tfh, although this did not reach statistical significance. There was no correlation with total R21 antibodies and a weak negative correlation with C-terminus.

Furthermore, I ran a correlation analysis between the different Tfh subsets (Tfh2 (CXCR3-CCR6-); Tfh17 (CXCR3-CCR6+) and Tfh1 (CXCR3+CCR6-)) at day 14 against IgG MBC and total IgG antibodies at day 84 to determine if any specific subset was associated with them. However, I observed little or no correlation with any of the Tfh subsets (Tfh1, Tfh2, and Tfh17).

Table 5. 4: Association of cTfh and subsets at day 14 with total IgG anti-CSP antibodies

	Anti-R21 IgG at day 84			**Anti-NANP IgG at day 84**			**Anti-C-terminus IgG at day 84**		
	r	**p**	**n**	**r**	**p**	**n**	**r**	**p**	**n**
Total cTfh (CXCR5+/PD1+)	0.054	0.797	25	0.245	0.237	25	-0.155	0.462	25
Tfh2	0.103	0.624	25	-0.086	0.682	25	0.119	0.570	25
Tfh17	0.105	0.611	25	0.016	0.937	25	0.019	0.924	25
Tfh1	-0.193	0.355	25	0.05	0.81	25	-0.229	0.270	25

5.6 Discussion

Memory B cells (MBCs) and T follicular helper (Tfh) cells are crucial for durable immune responses and long-term protection against pathogens. In this chapter, I assessed Tfh and MBC interactions and the persistence of vaccine-induced antibodies and characterized the Tfh subsets after R21/MM vaccination. This study is the first to examine Tfh cell responses and antigen-specific MBCs induced by R21 vaccination in malaria-exposed adults.

Results from Chapter 4 revealed that R21/MM vaccination elicited significant anti-IgG, anti-IgA, and anti-IgM CSP responses. I used the FluroSpot assay, which simultaneously detects various immunoglobulin isotypes, to measure antigen-specific MBCs. First, I examined CSP-specific MBC at a steady state, that is, acquired from natural exposure. The frequency of CSP-specific memory B cells was low in all three isotypes. These findings are consistent with those of previous studies (Agnandji et al., 2011; Jahnmatz et al., 2022), suggesting that malaria exposure does not necessarily lead to the development of persistent CSP circulating MBCs. Similar to antibodies, MBCs also increases with age and exposure (Weiss et al., 2010). Therefore, it is unsurprising to observe low MBCs in this population with little or no malaria exposure. Additionally, sporozoites spend only a short time in the skin and circulation (Ménard et al., 2013), limiting the opportunity of the immune system to mount a robust response, leading to low pre-existing immunity to CSP.

After vaccination, I observed a significant increase in IgG MBC responses to both R21 and C-terminus two weeks post the second dose, but no further boosting effect was seen after the third dose. This aligns with Agnandji findings, where they reported CS-specific MBCs responses after two vaccine administrations of RTS, S/AS01$_E$, in young children (Agnandji et al., 2011). The lack of increase in responses following the third dose was explored by Macnamara et al., who suggested that the feedback mechanism created by antibodies influences the immune response to vaccination by limiting the expansion of MBC responses to the immunodominant NANP repeat region, allowing for the expansion of subdominant B cell responses to other regions of the vaccine, potentially through epitope masking (McNamara et al., 2020). This could potentially explain why I did not observe an additional boost in antibodies and MBC after the third vaccination. Though these studies were conducted in naïve individuals vaccinated with PfSPZ and in mouse models (McNamara et al., 2020). Strategies such as delaying booster doses until antibody levels decline could help reduce the suppression effects and enhance B-cell activation, potentially leading to improved vaccine outcomes. Some studies have shown that delaying the interval and lowering the dosage of the third RTS,S/AS01 vaccine dose can enhance immunogenicity (Dennison

et al., 2021; Pallikkuth et al., 2020). Interestingly, a significant increase in IgA MBCs was noted following the first two doses of R21/MM, although these responses were short-lived, and by day 84, there was no significant difference in IgA MBC compared to baseline. The lack of detectable IgA memory B cells in circulation may be due to their migration back to the bone marrow or secondary lymphoid organs. IgA antibodies are primarily associated with mucosal immunity; however, they are also present in high quantities in blood (Prigent et al., 2016). IgA antibodies could prevent the entry of *Plasmodium* parasites through mucosal surfaces (Suau et al., 2021). The presence of IgA MBCs suggests long-term immune memory, which is capable of responding quickly to subsequent infections. Recent studies have highlighted the potential role of IgA in malaria protection following RTS,S vaccination or sporozoite challenge (Suau et al., 2021; Tan et al., 2021). However, it remains unclear whether the increase in MBCs observed in this study contributes to protection, and further investigations using functional assays are required to clarify this. Furthermore, we cannot say for certain that the response observed is only specific to the CSP portion of the vaccine or whether it is to the hepatitis core B antigen driving the responses. Unfortunately, I was unable to examine NANP-specific MBC responses due to logistical and time constraints to determine whether these memory IgA responses are also NANP-specific. Nonetheless, I did observe NANP-specific IgA antibodies and, to a lesser extent, C-terminus-specific IgA. The relationship between IgA levels and malaria infection suggests that IgA plays a role in the immune response to malaria, potentially providing protection (Berry et al., 2021; Suau et al., 2021; Tan et al., 2021)

I also observed R21- and C-terminus-specific MBC at C+90, which is a notable finding, as the importance of MBC lies in its ability to provide long-lasting immunity against previously encountered pathogens. They respond quickly to reinfection by producing antibodies which are crucial for preventing infection and reducing disease severity which is why the generation and maintenance of MBC are critical for the success of vaccines. It is reassuring that we observed MBCs after vaccination with R21/MM up to day 84 which was significant compared to baseline.

Previous studies have found correlations between the frequency of MBC and antibody titres for diseases such as smallpox (Crotty et al., 2003) and blood-stage malarial antigens from natural exposure (Dorfman et al., 2005; Jahnmatz et al., 2022). This suggests that MBCs divide and differentiate into plasma cells even in the absence of antigen/exposure. Agnandji and colleagues did observe a correlation between the frequency of CS-specific circulating MBC and CS-specific antibody titres in children following vaccination with RTS, S (Agnandji et al., 2011). However, in this study, I observed either no correlation or a modestly negative correlation between MBCs for R21 and C-terminus and overall IgG antibody levels

at the various time intervals analysed. It is important to note that memory B cells take 4 -7 days to produce detectable antibodies following re-exposure to their specific antigen, so they may not necessarily align with antibody levels during the same time; measuring plasma cells may be a more suitable parameter when examining the relationship between these two factors.

IgM memory B cell responses are challenging to interpret owing to their polyreactive characteristics. MBCs producing IgM displayed non-specific binding to the assay plates, as indicated by the high background binding observed in the negative well with no antigen (PBS). This is one of the limitations of the FluroSpot assay, which could not be addressed within time and resource constraints. One potential alternative would involve purifying B cells before culturing, aiming for a more specific and cleaner result.

Tfh cells play a critical role in the adaptive immune response, particularly in the context of humoral immunity. Their main function is to provide essential signals to B cells to facilitate their activation, differentiation, and production of high-affinity antibodies (Crotty, 2019; Deenick & Cindy, 2011). Monitoring cTfh cells can serve as a biomarker to assess GC-Tfh responses in vaccination studies (Nielsen et al., 2021), offering insights into the effectiveness of vaccines and the immune response in humans. Here, I initially examined the role of circulating Tfh cells (CD4+ CD45RA-) following vaccination with R21 based on the expression of CXCR5+ and/or CXCR5+/PD1. In this study, a significant increase in the total cTfh frequency was observed 14 days after a single dose. Suggesting, R21/MM vaccination stimulates an effective immune response, which may lead to the development of long-term adaptive immunity. I did not observe an increase in total cTfh following additional vaccine doses, suggesting that a single R21 dose is sufficient to activate and differentiate Tfh cells.

Additionally, they demonstrated that $CXCR3^-$ (Tfh2/Tfh17) subsets are superior in providing help to B cells compared to $CXCR3^+$ (Tfh1) (Obeng-Adjei et al., 2015). Bowyer et al. reported that co-administration of RTS and S/AS01B with viral vectored vaccines also led to an increase in the frequencies of CXCR3+ cTfh1 which was associated with reduced immunogenicity compared with RTS and S/AS01B administered alone in U.K adults (Bowyer, Grobbelaar, et al., 2018). Recently, Chan and colleagues observed that during *P. falciparum* infection using the induced blood-stage malaria (IBSM) system in malaria-naïve adults, Tfh cells are activated with distinct dynamics. Specifically, Th2-Tfh cells activate early during peak infection (around day 8), whereas Th1-Tfh cells activate later (approximately day 14/15) after peak infection and treatment. Highlighting the contrasting roles of Th2 and Th1 Tfh cells in malaria infection. They also observed that the frequency of parasite-specific Tfh2 cells was correlated

with the functional breadth and magnitude of parasite antibodies (Chan et al., 2020). A study in Uganda noted an age-dependent change in Tfh subsets, with a decrease in Tfh2 cells and an increase in Tfh 1 cells in early childhood (<7 years) independent of malaria infection. Through clustering analysis, they also observed that children with the highest antibody levels showed increased Tfh cell activation and Tfh2 proliferation. (Chan et al., 2022). These studies highlight the role of Tfh2 in antibody development. In this study, distinct subsets of activated Tfh cells were identified based on CXCR3 and CCR6 expression. Analysis revealed no significant upregulation within the Th1, Th2, or Th17 subsets post-vaccination, and Th2 and Th17 were the predominant subsets in the circulation. Given the positive association between $CXCR3^{-}$ subsets in stimulating naïve B cells *in vitro* and promoting the secretion of high-quality antibodies (Chevalier et al., 2011; Gao et al., 2023; Morita et al., 2011), it is encouraging to observe that Tfh2 and Tfh17 are the predominant subtypes, suggesting that the presence of both subsets can lead to more robust humoral immunity. The increase in Tfh2 levels after vaccination suggests that R21 stimulates these more effective Tfh subsets which may lead to sustained antibody responses. Some of the studies highlighted, have shown a Tfh1 response following CSP vaccination or during malaria infection though these studies were focused on naïve adults or children. The authors speculated that children and naïve adults produced high levels of IFN-γ and TNF-α as a result of infection, which promoted the activation of Tfh1 subsets. Although a slight increase in the Tfh1 subset was observed following vaccination, this increase was not significant. The IBSM study by Chan and colleagues demonstrated that there is also a difference in the distribution of Tfh subsets when looking at the different memory compartments, with central memory (CM) T cells having higher frequencies of Tfh2/Tfh17 to Tfh1, while effector memory (EM) cells had higher frequencies of Tfh1 cells compared to Tfh2/Tfh17. Unfortunately, in this study, I was unable to assess memory T cell compartments which may explain the higher Tfh2/Tfh17 frequencies that I observed in this study. CM cells offer a long-term memory resource that can proliferate and generate effector cells, whereas EM cells provide immediate, localised immunity to rapidly control infections at sites of re-exposure by producing cytokines such as IFN-γ and TNF-α.

It would be useful to analyse vaccine-specific responses using, for example, the activation-induced marker (AIM) assay to determine whether these increases are due to the CSP components of the vaccine and not the hepatitis core B antigen or Matrix M. AIM assay is designed to identify and quantify antigen-specific T cells based on the upregulation of activation markers following T-cell receptor (TCR) stimulation. This allows for detailed analysis of T cell frequency, phenotype, and function in response to specific antigens (Bowyer, Rampling, et al., 2018; Poloni et al., 2023).

Associations have been noted between vaccine IgG antibodies, MBC, and Tfh cells (Chan et al., 2020; Osier, 2018; Pallikkuth et al., 2020). Nielsen and colleagues observed a positive correlation between anti-RH5 IgG antibodies (at peak timepoint (day 84) and MBC (at later timepoint day 140) and RH5 specific Tfh cells at day 14 following vaccination with RH5, a potential blood stage vaccine candidate. They suggested that early Tfh cell responses might be predictive of long-lasting humoral responses (Nielsen et al., 2021). Other studies have shown that following influenza vaccination, circulating ICOS+ CXCR3+ CXCR5+ Tfh cells correlate with antibody responses (Bentebibel et al., 2013), while Locci and colleagues PD-1+CXCR3–CXCR5+ Tfh cells positively correlate with broadly neutralizing antibodies against HIV (Locci et al., 2013). In this study, correlation analysis revealed that the frequency of total Tfh cells at day 14 did not correlate with the total IgG antibody responses to any of the vaccine components (R21, C-terminus, and NANP) at day 84, indicating that other factors influence antibody responses beyond the Tfh cell quantity. However, there was a positive association between R21 MBC and total Tfh cells at day 84, suggesting that Tfh cells may play a role in the generation or maintenance of memory B cells specific to the vaccine, supporting long-term humoral immunity. Although the sample size for this analysis was small (n = 13) limiting the conclusion that can be drawn. There was no association between Tfh subsets (Tfh2, Tfh17, and Tfh1) and the R21 IgG MBC on day 84. Using antigen-specific Tfh cells may provide a more accurate picture of vaccine responses and, in turn, correlate better with MBCs and total IgG titres. It is also possible that the timing of sampling used in this study was not optimal, as Tfh cells are often present in circulation as early as day 3 (Pallikkuth et al., 2020), and we may have missed the peak window of Tfh cells in this study. Chan et al., observed that Tfh2 cells were activated earlier than Tfh1 cells following a blood-stage challenge in naïve individuals. (Chan et al., 2020). This finding also highlights the importance of sampling time when studying specific immune cell populations.

5.6.1 Limitations

As indicated in Chapter 4, the small sample size is a significant limitation of this study. Moreover, the limited number of assays that were conducted poses challenges in drawing conclusions about certain associations observed. The sample size was further reduced by contamination during the 5-day culture of PBMCs to produce memory B cells. Another alternative approach for investigating MBC would have been to use antigen specific tetramers and analysed cells using flow cytometry, however the starting frequency of MBCs may have made such an approach technically challenging. While cTfh cells provide valuable insights into the immune response, using them as a proxy for GC Tfh cells has limitations due to lack of GC context-specific interactions, cTfh may not accurately represent the specific microenvironment and interactions that occur within the germinal centres, and individual variability. Furthermore, time constraints and logistical difficulties in obtaining reagents prevented the completion of certain planned assays such as the antigen specific approach of identifying cTfh cells as well as the B cell phenotyping using flow cytometry mentioned.

5.6.2 Conclusions

Evidence from this chapter shows that R21/MM vaccination can induce strong long lasting memory B cell response, particularly to IgG and to some extent IgA, as well as robust cTfh cell responses. Further research into the interaction between MBCs and Tfh cells, as well as the dynamics and functionalities of different subsets of Tfh cells, in the context of malaria vaccination is crucial for a comprehensive understanding of the immune response to the malaria parasite and to vaccination.

6 CHAPTER SIX – Sporozoite challenge outcome

6.1 Introduction

The prevalence of *P. falciparum* has declined in the Kenyan coastal region since 1998 (Snow et al., 2015). The decline has been patchy, and transmission in one sublocation (Ngerenya, Kilifi) has continued to decline and is now considered a low-to-no transmission area for malaria in the past 10 years (Njuguna et al., 2019). This presents a unique opportunity to examine vaccine efficacy using the CHMI model in individuals residing in malarial regions. By recruiting participants from this region, we could gather a group of individuals with limited or no exposure to malaria. Studies of vaccine efficacy are often conducted based on natural exposure in the field, but this exposure is heterogeneous, which complicates the interpretation of results. When testing vaccine efficacy through the CHMI model, we can be more confident that the variability in natural exposure has a minimal impact on the results, since the exposure to the challenge agent is controlled and uniform across the group.

CHMI models are valuable tools for predicting correlates of vaccine-induced protection. These models involve vaccinating healthy human volunteers, exposing them to the parasite, and closely monitoring them for signs and symptoms of malaria infection. CHMI models using sporozoites and blood stage challenges are cost-effective and time-saving methods for assessing vaccine efficacy and exploring immunological host-parasite responses (Stanisic et al., 2018; Yap et al., 2020). To date, in the African continent, only six countries have carried out human malaria infection studies using cryopreserved *P. falciparum* sporozoites from Sanaria (reviewed in Chapter 2). This is the first clinical trial to investigate and assess vaccine efficacy to R21/MM in adults living in a malaria region using the CHMI model. PfSPZ challenges have been administered by intradermal (ID), direct venous injection (DVI), or intramuscular (IM) routes, all of which have been shown to be safe, well-tolerated, and infectious; DVI is the most efficient route (Bastiaens et al., 2016; Gómez-Pérez et al., 2015; Lyke et al., 2015; Roestenberg et al., 2013; Shekalaghe et al., 2014; Steinhardt et al., 2019). In this study, volunteers received three doses of R21/MM and were challenged with either ID or DVI to determine whether the route of challenge would impact protection outcomes. Parasite monitoring was carried out using qPCR twice daily from day 7 post challenge. The data generated from CHMI models can be used to understand immune responses against malaria and help predict the correlates of protection by identifying immune responses associated with protection against malaria.

To date, there is no clear understanding of the specific targets and effector mechanisms responsible for the immunological protection against malaria. The identification of correlates of protection and the

development of *in vitro* assays that provide an accurate measure of protection are critical for evaluating vaccine efficacy and informing future vaccine design. This study presents a unique opportunity to deepen our understanding of R21s vaccine-induced protection against malaria and explore new avenues for addressing the challenges associated with developing effective vaccines for populations in malaria-endemic regions.

6.2 Aim

My main objective here was to identify immune responses associated with protection in semi-immune adults using the human challenge model. This chapter will assess the various immunological assays conducted in previous chapters to identify potential correlates of vaccine-induced protection following vaccination and challenge with PfSPZ. The study design will also allow investigation into whether the route of challenge, , plays a role in protection.

6.3 Materials and Methods

6.3.1 VAC074 trial design

The design of the trial and the bleeding schedules are described in detail in Chapter 4. Regrettably, due to safety concerns identified by the FDA, there was a hold on the PfSPZ challenge (Sanaria), therefore this chapter only includes data for the first cohort. As a result, the study's sample sizes were not sufficiently powered to evaluate the immunological correlations with protection.

6.3.2 qPCR and immunological assays

All molecular and immunological assays used in this chapter for analysis have been described in the above chapters.

6.3.3 Challenge preparation and administration.

Immediately prior to challenge, PfSPZ cryovials were individually thawed by partially submerging the vials in a 37°C ± 1°C water bath for 30 seconds. Trained and designated study personnel then prepared and dispensed the PfSPZ Challenge to the clinical staff. Sanaria Inc. provided aliquots of the diluent phosphate-buffered saline (PBS) and 25% human serum albumin (HSA) to the clinical sites. The PfSPZ Challenge was administered using a needle and syringe by either direct venous injection (3200 parasites) or intradermal (22,500 parasites). Clinical staff administered the PfSPZ Challenge.

6.3.4 Pre-challenge clinic and blood sampling for malaria qPCR

Participants attended a pre-challenge clinic visit to assess any medical issues including general health, complete blood counts, biochemistry, Covid-19 screening, pregnancy screening for women, and malaria screening before challenge. Following the all-clear, participants were challenged, and 4 mL of blood was collected in EDTA-treated vacutainers twice a day (morning and evening) to monitor parasitaemia from days 7 to 14 post-challenge, and once a day from days 15 to 21. Parasitaemia was monitored using a validated qPCR described in chapter 3. Anti-malarials were given at a threshold of 500 parasites per µl or if a participant became symptomatic.

6.4 Statistical analysis

Data analysis was performed in GraphPad Prism version 10.2.1 (GraphPad Software, San Diego, CA, USA). The Mann–Whitney U test was used to compare data from two groups of subjects (unpaired). Non-parametric tests were performed for all analysis. Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison was used to compare data from more than 2 groups. Statistical significance was set as <0.05.

6.5 Results

6.5.1 Anti-CSP antibodies wane over time, with no boost from sporozoite challenge.

I assessed the durability of vaccine-induced antibodies and the impact of sporozoite challenge on both vaccinated and non-vaccinated participants, focusing on cohort 1. Two of the 19 vaccinated participants withdrew before the challenge. Antibody responses, including total IgM and IgA, were measured using a standardized ELISA method.

Total IgG responses to R21 and C-terminus gradually declined from day 84 post sporozoite challenge. The R21 GMT was 8086 at day 84 (95% CI: 6081–10753, n = 17) and dropped to 7457 at 7 days post-challenge (95% CI: 5501–10108, n = 17). By the day of diagnosis, GMT further decreased to 7233 (95% CI: 5413–9665, n = 16) and to 6801 by 35 days post-challenge (95% CI: 4879–9480, n = 16). By 90 days post-challenge, GMT fell to 4930 (95% CI: 3174–7657, n = 16). The decrease between day 84- and 90-days post-challenge was significant ($p = 0.019$, Kruskal-Wallis test with Dunn's multiple comparisons).

C-terminus responses also declined post-challenge. At day 84, the GMT was 2360 (95% CI: 1733 – 3215, n = 17), dropping to 1631 at 7 days post-challenge (95% CI: 1104 – 1946, n = 17). By the day of diagnosis, the GMT fell to 763.2 (95% CI: 385.8 – 1510, n = 16), but increased slightly to 1185 (95% CI: 817.6 – 1718, n = 17), though not significantly different from subsequent time points. By C+90, GMT had decreased further to 606.6 (95% CI: 438.4 – 839.4, n = 16). Responses at 35- and 90-days post challenge were significantly lower than at day 84 ($p = 0.004$ and $p < 0.0001$ respectively, Kruskal-Wallis test with Dunn's multiple comparison). This suggests that sporozoite challenge does not appear to boost the R21 or C-terminus responses. However, the responses to both antigens were still above the positivity threshold at 90 days post-challenge.

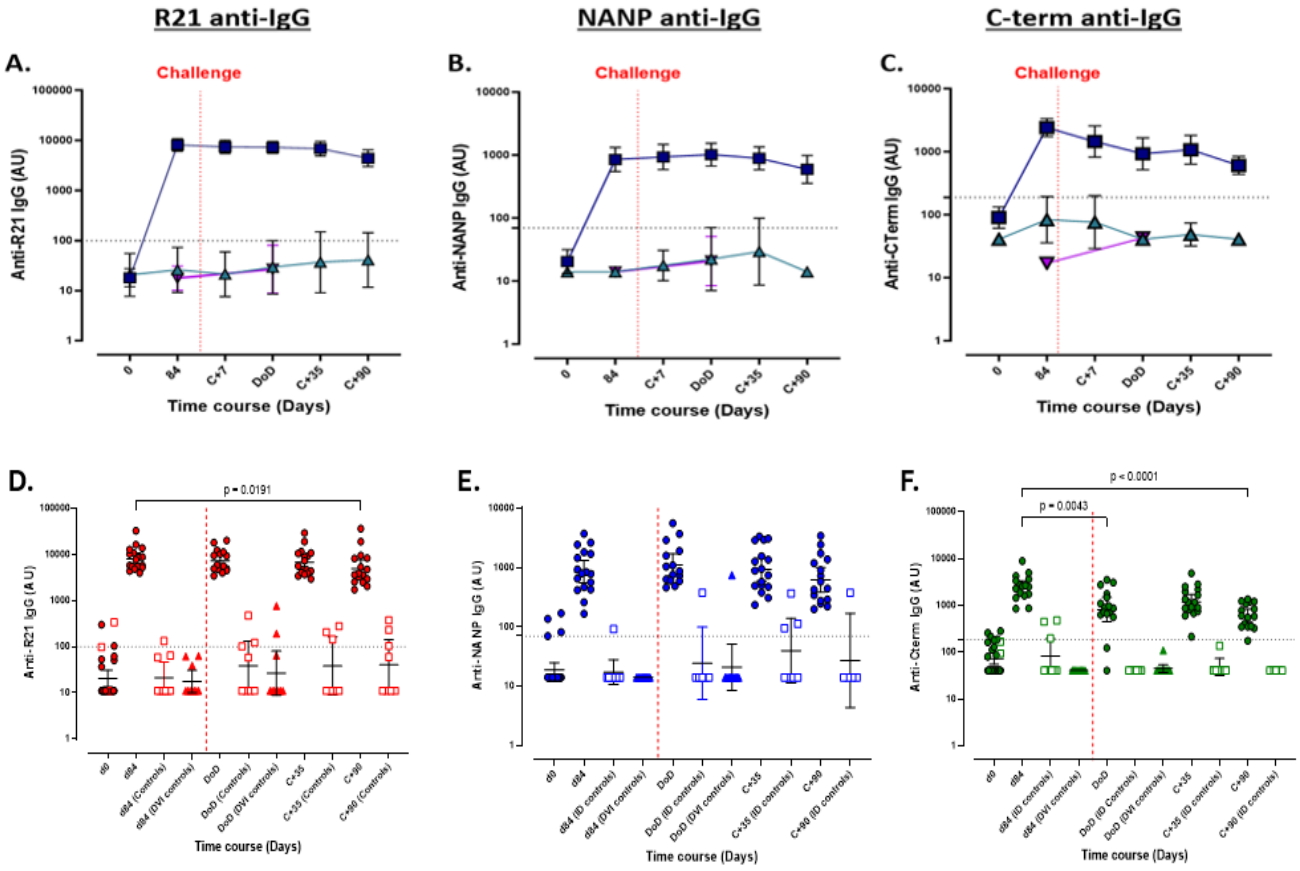

Figure 6. 1: Anti-IgG responses post vaccination and post sporozoite challenge.

Total IgG responses to R21, NANP and C-terminus were measured by a standardized ELISA at day 0, 84, C+7, DoD, C+35 and C+90 to demonstrate the kinetics of antibody responses following vaccination and challenge. (A) summary kinetics of anti-R21 IgG responses (B) summary kinetics of anti-NANP and (C) summary kinetics of anti-C Terminus. (D) Individual IgG responses to R21. (E) Individual IgG responses to NANP. (F) Individual IgG responses to C-terminus. In graphs A, B and C vaccinees (n = 17, blue squares); Non-vaccinees (ID, n = 9, green triangles); Non-vaccinees (DVI, n = 10, purple triangles). In graph D, vaccinees are represented by red filled circles; non-vaccinees challenged by ID are open red squares (n = 9) and non-vaccinees challenged by DVI are red triangles (n = 10). In graph E, vaccinees are represented by blue filled circles; non-vaccinees challenged by ID are open blue squares (n = 9) and non-vaccinees challenged by DVI are blue triangles (n = 10). In graph F, vaccinees are represented by green filled circles; non-vaccinees challenged by ID are open green squares (n = 9) and non-vaccinees challenged by DVI are green triangles (n = 10). Responses within the groups was compared Kruskal-Wallis with Dunns comparison test (Graphs D, E, and F). GMT ±95% CI values reported. Horizontal dotted line represents the seropositivity threshold (Mean + 3x SD of UK malaria naïve sera). Vertical red line represents challenge day. Day 84 = day before challenge; C+7 = 7 days post challenge; DoD = day of diagnosis; C+35 = 35 days post challenge and C+90 = 90 days post challenge.

I observed a slight increase in responses to NANP following the challenge. On day 84 (the day before challenge), the GMT was 851 (549.7 – 1318), 7 days post-challenge, and the responses were 932 (95% CI: 589.4 – 1475), peaking on the day of diagnosis with a GMT of 1110 (95% CI: 724.8 – 1700). The responses gradually fell to GMT 921.6 (95% CI: 595 – 1427) at 35 days post-challenge, and by 90 days post-challenge, the GMT was 619 (95% CI: 383.5 – 1000). Although there was a trend towards an increase in NANP responses following sporozoite challenge, none of the responses after challenge were significantly different from the responses seen on day 84.

Non-vaccinated individuals showed a trend of increasing anti-R21 and anti-NANP IgG responses post-challenge, indicating a boost from malaria exposure despite not being vaccinated. R21 responses increased from GMT 25.82 (95% CI: 9.17 – 72.69, n = 8) at day 84 to GMT 29.27 (95% CI: 8.59 – 149.6, n = 7) at day of diagnosis to GMT 36.81 (95% CI: 9.06 – 149.6, n = 7) at 35 days post challenge and finally GMT 40.83 (95% CI: 11.64 – 143.2, n = 7) at 90 days post challenge. Though these increases were not significant. NANP responses increased from GMT 14 at day 84, to 22.40 (14 – 70.71, n = 8) on the day of diagnosis, to 22.31 (95% CI: 7.13 – 69.8, n = 7) at 35 days post-challenge before returning to baseline levels by 90 days post-challenge GMT 14 (95% CI: 14 – 14, n = 7). Despite the increases, antibody levels remained below the positivity threshold.

Overall, total IgG responses to all the antigens remained significantly different in the vaccinated participants compared to the non-vaccinated group after challenge. Although responses to R21 and C-terminus showed a gradual decrease after challenge they were still well above the seropositivity line. NANP responses showed a slight rise immediately post challenge, total responses remained well above the positivity threshold and higher that those of the unvaccinated group. Indicating that the R21/MM vaccination effectively induced and maintained IgG responses against all CSP antigens even after exposure to malaria.

6.5.2 Gradual decline of IgA and IgM antibody responses to CSP post challenge, with increased anti-NANP IgM in unvaccinated controls post challenge.

6.5.2.1 Total IgA responses

Next, I examined the impact of challenge on IgA and IgM antibody responses using a standardized ELISA at day 0, day 84, the day of diagnosis, as well as 35- and 90-days post challenge. Similar to IgG responses, IgA and IgM antibody titres gradually decrease for all three antigens following challenge. For R21 IgA, GMT was 1845 (95% CI: 1172 – 2904, n =17) at day 84, dropping to 1391 (95% CI: 711.6 – 2721, n = 16) at day of diagnosis and by C+90 responses were 932 (95% CI: 458.6 – 1894, n = 15). NANP IgA responses decreased from GMT 410.2 (95% CI: 225 – 746.9, n =17) at day 84, to GMT 322.7 (95% CI: 172.8 – 602.7, n =16) at the time of diagnosis, and further to GMT 182.1 (95% CI: 91.99 – 360.4, n =16) at 90 days post challenge. C-terminus IgA responses were GMT 110.7 (95% CI: 50.8 – 241.2, n = 17) at day 84, GMT 108.4 (95% CI: 54.07 – 217.5, n =17) at the time of diagnosis and reached nearly identical levels to the positivity threshold GMT 22.22 (95% CI: 7.17 – 68.88, n =16) at C+90.

R21 IgA responses remained above the positivity threshold at C+90, NANP and C-terminus responses had dropped to the positivity threshold, which suggests that IgA responses may not be effectively maintained for the smaller epitopes present in the vaccine. The non-vaccinees remained negative for all antigens throughout the study.

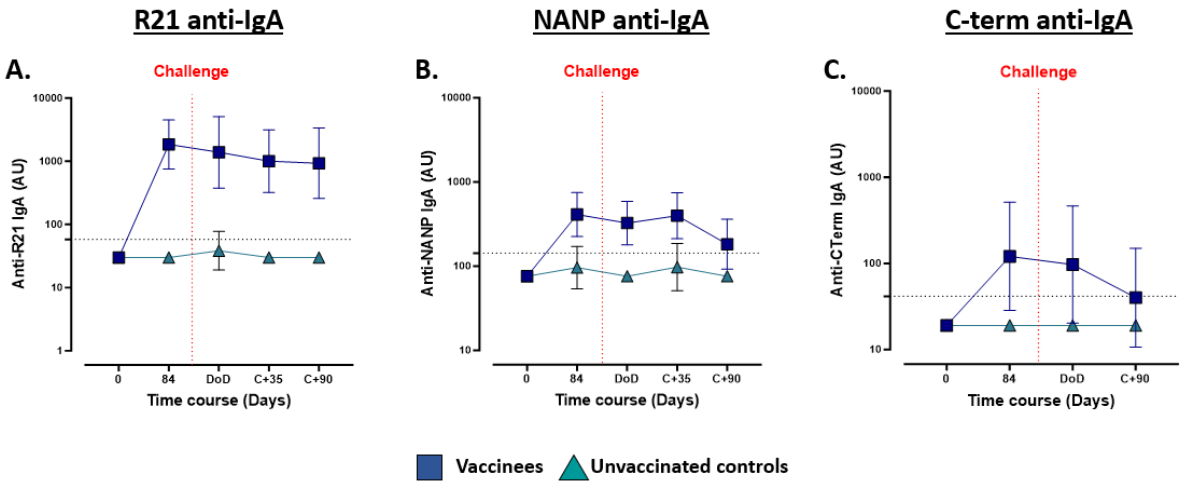

Figure 6. 2: Anti-IgA responses post vaccination and post sporozoite challenge.

IgM responses were measured by standardized ELISA at day 0 (n = 19), day 84 (n=17), 35 days post-challenge (n = 16, C+35) and 90 days post-challenge (n = 16, C+90). A) R21 IgA responses and (B) NANP IgA responses. GMT ±95% CI values reported. Red vertical dashed line represents challenge day. Horizontal dotted line represents the seropositivity threshold (Mean + 3x SD of UK malaria naïve sera). Day 84 = day before challenge; C+7 = 7 days post challenge; DoD = day of diagnosis; C+35 = 35 days post challenge and C+90 = 90 days post challenge.

6.5.2.2 Total IgM responses

IgM antibody responses to both R21 and NANP gradually decreased from day 84 to C+90, although this trend was not significant compared to day 84. At day 84, IgM responses were GMT 2155 (94% CI: 1174 – 3954, n = 17). However, they had fallen to GMT 1118 (95% CI: 683.6 – 1827, n =16) by C+90. Similarly, responses to NANP decreased from GMT 724.3 (95% CI: 475.2 – 1104) at day 84 to GMT 475.1 (95% CI: 328.9 – 686.2) at C+90.

Interestingly, IgM responses to NANP in non-vaccinated individuals increased even before the challenge. At baseline, the GMT was 88.63 (95% CI: 45.34 – 173.2, n = 9), rising to GMT of 157.8 (95% CI: 77.62 – 320.7, n = 9) at day 84, and reaching 207.6 (95% CI: 108.7 – 396.5, n =7) by C+90. By C+90, there was no statistical difference in the NANP between the vaccinated and unvaccinated groups. Similarly, R21 IgM responses in the control group increased post-challenge, from GMT 38 at day 84, to GMT 289 (95% CI: 164.0 – 509.7, n = 9) at day of diagnosis. However, these responses declined by C+90, with a GMT of 103.8 (95% CI: 32.79 – 328.4, n =5). This suggests that the sporozoite challenge influences IgM responses to both R21 and NANP.

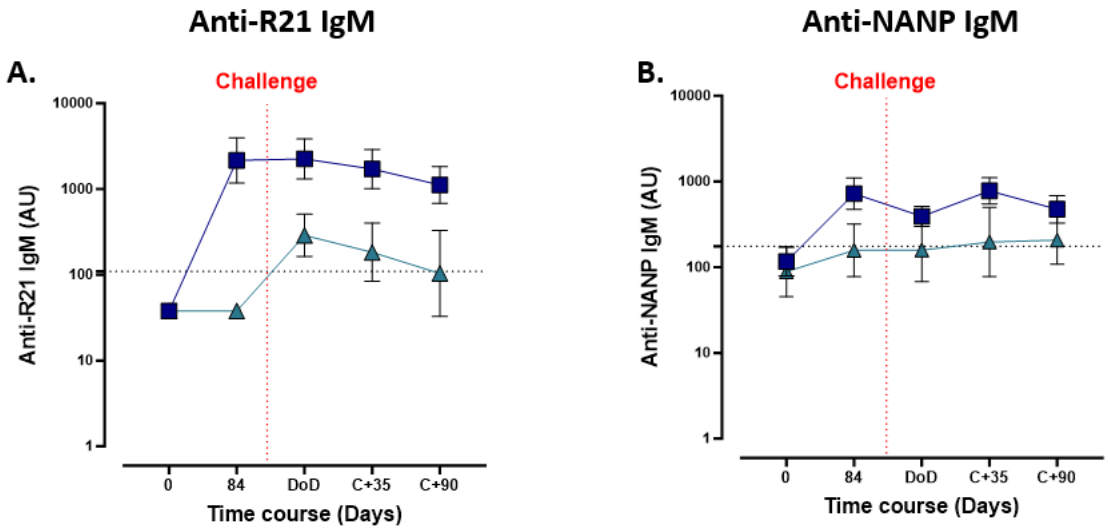

Figure 6. 3: Anti IgM responses post vaccination and post sporozoite challenge.

IgM responses were measured by standardized ELISA at day 0, day 84, day of diagnosis, 35 days post-challenge (C+35) and 90 days post-challenge (C+90). (A) R21 IgM responses and (B) NANP IgM responses. GMT ±95% CI values reported. Red vertical dashed line represents sporozoite challenge. Black horizontal dotted line represents the seropositivity threshold (Mean + 3x SD of UK naïve immune sera). Day 84 = day before challenge; C+7 = 7 days post challenge; DoD = day of diagnosis; C+35 = 35 days post challenge and C+90 = 90 days post challenge.

6.5.3 Vaccine efficacy and route of challenge.

The qPCR results were surprising, as a noticeable impact of the challenge route on vaccine efficacy was observed . All participants who were immunized and challenged through the skin (ID) were protected by R21 vaccination, while those who were challenged intravenously were susceptible despite vaccination with R21, and all had detectable parasites by day 15. As expected, the non-vaccinated controls were all unprotected whether challenge via ID or DVI. I used the previous CHMI-SIKA, Ngerenya (Kapulu et al., 2022) participants as a control group for the DVI challenge. In the vaccinated group challenged by ID, three individuals had detectable parasites, but they were able to clear the parasites without requiring treatment. As the effect of challenge route was so strong it was difficult to look at the impact of specific immune responses on outcome when combining the ID and DVI groups. Furthermore, the DVI group were all susceptible to infection and there was no variation in outcome within this group. Therefore, I focused on the ID group within which three individuals were briefly parasite positive and could be compared with the rest of the group participants who were PCR negative (n = 9).

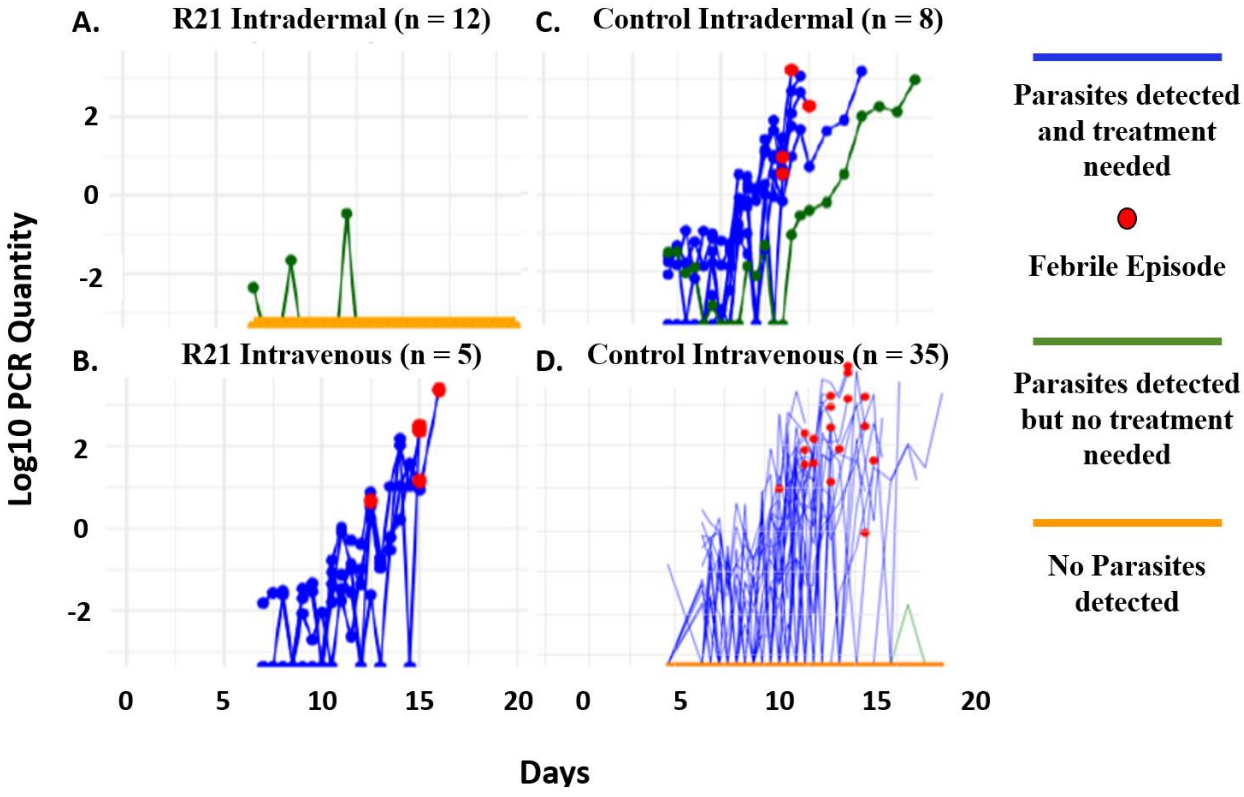

Figure 6. 4: qPCR outcome based on vaccination status and challenge route.

Participant received 3 doses of R21/MM and four weeks following their last dose they were challenged with *P.falciparum* sporozoites. Parasitaemia was monitored by qPCR. (A) R21 vaccinees challenged intradermally. (B) R21 vaccinees challenged intravenously. (C) Unvaccinated control challenged intradermally. (D) Unvaccinated controls challenged intravenously. PCR data was generated and provided by Prof. M Kapulu.

6.5.4 Antibody Response Patterns in Parasite-Positive Individuals Following ID Challenge

I attempted to identify a pattern among the three individuals who had a positive qPCR result and those who remained negative, focusing on their antibody responses. This analysis aimed to evaluate whether these individuals exhibited distinct antibody profiles, such as differences in the magnitude or specificity of antibody responses, compared to those who remained parasite negative. However, no significant difference was evident between the two groups in terms of antibody titres, antibody isotypes, or any antigen that could explain why these specific individuals did not achieve sterile protection.

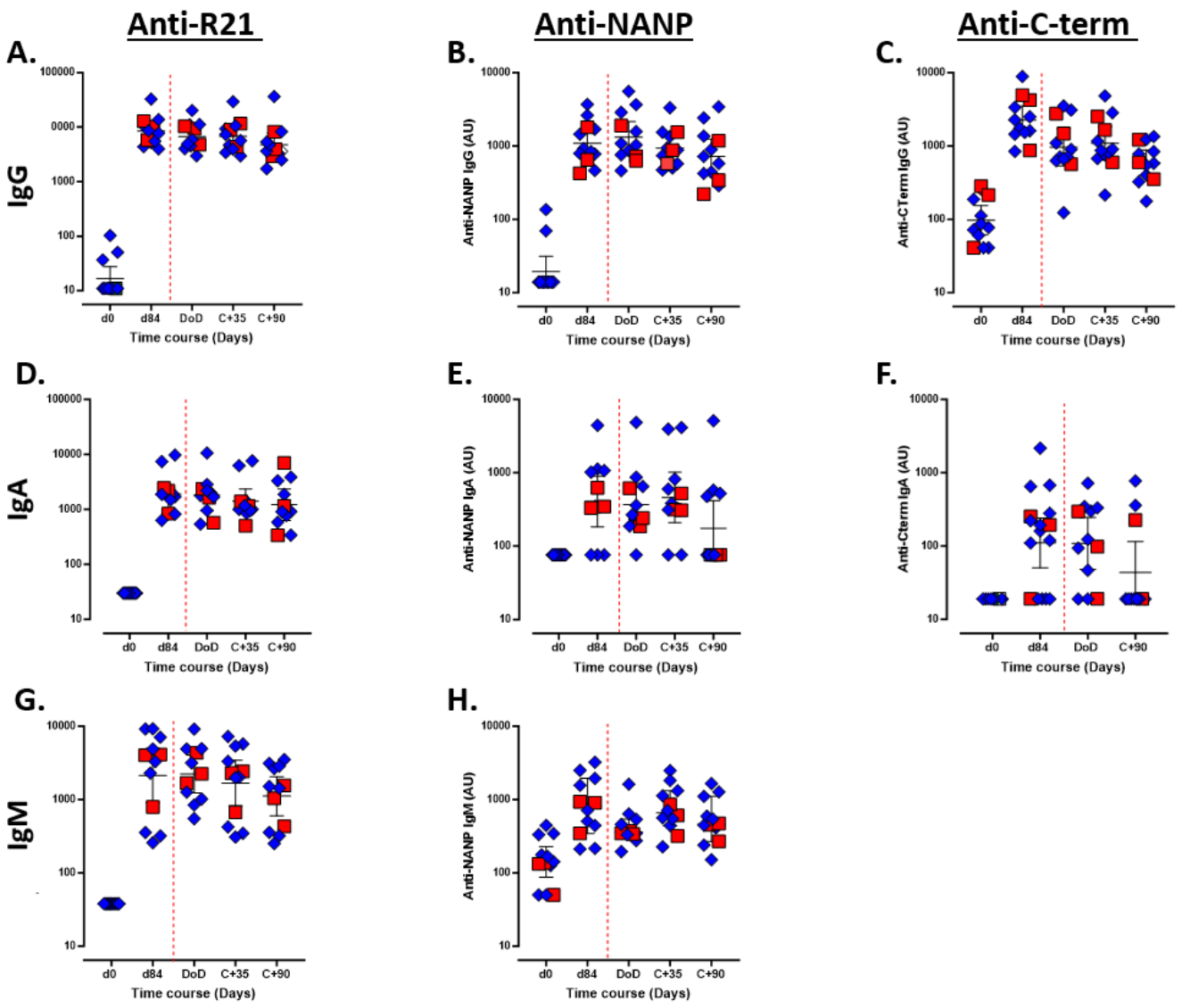

Figure 6. 5*:* Antibody responses in the vaccinees challenged intradermally.

Antibody responses were assessed using a standardized ELISA to determine if there were any specific patterns or trends in the immune response among individuals who tested parasite-positive in the ID challenge group. (A) Anti-R21 IgG responses. (B) Anti-NANP IgG responses. (C) Anti-C-terminus IgG responses. (D) Anti-R21 IgA responses (E) Anti-NANP IgA responses. (F) Anti-C-terminus IgG responses. (G) Anti-R21 IgM responses and (H) Anti-NANP IgM responses. Individual responses and GMT ±95% CI are shown. Blue rhombus represents individuals challenged by ID (n = 9) and red squares represent individuals (n =3) who were challenge via ID but had detectable parasites within the group. Vertical red dashed line represents challenge day.

6.5.5 Sporozoite challenge increases anti-R21 IgG avidity, no effect on NANP or C-terminus avidity

Avidity was measured in the vaccinees using the NaSCN displacement of IgG as described in chapter 4. Avidity indices was measured to determine the maintenance of avidity and any potential impact of sporozoite challenge on antibody avidity.

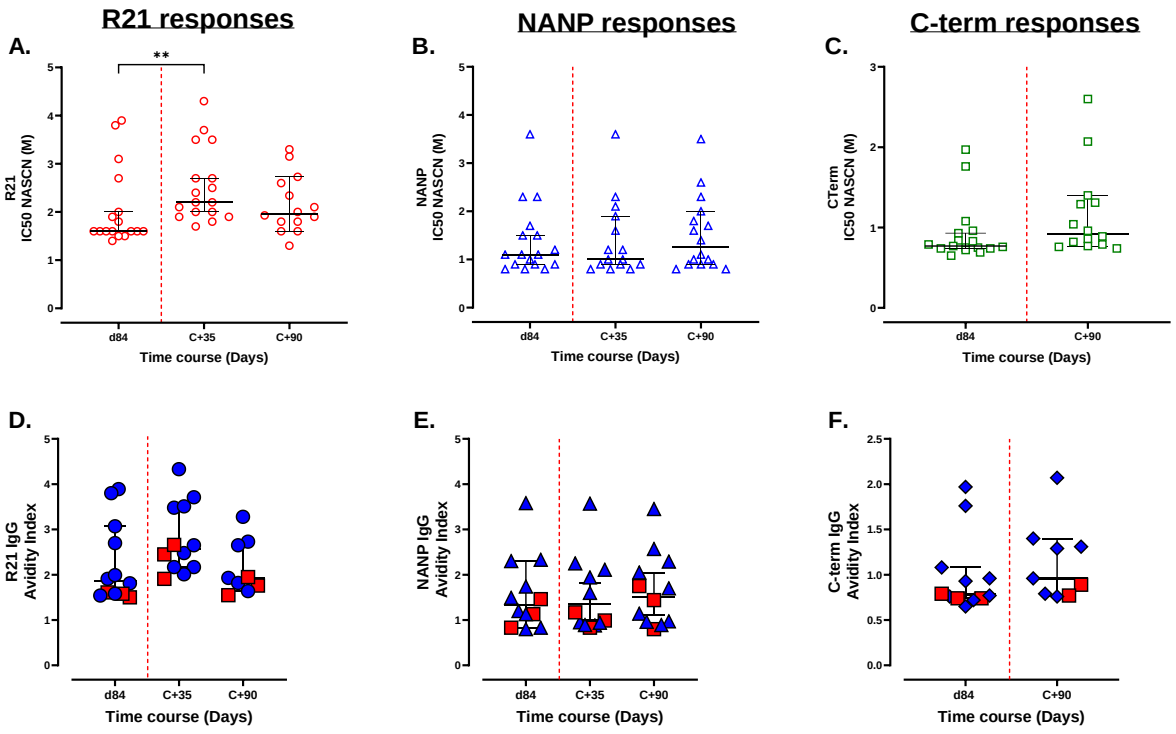

Figure 6. 6: Avidity to CSP antigens post vaccination and post challenge in the ID challenged group

Avidity was analysed on day 84 (before challenge) and 35- and 90-days post challenge. (A) anti-R21. (B) anti-NANP. (C) C-terminus. (D) R21 avidity in the ID challenged group. (E) NANP avidity in the ID challenged group. (F) C-terminus avidity in the ID challenged group. Blue symbols in graph D, E and F represents individuals challenged in the ID group that were fully protected (n = 9) while red represents those that had detectable parasites with the ID challenge group (n = 3). Individual responses and median ±95% CI are shown (Graphs A-C). Vertical red dashed line represents challenge day Individual responses and median ±95% CI is shown (at day 84 n = 17, at C+35 n = 17 and at C+90 n = 14). Responses within the groups was compared using Kruskal-Wallis with Dunns comparison test.

A significant increase in anti-R21 avidity was observed following challenge, with the avidity index increasing from a median of 1.6 (95% CI: 1.6 – 2.5, n = 17) to 2.2 (95% CI: 2.1 – 2.9, n = 17) at C+35. Avidity to NANP, and C-terminus remained constant from day 84, C+35 to C+90 with little or no significant change following parasite exposure.

I conducted a further analysis to investigate potential patterns among individuals who exhibited transient parasite positivity in the ID challenge group. The findings suggest a trend towards lower avidity indices prior to challenge (day 84) for all three antigens in the three individuals within the ID group who demonstrated a positive qPCR (represented by red squares). Although the small sample size limits the ability to draw definitive conclusions, this observation may indicate that individuals with lower avidity responses prior to the challenge were more likely to exhibit transient parasitaemia post-challenge.

6.5.6 Sporozoite challenge increases NANP anti-C1q responses in the unvaccinated group

In chapter 4, I demonstrated that vaccination leads to the development of complement fixing antibodies particularly to the NANP region of the vaccine. Here I investigated whether sporozoite challenge affects these complement fixing antibodies as well as the durability of these antibodies using the C1q ELISA.

Following challenge, I observed no significant increase in C1q fixing antibodies to NANP in the vaccinated group from day 84 (median OD_{450nm} = 0.478, 95% CI: 0.447 – 0.572, n =17) to C+5 (median OD_{450nm} = 0.532, 95% CI: 0.392 – 0.471, n = 17). Suggesting sporozoite challenge does not enhance their complement fixing capabilities. By C+90, these antibodies had declined to a median OD_{450nm} of 0.424, (95% CI: 0.392 – 0.472, n = 16). Despite this decline, it is reassuring that the C1q-fixing antibody responses remain above the positivity threshold for all vaccinees up to 90 days post-challenge, suggesting that the antibody response is maintained over time

Interestingly, sporozoite challenge led to an increase C1q NANP antibodies in the unvaccinated individuals at C+5 (median OD_{450nm} = 0.416, 95% CI: 0.269 – 0.519, n = 3) compared to day 84 (median OD_{450nm} = 0.14, 95% CI: 0.137 – 0.159, n = 9) and this increase is significant (Mann Whitney p = 0.001). Then again, the samples sizes were small, and these results may not be significantly powered to detect a difference between the groups.

C-terminus C1q responses had started to decrease by day 84. Unfortunately, I could not measure responses between day 84 and C+90 and in the control group due to unavailability of reagents. By C+90, vaccinee responses were comparable to unvaccinated controls at day 84, both falling below the positivity threshold (figure 6.7 (B)). This suggests that C-terminus C1q responses are short-lived irrespective of the challenge.

A similar gradual decline in antibody titres was also observed when examining total anti-C-terminus IgG data.

In the ID challenged group, the three individuals who were not sterilely protected showed a potential trend of lower anti-C1q antibodies to NANP at day 84, as indicated by the red squares in Figure 6.7 (C). However, due to the small sample size, it is challenging to determine whether this observation represents a statistically significant trend.

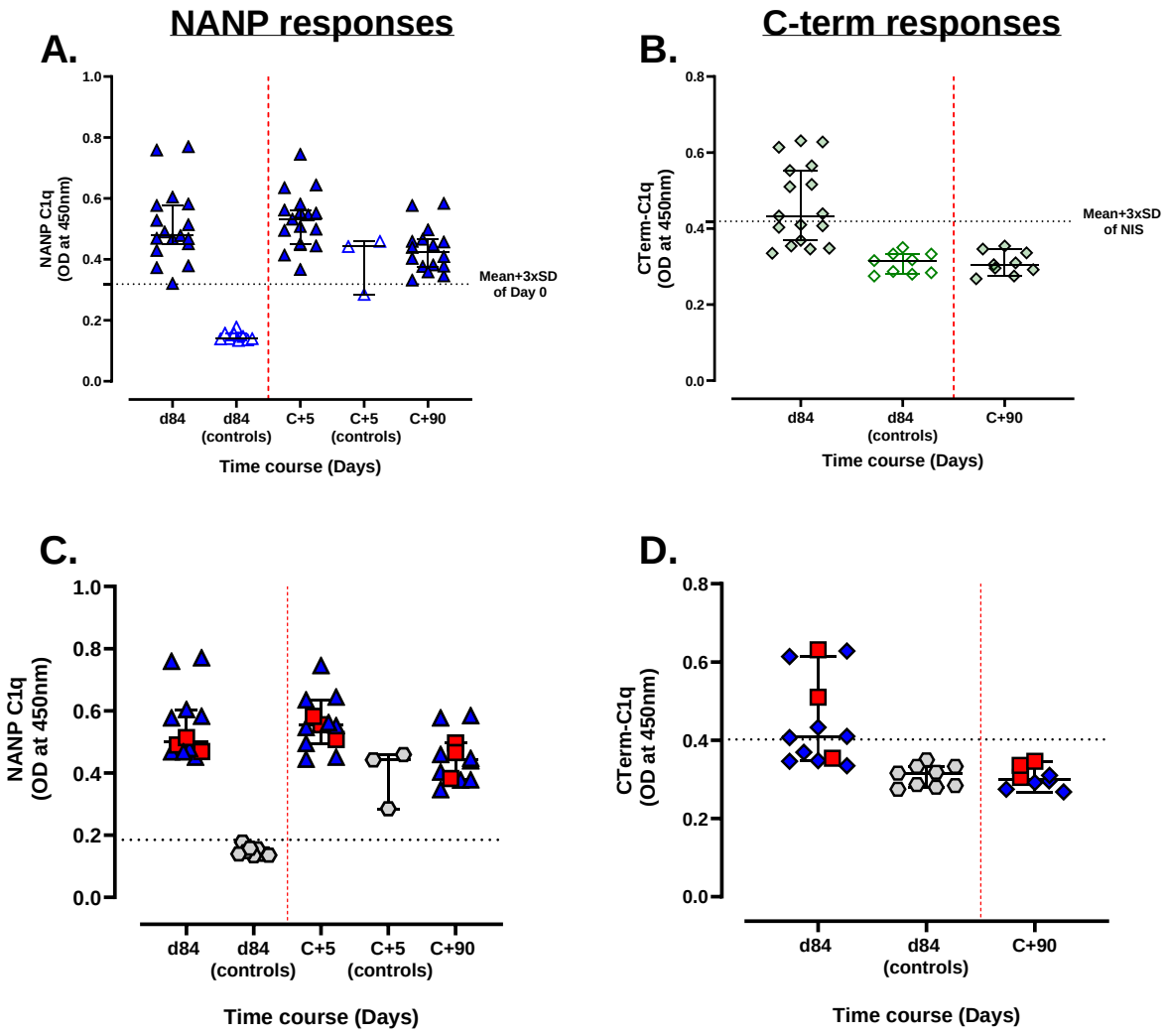

Figure 6. 7: Acquisition of vaccine induced C1q fixing antibodies following sporozoite challenge.

(A) C1q antibodies to NANP were measured at day 84 (n = 17) and 5 days (n = 17) and 90 days (n = 16) post-challenge in the vaccinees and at day 84 (n = 9) and C+5 (n = 3) in the controls. (B) C1q antibodies to C-terminus were quantified on day 84 (n = 17) and 90 (n – 16) days following challenge. (C - D) Acquisition of vaccine induced C1q fixing antibodies in the ID challenged group. (C) C1q antibodies to NANP in the ID challenged group (n = 9).

(D) C1q antibodies to C-terminus in the ID challenged group. Blue symbols in graph C and D represents individuals challenged in the ID group that were fully protected (n = 9) while red represents those that had detectable parasites with the ID challenge group (n = 3). Individual responses and median ±95% CI are presented. Responses between the groups were analysed using Kruskal-Wallis with Dunn's multiple comparisons test. The horizontal line represents the positivity threshold (Mean + 3x standard deviation of day 0). The vertical red dashed line indicates the sporozoite challenge day.

6.5.7 Association and clustering of total IgG titres to R21 and C-Terminus with memory B Cells at C+90.

Next, I wanted to determine if there was any association between total IgG antibody titres and MBCs at C+90 within the ID challenged group. Additionally, I aimed to determine if the three parasite-positive individuals exhibited any clustering in their immune responses. I aimed to explore potential patterns or correlations that could shed light on the immune dynamics of these individuals. I noted a moderately positive association to R21 ($r = 0.582$, $p = 0.06$, $n = 11$) and little association to C-terminus ($r = 0.1$, $p = 0.776$, $n = 11$). Unfortunately, data on IgM and IgA responses to R21 and C-terminus were unavailable due to several participants falling below the limit of detection in the ELISA. No clear pattern was observed in the three individuals (shown as red squares) regarding total IgG antibodies or memory B cells 90 days post-challenge (Figure 6.8).

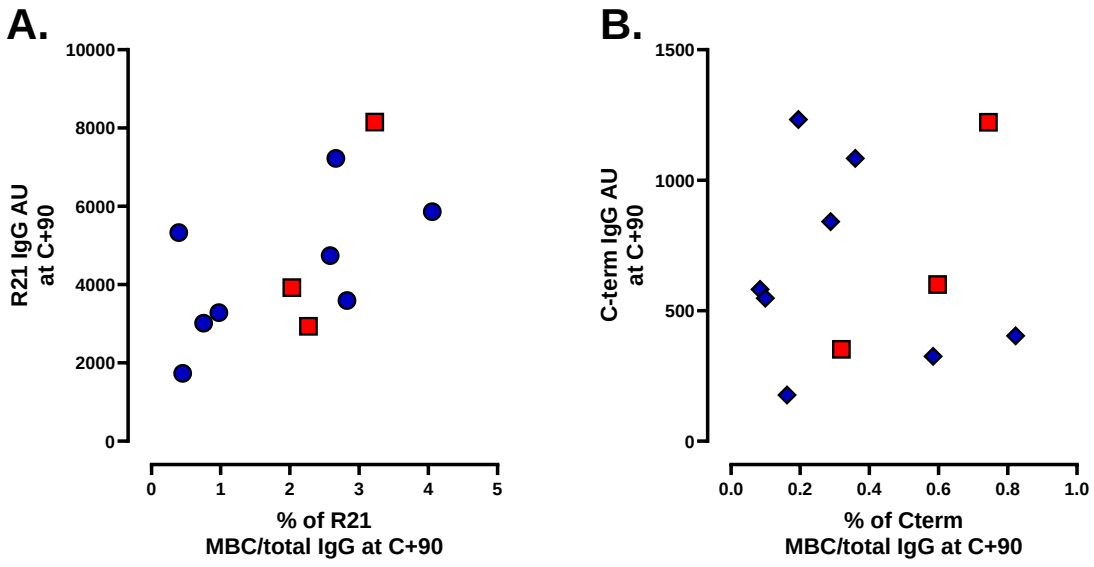

Figure 6. 8 Relationship between memory B cells responses and total IgG antibody titres at C+90.

(A) R21 responses (B) C-terminus responses. Blue circles represent individuals challenged by ID (n = 8) and red squares represent individuals who had detectable parasites within the ID group (n = 3).

6.5.8 The frequency of total Tfh cells expands after challenge.

In chapter 4, I demonstrated a significant increase in total cTfh cells from baseline following a single dose of R21/MM. Here, I aimed to investigate the effect of sporozoite challenge on cTfh and whether there is a rise in specific sub-populations, and whether these coincide with the subsets that are impacted by vaccination. I used CXCR5+/PD1+ expression on memory (CD45RA-/CD4+) T cells to identify total cTfh cells by flow cytometry.

Regrettably, I did not have data at day 84 (a day before challenge) as such I used day 70 as the baseline levels before challenge which was two weeks following 3rd vaccination and one month before challenge. I performed a comparison analysis and saw a notable rise in the total number of cTfh cells from day 70 to C+5 (Mann Whitney U test, $p = 0.039$, $n = 15$) following malaria infection, which was similar to the response I observed following a single vaccination with R21/MM. Though, I used an earlier timepoint for this analysis following challenge (C+5), previously I used 14 days after vaccination due to the study design I did not have samples at day 14 post-challenge, but from the literature Tfh cells are in circulation from day 3 and peak at day 14 following vaccination with yellow fever (J. Huber, 2020).

I also analysed the Tfh sub-populations based on their expression of CCR6 and CXCR3. The results showed that the predominant subsets were Tfh2 and Tfh17. There was a slight increase in Tfh1 and Tfh2 subsets from day 70 to C+5 and a slight decrease in TfH17 sub-populations, though neither was significant. These findings suggest that sporozoite challenge may lead to modest shifts in Tfh subset distribution, but the changes do not appear to be substantial and the overall anti-sporozoite cTfh cell responses are dominated by Tfh2 and Tfh17 subsets. I conducted a sub-analysis to explore whether the three individuals in the ID challenged group who were not sterilely protected exhibited any distinct pattern or clustering in terms of total cTfh cells and their subsets. However, no significant clustering or clear pattern emerged from the data (data no shown).

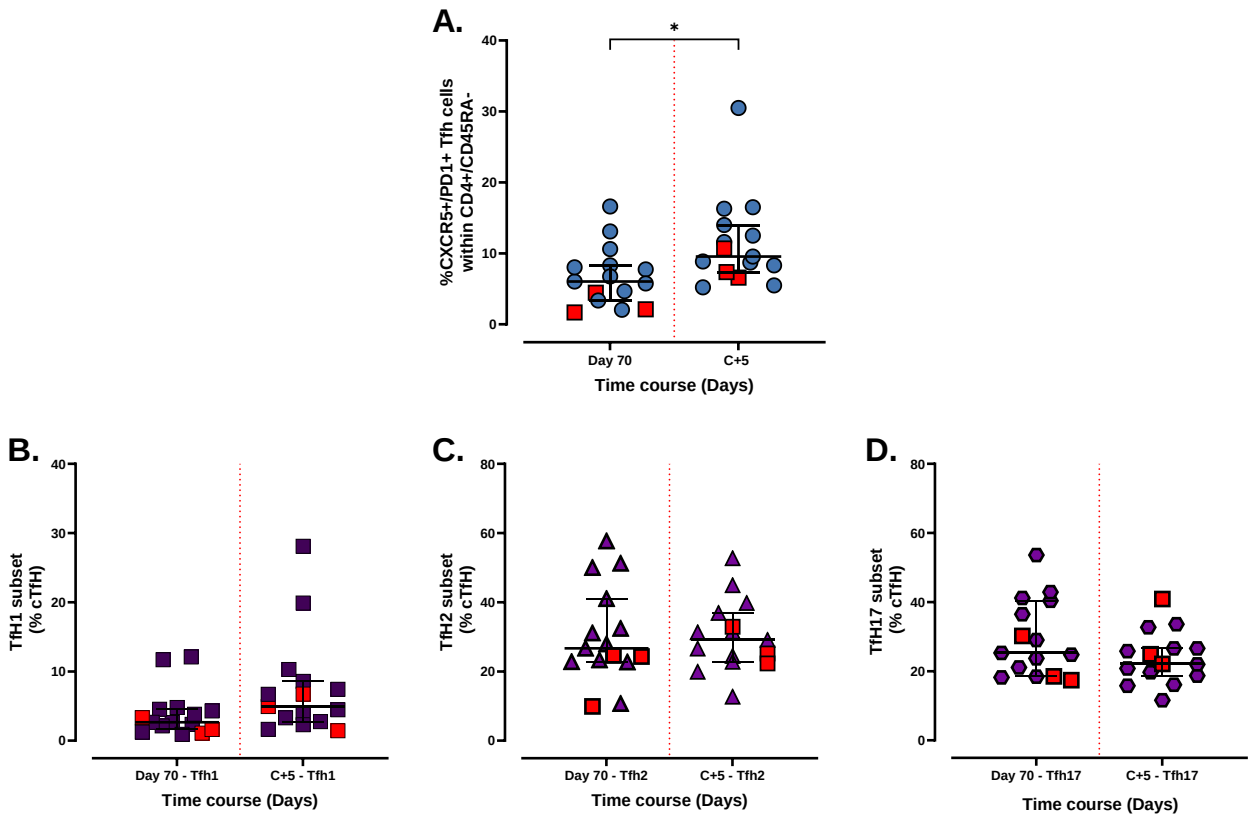

Figure 6. 9: Increase in the frequencies of total cTfh following sporozoite challenge.

One month following 3 doses of R21/MM participants were challenge with *P.falciparum* sporozoites. Tfh phenotyping was carried out 5 days post challenge (A) total CXCR5+/PD1+ Tfh cells. (B) frequency of Tfh1 cells (CXCR3+ CCR6-). (C) frequency of Tfh2 cells (CXCR3- CCR6-). (D) frequency of Tfh17 cells (CXCR3- CCR6+). Responses between the groups were compared using the Kruskal-Wallis Test with Dunns multiple comparison. Individual responses and median ±95% CI are shown. Data are from 15 vaccinated individuals at days 0 (before immunization); day 70 (2 weeks post 3rd dose) and C+5 (5 days post challenge). Red squares represent individuals who had detectable parasites within the ID group (n = 3). Red vertical dashed lines represent sporozoite challenge.

6.5.9 Total Tfh cells at day 14 correlate with total R21 IgG antibodies at C+90.

I ran a correlation analysis to determine potential association between total Tfh cells at day 14 and total IgG antibodies at C+90. Additionally, individuals who were briefly parasite positive in the ID challenged group were examined to ascertain if they clustered in a particular pattern. I concentrated on day 14 as this time point showed a significant difference from baseline. I observed a negative association that was significant between total cTfh responses and total anti-R21 IgG titres at C+90 , and a general negative association between anti-NANP and C-terminus. Suggesting a decrease in antibody titres as the frequency of total Tfh cells increases. I also observed little or no correlation with the Tfh subsets at day 14 and total IgG antibodies at C+90 to any of the three antigens (data not shown).

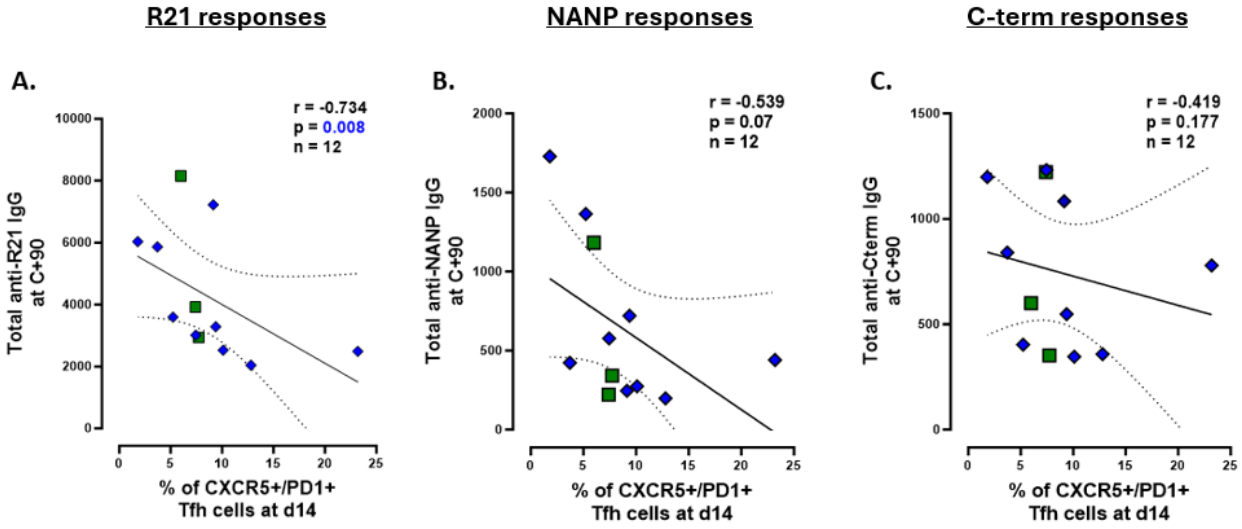

Figure 6. 10: Correlation analysis of total Tfh frequencies and total IgG titres

Spearman's correlation was performed to determine any relationship between the frequency of total cTfh at day 14 and antibody titres at C+90. (A) R21 responses (B) NANP responses and (C) C-terminus responses. Data shown represents all individuals vaccinated with R21/MM. Green squares represent individuals who had detectable parasites within the ID group (n = 3).

6.6 Discussion

The goal of this chapter was to explore potential immune responses that could be associated with vaccine-induced protection after sporozoite challenge. Adult volunteers were immunized three times with R21/Matrix M and then challenged one month later. Parasite monitoring began 7 days after the challenge and was performed using qPCR. Unfortunately, the study was not adequately powered to identify correlates since a) only one cohort was challenged due to manufacture issues with the PfSPZ challenge reagent and b) even within that cohort, the major difference was due to route of challenge, with only limited variation within challenge groups.

The qPCR analysis revealed that the route of challenge had a significant influence on protection outcomes. Individuals who were vaccinated and challenged through the skin were immune until the end of the study at C+21, but those who were vaccinated and challenged intravenously all acquired malaria. The unvaccinated participants that were challenged via the skin all had positive qPCR results, indicating that R21/MM vaccination has a role in providing protection between the ID challenged groups. Although the outcome was slightly surprising, it is not entirely unexpected. In addition to acting as a physical barrier, the skin recognizes sporozoites and mounts an immunological response to them, triggering both innate and adaptive immune defences. This makes the skin the first line of defence against infections (Daily, 2018; Flores-Garcia et al., 2018; Ménard et al., 2013; Mitchell et al., 2022).

In a study conducted by Flores-Garcia and colleagues, they demonstrated that antibodies induced from immunizing mice with radiated-attenuated *P. berghei* sporozoites, targeted migrating sporozoites and offered significant protection at the injection site by impeding sporozoite mobility. Additionally, they found that challenges through the skin offered greater protection compared to intravenous challenges. (Flores-Garcia et al., 2018). Furthermore, when mosquitoes inject sporozoites into the skin, these sporozoites move slowly and gradually entering the bloodstream within approximately 1-3 hours post inoculation (Amino et al., 2006; Yamauchi et al., 2007). Similarly, when challenge was administered through the skin it can be assumed that sporozoites gradually entered the bloodstream. I speculate that the high antibody titres, along with the various isotypes induced and the functional quality (complement fixation) of antibodies against CSP induced by vaccination contributed to protection by either neutralizing sporozoites or inhibiting their motility when challenge is administered through the skin.

Overall, there was a gradual decline in anti-R21, NANP and C-terminus (IgG, IgA and IgM) responses from day 84 to C+90, sporozoite challenge did not significantly boost CSP antibody responses in the vaccinated participants. It is possible that while the quantity of antibodies does not increase, their quality

(e.g., avidity or functionality) may still be sufficient to provide protection. I noted a subtle increase in IgG and IgM antibody titres in the unvaccinated participants following challenge. I attempted to identify a pattern or trend among the three participants who were qPCR positive to understand the reason why they were unable to control their parasites, albeit briefly. However, I did not observe a particular trend or clustering among the three participants when I looked at their anti-IgG, -IgA or -IgM responses to any of the three antigens analysed.

When I analysed the avidity in all the vaccinees following challenge, I observed a significant increase in R21mavidity following challenge, suggesting that the antibodies have undergone further affinity maturation, I did not see the same effect towards the smaller epitopes (NANP or C-terminus). This could be due to differences in the immunogenicity or structural complexity of these epitopes. The larger R21 antigen could trigger a stronger immune response, promoting affinity maturation, whereas smaller epitopes might not generate as robust a response or experience comparable affinity enhancements. I also observed a trend towards lower avidity indices in the three individuals who developed parasitaemia in the ID challenged group. However, as the numbers were small in this analysis, it makes it challenging to drawn statistically significant conclusions, though this trend warrants further investigation. Some studies have demonstrated an association between high avidity NANP antibodies and protection in CHMI studies (Regules et al., 2016; Seaton et al., 2021) as well as in field studies (Dobaño, Ubillos, et al., 2019) following vaccination with RTS, S. These findings imply that the quantity and avidity of vaccine induced functional antibodies play a role in contributing to protection. However, as highlighted above I was unable to link any potential changes in avidity to protection. A larger sample size would be necessary to thoroughly evaluate the potential contribution of avidity to protective immunity.

The complement system is a crucial part of the innate immune system. Research has indicated that its activation against *Pf* sporozoites is associated with protection in children (Behet et al., 2014; Kurtovic et al., 2018; Kurtovic, Drew, et al., 2021). In this study, I showed that vaccination leads to the production of complement fixing antibodies targeting NANP, which remained present 90 days after challenge. There was a tendency towards lower levels of NANP C1q antibodies in the three participants. While the data suggest a possible association between lower anti-C1q antibody levels and lack of sterile protection, further investigation with a larger cohort would be needed to confirm this potential relationship. Sporozoites propel themselves through the dermis by gliding motility to successfully establish infection in the liver. It is well known that CSP antibodies can inhibit sporozoite transversal and this inhibitory activity is enhanced by human complement (Behet et al., 2014; Kurtovic et al., 2018; Opi et al., 2021). It

is therefore plausible that these complement fixing antibodies act as an additional immune effector mechanism particularly in the skin. Sporozoites injected through the skin had a reasonable more amount of time to interact with the complement system which could potentially explain the difference that was observed in protection outcomes. Zenklusen and colleagues have also suggested a potential role for vaccine induced IgM in complement fixing and inhibiting sporozoite invasion *in vitro* (Zenklusen et al., 2018). Here I also observed high durable IgM CSP, although I was unable to determine whether these IgM antibodies fix complement. However, from the literature IgM antibodies are potent complement activators (W. Hoffman et al., 2016), one can therefore speculate that the presence of vaccine induced IgM may also contribute to protection.

Furthermore, I cannot state that antibodies are the only immune mechanism involved in protection, as the protection against *P. falciparum* is intricate and multifaceted. Therefore, it is essential to investigate the functions of innate immunity, such as neutrophils, natural killer cells, monocytes, and cytokines as these would be the first responders during an infection (Opi et al., 2021; Suscovich et al., 2020). As well as some more antibody functional assays such as the sporozoite gliding assays to assess sporozoite motility *in vitro* or transversal assays to determine the overall functional capabilities of these vaccine antibodies to understand their role in protection.

The frequency and activity of Tfh cells are closely related to the strength of antibody responses with higher Tfh frequencies correlating strongly with antibody responses particularly Tfh2 and Tfh17 subsets (Chan et al., 2020; J. E. Huber et al., 2020; Nielsen et al., 2021; Oyong et al., 2022). In chapter 5, I demonstrated an increase in total cTfh following vaccination with a predominant Tfh2 and Tfh17 subsets. Here I assessed the impact of challenge on Tfh cells. Due to the reasons highlighted in the beginning I was unable to assess the impact of Tfh cells on outcome. In this study I focused on memory T cells and found an overall increase in total cTfh frequencies after challenge which was significant. Additionally, the Tfh1 subset demonstrated a modestly higher level of activation after challenge followed by the Thf2 subset, while the Tfh17 subset showed a decrease immediately after the challenge. This pattern mirrors the response observed post-vaccination. Some studies have demonstrated a shift towards the unfavourable Tfh1 subset following PfSPZ (Wahl et al., 2022) and RTS, S/AS01B vaccination (Bowyer, Grobbelaar, et al., 2018), though these studies were carried out in malaria naïve individuals. Although, I observed a slight increase in the Tfh1 subset, this was not statistically significant, and neither was it greater in frequency than Tfh2 or Tfh17. As mentioned, I was unable to determine the distribution of CM T cells

(CCR7+) and EM memory (CCR7-) in this study which may explain the dominant Tfh2/17 subsets. Vaccines that promote the generation of central memory T cells may lead to better long-term protection. Nielsen and colleagues observed that frequencies of antigen-specific cTfh cells, particularly Th2-cTfh cells, correlate positively with peak anti-PfRH5 IgG concentrations (Nielsen et al., 2021). Here I noted a negative correlation between total Tfh responses and IgG antibody responses at C+90 to the three CSP antigens. However, these findings should be interpreted with caution, given the small sample size, it appears that a single individual may be driving the observed negative association, potentially skewing the results. Additionally, these findings suggest that while Tfh cells are critical for B cell help, their presence alone may not be sufficient for optimal antibody production, highlighting the need for a deeper understanding of immune dynamics. An antigen specific approach to analysing Tfh cell responses could prove crucial when examining vaccine-induced responses. In addition, the incorporation of commonly used activation markers for example Inducible T-cell COStimulator (ICOS) and CD38 as they help identify Tfh cell subsets that are actively participating in immune response. These markers are upregulated in Tfh cells that are actively engaged in providing B cell help and participating in the germinal centre reaction (Crotty, 2019). This approach allows for a clearer understanding of the Tfh activation.

6.6.1 Conclusion

In summary, the findings of this chapter indicate that the route of infection significantly influences the outcome of protection following CHMI. Although the route of challenge is a key factor in determining protection, it is also apparent that vaccination plays a role, as evidenced by the inability of unvaccinated participants to control parasites. The observed differences in protection outcomes based on the route of challenge raises important questions about the mechanisms responsible for the protective effect of the R21/MM vaccine and the importance of considering the route of challenge when using CHMI models to measure vaccine efficacy. Furthermore, I noted high and long-lasting total IgG antibodies to R21 and NANP C-terminus, along with C1q complement fixing antibodies targeting the NANP region, potentially all contributing to sustained defence against infection.

7 CHAPTER SEVEN - Concluding remarks and future directions.

There have been great strides towards beating malaria over the years. Malaria case incidence has declined from 81 per 1000 population at risk in 2000, to 58 per 1000 population at risk in 2023 (WHO, 2023). However, global malaria progress has stalled in recent years, despite extensive efforts in vector control, such as insecticide treated bed nets and insecticide spraying, and treatment with antimalarial drugs, there was still an estimated 249 million malaria cases in 2022, and Sub-Saharan Africa bears the biggest burden of disease (WHO, 2023). After decades of research two malaria vaccines are now recommended for use in children RTS, S and R21. These vaccines will fill a critical gap in the current prevention and control strategies.

The R21/Matrix-M vaccine represents a promising tool in the advancement of malaria control and elimination. Phase III trials have demonstrated high efficacy and robust antibody responses to NANP (Datoo et al., 2024). It has been well documented that CSP antibodies play a role in protection with CSP based vaccines (Abuga et al., 2021; Goh et al., 2019; Kurtovic, Atre, et al., 2021; Moita et al., 2022; Stanisic & McCall, 2021), however, the underlying immune mechanisms that are involved with R21/MM vaccine induced protection have yet to be clearly identified.

In this thesis, my overall aim was to characterize the immune responses to R21/MM and use the CHMI model to identify potential immune markers that associate with protective challenge outcomes.

To improve our knowledge on the CHMI model, I carried out a review of the existing literature on CHMI studies and its application in malaria endemic regions, specifically in Africa. My objective was to review and address the challenges of using this model in individuals with pre-existing immunity, as well as to determine the model's effectiveness in assessing vaccine efficacy in this particular population, and to identify any gaps in the literature. My research revealed that the CHMI model has been established successfully and is currently being used in six African countries to study VE and NAI, with varying malaria exposure. Furthermore, I observed that all the studies used Sanaria's PfSPZ as challenge material, and the most commonly used route of challenge was direct venous injection. This VAC074 study was the first study to assess R21s VE via ID and DVI challenge in Africa. In chapter 3, I validated an existing qPCR assay being used at the KEMRI-Wellcome Trust research specifically for the quantification of malaria parasites following CHMI. The assay was validated according to the ICH guidelines (ICH, 2014). In this chapter I demonstrated that the assay was fit for purpose with high sensitivity, reproducibility, and accuracy at blood volumes of <500 µl.

In chapter 4, I carried a comprehensive analysis on the quantity and quality of vaccine induced antibody responses. I firstly optimized robust ELISA protocols to improve the effectiveness, efficiency, and reproducibility of the assays used. Here, I observed not only does R21/MM induce high, durable IgG NANP responses, but it also induces robust IgG, IgA, and IgM responses to the R21 protein, NANP and the subdominant C-terminus portion. I noted there was no significant increase in antibody titres after the second dose of the vaccine, and prior exposure does not significantly impact vaccine induced responses. Using the Meso-Scale Discovery assay platform, I found that vaccination induced low hepatitis B surface antigen antibody responses, which was encouraging, and they were above the WHO protection threshold of 0.01 IU/mL. This was particularly notable because the main immune responses were directed against CSP, which is the primary target of the vaccine. The ability of the vaccine to elicit protective HBsAg-specific responses, despite its design prioritizing CSP-specific immunity, highlights its dual functionality. I also noted the induction of complement fixing antibodies to NANP and to a lesser extent C-terminus, implying that the vaccine induced antibodies could potentially initiate the activation of the complement system, which is part of the innate immune response. I did not observe a significant increase in avidity to any of the vaccine antigens with additional vaccine doses, neither did I observe a boost to avidity towards NANP and C-terminus following sporozoite challenge which is similar to what others have observed (Ajua et al., 2015; Olotu et al., 2014). However, I did note an increase in avidity towards the R21 protein. Using the FluroSpot assay (chapter 5), I carried out a multiplex analysis on antigen specific memory B cells. I showed not only does R21/MM vaccination induce IgG MBCs to R21 and C-terminus, but it also appears to induce antigen specific memory cells to IgA. These responses are sustained to IgG but are short-lived for IgA. These findings underscore the complexity of MBC development and the need to consider factors such as antigenic variation, transmission intensity, and other immune regulatory mechanisms. Additionally, I characterized the circulating Tfh cells showing a more Thf2 and Tfh17 population, which are essential in producing high-affinity antibodies and the development of long-lasting immunity (Chevalier et al., 2011; Gao et al., 2023; Morita et al., 2011).

Chapter 6, I aimed to identify whether any of the immune responses I characterized was associated with protection. This was the first study in a malaria endemic area to use CHMI to measure vaccine efficacy using different challenge routes. We immediately observed a significant impact of challenge route in protection outcome as the vaccinees challenged through the skin were all protected. Unfortunately, due to the significant impact on challenge and the small sample size it was difficult to tease out any immunological marker or responses that could potentially correlated with vaccine induced protection.

Nonetheless, this finding highlights the significance of the skin in malaria protection. Studies have demonstrated that sporozoites can remain in the inoculation site for several hours before entering the blood stream (Flores-Garcia et al., 2018; Sinnis & Zavala, 2012). Consequently, I speculate that the extended period in the skin allows the high concentration of antibodies induced from vaccination ample time to act in neutralizing the sporozoites. Furthermore, I have also demonstrated that these vaccine induced antibodies, can interact with the complement system, potentially acting as an additional mechanism in inhibiting sporozoites from exiting the dermis. Additionally, these antibodies may activate other effector mechanisms that enhance the clearance of sporozoites from the skin, such as antibody-dependent phagocytosis (Leitner et al., 2020; Musasia et al., 2022), antibody-dependent complement deposition (Behet et al., 2018; Kurtovic, Agius, et al., 2019; Kurtovic, Drew, et al., 2021), and antibody-dependent cytotoxicity (Goaqian Feng & Beeson, 2024). Lastly, it was interesting to note that sporozoite challenge did not significantly increase antibody titres directed against the vaccine induced antibodies.

7.1 Suggestions for future studies

7.1.1 Limitations of sample size

The primary limitation of this thesis was the restricted sample size, which made it difficult to detect statistically significance differences in the immune responses among the vaccinees. This was a Phase IIb clinical trial which was adequately powered with a minimum participants of 64. However, due to manufacturers issues with the challenge agent, only half the participants were challenged. Therefore, it is necessary to interpret these findings with caution. There is need to replicate this study with larger, adequately powered studies. This would strengthen the robustness and improve the precision of the results and provide more conclusive evidence regarding R21/MM vaccine induced responses.

7.1.2 Investigating the role of the skin and innate immunity in malaria protection

Here we observed a significant impact on challenge route, investigating the role of immune responses at the skin would provide more insights on why we observed such a strong effect in challenge route. Limited studies have explored the role of the skin in vaccine induced immunity and studies have mainly utilized mouse models but they highlight the skin as the site where the host immune response begins and its importance in priming for subsequent immune responses (Daily, 2018; Flores-Garcia et al., 2018; Hopp et al., 2015; Ménard et al., 2013; Sinnis & Zavala, 2012). It would therefore be beneficial to assess the impact of vaccine induced antibodies on the motility and infectivity of sporozoites, using *in vitro* assays, such as sporozoite gliding assays or hepatocyte invasion inhibition assays. These experiments may help

provide a better understanding on the protective measures produced by R21/MM vaccination. Innate immunity plays a crucial role in the initial response to malaria infection and likely also contributes to vaccine-induced protection (Minkah et al., 2019; Pohl & Cockburn, 2022; Stevenson & Riley, 2004). In addition to complement, cytokines and chemokines associated with the innate system should be studied. By exploring the interface between innate and adaptive immunity, we can gain a comprehensive understanding of the host immune response to R21/MM vaccination.

7.1.3 Comparing these results with naïve individuals

To account for the role of NAI in this study it would be interesting to compare individuals from a naïve population who have also received R21/MM and been challenged. By comparing the responses induced in naïve individuals versus these participants we can better understand the impact of previous exposure on the immune response to the vaccine. This analysis may reveal differences in both antibody and cellular immune responses and give a better understanding on what roles they potentially play in vaccine induced protection.

7.1.4 Exploring functional assays.

Recent studies have emphasized the importance of studying multiple antibody effector functions in immunity to malaria, particularly in the context of CSP based vaccines (Kurtovic, Atre, et al., 2021; Opi et al., 2021; Suscovich et al., 2020). Antibodies are the key mediators in immunity for CSP vaccines, including R21/MM and given the diverse functioning of antibodies, further assays should be considered for exploration. Here I measured the quantities, isotypes, avidity and complement fixing antibodies. Functional assays such as antibody-dependent cellular cytotoxicity, antibody-dependent respiratory burst, and antibody-dependent phagocytosis may help to understand the various mechanisms through which vaccine-induced antibodies exert their protective effects. These assays can shed light into the ability of antibodies to recruit other immune cells that enable the clearance of parasites, thereby enhancing our understanding of the functional attributes of the antibody response elicited by the R21/MM vaccine.

7.1.5 Multistage vaccines

It is highly likely for a malaria vaccine to be successful we would need a multistage vaccine that target different stages of the parasite life cycle. Combination vaccines have shown to be very successful such as the measles, mumps and rubella (MMR) vaccine and the diphtheria, pertussis and tetanus [DPT] vaccine (Halsey, 2001). In malaria, combining a pre-erythrocytic vaccine like R21, with a transmission blocking vaccine could potentially provide a dual protective effect by targeting both the infection and transmission stages of the malaria parasite. Recently, Hayashi and colleagues evaluated the immunogenicity of two

leading *P. falciparum* vaccine candidates, Pfs25 (transmission blocking vaccine) and PfCSP, delivered as mRNA-LNP vaccines. They found that co-administration of Pfs25 mRNA-LNP and PfCSP mRNA-LNPs elicited comparable antigen-specific antibody responses to the single antigen mRNA-LNPs. Suggesting that this combination approach could be a viable strategy for achieving more comprehensive malaria protection (Hayashi et al., 2022). Future studies are needed to investigate more efficacious combinations and what functional roles they may play in protection.

7.1.6 Transcriptomics

Transcriptomics involves the comprehensive analysis of RNA transcripts within a cell, providing valuable insights into the gene expression profile and molecular pathways activated in response to vaccination (Stanisic & McCall, 2021). Integrating transcriptomic analyses into the evaluation of vaccine-induced protection can offer a holistic understanding of the host immune response at the molecular level. Transcriptomic profiling can facilitate the identification of key genes and pathways associated with protective R21/MM induced immunity (Oyong et al., 2023). We can compare gene expression profiles before and after vaccination as well as in vaccinated naive individuals and in malaria exposed individuals. This could potentially demonstrate unique gene expression patterns that are upregulated or downregulated in response to vaccination and could serve as a way to identify vaccine induced protective efficacy. This approach could also be helpful in uncovering exact transcriptional signature biomarkers, which may not only predict vaccine effectiveness but might also associate with protection.

7.2 Concluding Remarks

This study highlights the importance of adopting a holistic approach when studying immunity to malaria and vaccine induced protection. The immune response to malaria is complex, and it follows a multi-step, complex interaction between the innate and adaptive arms of the immune system. To enhance our understanding of vaccine induced immunity, it is essential to adopt a multidisciplinary approach from now onwards. By doing so we can make significant strides in developing more effective vaccines and potentially identify an immune marker that can be correlated with protection against malaria.

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