



Developing a new anti-sporozoite malaria vaccine

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Authorship statement

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I worked with the Chief Investigator (CI) (Professor Adrian Hill) to design the study and alongside Dr Alison Lawrie, Rachel Roberts, Nguyen Tran, Ian Poulton and Fernando Ramos Lopez to set up the study. This involved preparation of the study documents for ethical and regulatory approvals, local approvals at all NHS sites, SOPs and training of clinical trial staff involved. The immunology oversight and the immunology laboratory work were carried out by Professor Katie Ewer and Duncan Bellamy respectively. Nick Edwards and his team were responsible for carrying out the qPCR on the malaria challenge samples for malaria diagnosis. Screening, enrolment, vaccinations and follow-up visits were conducted by myself, Dr Meera Madhavan, Dr Daniel Jenkin, Dr Hazel Morrison and by the CCVTM nursing team. Screening, enrolment, vaccinations and follow-up visits for participants enrolled at other Clinical Research Facilities were conducted by local study teams. Clinical oversight of the trial was performed by the CI, myself, Dr Meera Madhavan, Ian Poulton. I carried out the analyses of all the safety, immunogenicity and efficacy data presented in this chapter.

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The work in this chapter would not have been possible without all the clinical trial team at the KEMRI|Wellcome Trust, Kilifi.

Dr Mainga Hamaluba was the PI at this site and we designed the trial together with the CI, Professor Adrian Hill and Professor Philip Bejon.

I worked with the PI, Dr Alison Lawrie, Rachel Roberts, Irene Njau, Dr Samuel Sang and Marianne Munene to set up the study. This involved preparation of the study documents for ethical and regulatory approvals in Kenya as well as ethical approval in Oxford. I also performed local training for the study at the site in Kenya and thereafter, I provided remote support and trouble-shooting assistance while the local clinical trial team were responsible for screening, enrolment, vaccinations and follow-up visits.

The immunology oversight and the immunology laboratory work were carried out by Professor Katie Ewer and Duncan Bellamy respectively.

I carried out the analyses of all the safety and immunogenicity data presented in this chapter.

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I worked with the PI, Dr Alison Lawrie, Rachel Roberts, Dr Hamtandi Magloire Natama, Dr Athanase Some, Florence Ouedraogo, Toussaint Rouamba and Fernando Ramos Lopez to

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As I am blinded for this study, the main statistical analyses in this chapter for safety, immunogenicity and efficacy were performed by Nicola Williams and Matthew Cairns. Nicola Williams has produced the figures for the efficacy data and based on the data I was provided, I have produced the figures for the safety and immunology data. I carried out the statistical analyses for the additional analyses that did not require unblinding.

I have also written the two publications that have arisen from the work presented in this chapter with editorial input from Dr Hamtandi Magloire Natama, Professor Adrian Hill, Professor Halidou Tinto, Professor Katie Ewer, Nicola Williams and Matthew Cairns.

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Abstract

Malaria is one of the leading infectious causes of morbidity and mortality. There was an estimated 241 million malaria cases and 627,000 deaths in 2020 across 85 malaria endemic countries. When compared with data from recent years, this suggests that global progress has stalled, and that malaria mortality is increasing. There is an urgent need to develop new tools to combat this.

Significant progress has been made in malaria vaccine development, with the first malaria vaccine, RTS,S/AS01, achieving prequalification this year (2022). However, the current production capacity of RTS,S/AS01 may not, at least initially, meet the needs of the target population. At the same time, the WHO goal of developing a malaria vaccine with protective efficacy of $\geq 75\%$ against clinical malaria, for at-risk groups in malaria-endemic areas, over at least 2 years, has not yet been met.

R21 is produced by using recombinant HBsAg particles expressing the central repeat and the C-terminus of the *Plasmodium falciparum* circumsporozoite protein. R21 was initially developed at the University of Oxford and is now manufactured by the Serum Institute of India Pvt. Ltd (SIPL). R21 is combined with an adjuvant, Matrix-M, manufactured by Novavax AB, to produce the vaccine candidate R21/Matrix-M which targets *P. falciparum* malaria.

In this thesis, I describe the clinical development assessing safety, immunogenicity and efficacy of the R21/Matrix-M malaria vaccine candidate across a phase I/IIa clinical trial in UK adults (chapter 3); a phase Ib clinical trial in Kenya in adults, young children and infants (chapter 4); and a phase IIb clinical trial in infants in Burkina Faso (chapter 5). I evaluate the composition of the gut microbiome of infants living in malaria endemic areas (the target population for R21/Matrix-M) to assess if this is affected by any specific participant factors. I also assess if the baseline gut microbiome suggests any correlation with antibody responses generated by R21/Matrix-M, and if any association exists between the gut microbiome

composition and the total number of malaria episodes experienced at 12 months following vaccinations (chapter 6).

The clinical development phases of R21/Matrix-M have demonstrated that the vaccine is safe, immunogenic and protective against malaria. Importantly, efficacy of $\geq 75\%$ against clinical malaria was noted over 2 years in the target population of African infants, in the phase IIb trial, where vaccinations were administered prior to or at the start of the malaria season. The composition of the gut microbiome varied significantly according to age of the infant but had no impact on the antibody responses to the R21/Matrix-M malaria vaccine or the number of malaria episodes experienced by an infant.

In my conclusions in this thesis, I have reflected on some of the key issues beyond the science that affect deployment and implementation of a successful malaria vaccine. In order to make strides in eliminating malaria in sub-Saharan Africa, it is necessary for there to be cooperation amongst multiple stakeholders; developments in manufacturing and delivery of vaccines; and grassroots participation in vaccine programmes.

The results from these trials have led to a phase III trial across five African sites, in East and West Africa, evaluating the safety and efficacy of R21/Matrix-M in areas of differing malaria transmission in a larger number of infants and children. The aim is licensure of a safe, inexpensive vaccine, with high efficacy, that will significantly reduce the malaria disease burden.

Publications resulting from this work

(At the time of DPhil submission)

Dattoo MS, Natama MH, Somé A, Traoré O, Rouamba T, Bellamy D, et al. Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. *Lancet* (London, England). 2021;397(10287):1809-18. DOI: 10.1016/S0140-6736(21)00943-0. Epub 2021 May 5. PMID: 33964223.

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Abbreviations

AE	Adverse Event
Ab	Antibody
ACT	Artemisinin-based Combination Therapy
AESI	Adverse Event of Special Interest
ALT	Alanine Transaminase
AQ	Amodiaquine
βHCG	Beta human chorionic gonadotrophin
CHMI	Controlled Human Malaria Infection
CI	Chief Investigator
CRF	Case Report Form
CNS	Central Nervous System
CSP	<i>Circumsporozoite</i> protein
CTRG	Clinical Trials Research Governance
DAIDS	Division of AIDS Table for Grading the Severity of Adult and Paediatric Adverse Events
DC	Dendritic cells
DSMB	Data Safety and Monitoring Board
EC	Ethics Committee
ECG	Electrocardiogram
ELISA	Enzyme-Linked Immunosorbent Assay

ELISpot	Enzyme-Linked Immunosorbent Spot Assay
EU	ELISA units
EMA	European Medicines Agency
EPI	Expanded Programme of Immunisation
GCP	Good Clinical Practice
GMM	Genetically Modified Mosquitoes
GMP	Good Manufacturing Practice
GSK	GlaxoSmithKline Plc.
GTS	Global Technical Strategy
HBsAg	Hepatitis B surface antigen
HICS	High-income countries
HIV	Human Immunodeficiency Virus
ICF	Informed Consent Form
ICH-GCP	International Conference on Harmonisation – Good Clinical Practice
IgG	Immunoglobulin G
IM	Intramuscular
IMP	Investigational medicinal product
IPTp	Intermittent preventive treatment in pregnancy
IRSS	Institut de Recherche en Sciences de la Santé
IRS	Indoor residual spraying
IRSS	Institut de Recherche en Sciences de la Santé, Nanoro, Burkina Faso

ITN	Insecticide-treated net
KEMRI	Kenya Medical Research Institute
LLIN	Long-lasting insecticidal nets
LMIC	Low-middle income countries
LSM	Local Safety Monitor
LSHTM	London School of Hygiene & Tropical Medicine
MAbs	Monoclonal antibodies
ME	Multiple epitope
MedDRA	Medical Dictionary for Regulatory Activities
MM	Matrix-M adjuvant
MMVC	Multi-stage Malaria Vaccine Consortium
MHRA	Medicines and Healthcare products Regulatory Agency
MPAC	Malaria Policy Advisory Committee
MVIP	Malaria Vaccine Implementation Programme
NANP	Asparagine – Alanine – Asparagine – Proline
NIHR	National Institute for Health Research
NMP	National Malaria Programme
OxTREC	Oxford Tropical Research Ethics Committee
PBS	Phosphate-buffered saline
PCoA	Principal Coordinate Analysis
PfSPZ	Attenuated, aseptic, purified, cryopreserved sporozoites

PI	Principal Investigator
PPB	Pharmacy and Poisons Board
qPCR	Quantitative polymerase chain reaction
RA	Regulatory authority
RCT	Randomised-controlled trial
RDT	Rapid Diagnostic Test
RTS,S/AS01	Malaria vaccine (Mosquirix™) manufactured by GSK
R21/MM	Malaria vaccine manufactured by Serum Institute of India, adjuvanted with Matrix-M
SAE	Serious Adverse Event
SAF	Sir Alexander Fleming Building, Imperial College
SAGE	Strategic Advisory Group of Experts
SERU	Scientific Ethics Review Unit
SFC	Spot forming cells
SIPL	Serum Institute of India Private Ltd
SMC	Seasonal malaria chemoprevention
SOP	Standard operating procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
SP	Sulfadoxine/pyrimethamine
TOPS	The Over-Volunteering Prevention System
TLR	Toll-like receptor

TRM	Tissue-resident memory
VE	Vaccine efficacy
VLP	Virus-like particle
WHO	World Health Organisation
WRAIR	Walter Reed Army Institute of Research

1 Introduction

1.1 Burden of malaria

1.1.1 Global trends

There were an estimated 241 million malaria cases and 627,000 deaths in 2020 across 85 malaria endemic countries. Seventy seven per cent of total malaria deaths were among children aged under 5 years (1).

The WHO African Region accounted for the highest proportion of malaria cases and deaths: 95% of malaria cases (228 million) and 96% of malaria deaths (602,000). This was then followed by the WHO South-East Asia Region, which contributed to 2% of global malaria cases and 1.4% of global malaria deaths. After this, other regions affected by malaria are the WHO Eastern Mediterranean Region, the WHO Western Pacific Region and the WHO Region of the Americas (1).

Progress in reducing the burden of malaria has stalled. Global malaria incidence declined by 27% between 2000 and 2015 but by less than 2% between 2015 and 2019. Although there were 204 million cases in 2000 compared with 229 million cases in 2019, malaria incidence reduced from 368 to 222 cases per 1000 population at risk. This reflects the increase in population: in the WHO African Region there were approximately 665 million people in 2000 compared with 1.1 billion in 2019. Malaria case incidence increased to 232 cases per 1000 population at risk in 2020 but this increase was associated with disruption to services during the COVID-19 pandemic. (1, 2).

Malaria is caused by five species: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (3). *P. falciparum* causes the highest mortality and is most prevalent in the WHO African Region. *P. vivax* is the dominant malaria parasite in most countries outside of sub-Saharan Africa. (4).

1.1.2 High-risk groups

1.1.2.1 Children

In 2020, children under 5 years accounted for 77% of all malaria deaths worldwide. They are one of the most vulnerable groups affected by malaria (1). They are more susceptible to malaria infection as they have not yet developed acquired immunity to the disease. Children who survive malaria may suffer from long-term effects as repeated illness and reduced appetite may restrict play, educational opportunities and social interaction leading to slower development (5). Those with severe malaria are at higher risk of developing long-term neurological impairment (6).

Mortality rates in children, of which malaria causes a significant proportion, are higher in poorer households and this causes significant economic burden when compared with households that are less poor (7).

1.1.2.2 Pregnant women

During pregnancy, malaria infection carries significant risk for the mother, her foetus and the new born child. Complications for the mother include severe disease and death, placental sequestration of *Plasmodium* leading to anaemia, and increased risk of death before and after childbirth. Malaria can contribute to stillbirths and preterm births. Placental malaria infection can cause poor foetal growth and low birth weight and is a major risk factor for perinatal, neonatal and infant mortality. In areas of moderate to high transmission, WHO recommends vector control and the use of intermittent preventative treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) as part of antenatal care (2).

Across the WHO African Region, there were an estimated 33.8 million pregnancies in 2020 and 34% (11.6 million) of these were exposed to malaria infection. It is thought that malaria infection resulted in 819,000 children with low birth weight (1).

1.1.2.3 Other populations

Patients with HIV/AIDS, non-immune migrants, mobile populations and travellers are all considered to be at a higher risk of contracting malaria and developing severe disease (4).

1.1.3 WHO global technical strategy (GTS) for malaria 2016-2030

The GTS provides a framework to guide and support all malaria endemic countries as they aim to achieve malaria control and elimination. Global targets include reducing malaria case incidence and mortality rates by at least 40% by 2020 and 90% by 2030 from a 2015 baseline, eliminating malaria in at least 35 countries by 2030, and preventing malaria in countries that are malaria-free (8).

The GTS 2020 milestones were not achieved globally despite significant progress. In 2015, there were 93 countries that were malaria endemic, and 32% of these met the 2020 GTS morbidity milestone. All countries in the WHO South-East Asia Region met the 2020 GTS milestones for both morbidity and mortality (1, 2).

1.1.4 Elimination

The path to malaria-free status is characterised by four distinct phases. These are control, pre-elimination, elimination and the prevention of reintroduction. Malaria elimination is defined as the interruption of local transmission of a specified malaria parasite in a defined geographic area. Continued measures are required to prevent the reestablishment of transmission (9). More countries are moving towards trying to achieve malaria elimination. When a country has had three consecutive years of zero indigenous malaria cases, they are eligible to apply for the 'WHO certification of malaria elimination' (4). Since 2000, 12 countries have been certified by the WHO as malaria-free. No country that was certified malaria free between 2000 and 2020 has seen a resurgence of malaria transmission (1, 2).

1.2 *Plasmodium falciparum* malaria

1.2.1 Life cycle

Malaria involves cyclical infection of humans and female *Anopheles* mosquitoes. In order for malaria transmission to occur, three components of the malaria life cycle are required. These are *Anopheles* mosquitoes (to feed on humans, and in which the parasites can complete the 'invertebrate host' part of their life cycle), humans (who can be bitten by the mosquitoes, and in whom the parasites can complete the 'vertebrate host' part of their life cycle) and malaria parasites (10, 11).

As mentioned above, there are two hosts in the malaria parasite life cycle (Figure 1A). When a malaria-infected female *Anopheles* mosquito takes a blood meal, they inject sporozoites into the human host. These infect hepatocytes and multiply asexually over 7-10 days, maturing into schizonts. There are no clinical signs or symptoms at this point. These schizonts rupture and release merozoites which invade the human red blood cells and multiply further until these cells burst, allowing more red blood cells to become infected. This cycle repeats and clinical manifestations such as fever occur each time the parasite breaks free and invades more red blood cells. Some of these infected blood cells leave this cycle of asexual multiplication and the merozoites differentiate into gametocytes (sexual erythrocytic stages). These male and female gametocytes are ingested by an *Anopheles* mosquito when they take a blood meal. In the mosquito's stomach, the male and female gametocytes generate zygotes. These become elongated and motile to form ookinetes and invade the midgut wall of the mosquito where they develop into oocysts, where thousands of active sporozoites develop. These oocysts grow, rupture and release sporozoites which travel to the mosquito's salivary glands (10, 11). In turn, with a new blood meal, these sporozoites are injected into a new human host, perpetuating the malaria life cycle (Figure 1A).

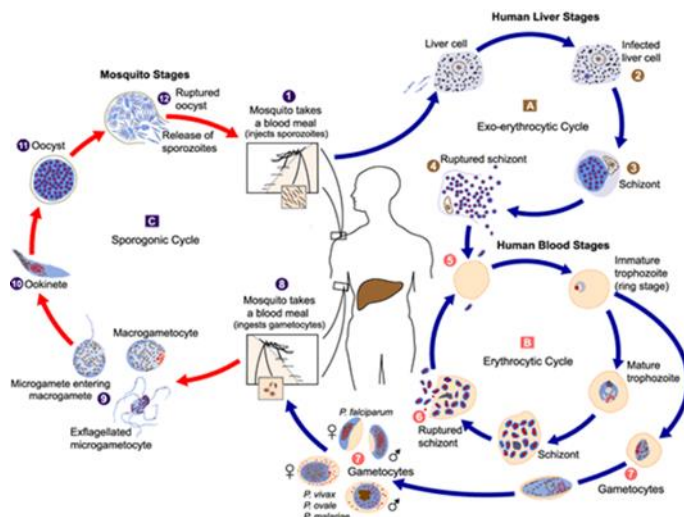


Figure 1A: The malaria parasite life cycle involving both hosts (10)

1.2.2 Disease

Symptoms of malaria can be non-specific and mimic most common viral illnesses. They include headache, fatigue, myalgia and arthralgia. These initial symptoms may then be followed by fever, chills, vomiting, anorexia and malaise. Young children may present with lethargy, poor feeding and cough. A quick, full recovery is expected if prompt antimalarial treatment is given and there are no signs of organ dysfunction. However, if treatment is delayed or ineffective, parasite burden will likely increase and disease progression to severe malaria may occur (12).

Severe malaria can present as cerebral malaria (coma), a metabolic acidosis, hypoglycaemia, acute renal failure, pulmonary oedema, or with severe anaemia. At this stage, if untreated, it will likely be fatal (12).

1.3 Current strategies for tackling malaria

For the control of malaria, WHO recommends a number of interventions which are discussed below. Unfortunately, coverage of most of these programmes have not met the target recommendations. The total funding for malaria control and elimination was around US\$ 3.3 billion in 2020 (1).

1.3.1 Insecticide-treated nets (ITN)

The aim of an ITN is to kill or repel any mosquitoes that come into contact with the insecticide of the net. WHO recommends ITNs in all malaria endemic settings, and these should be long-lasting insecticidal nets (LLINs) with pyrethroid. Pyrethroid-PBO (piperonyl butoxide) nets should only be used in areas where the main malaria vector exhibits full or intermediate-level pyrethroid resistance (13). Data from large-scale field trials and transmission models show that if coverage $\geq 50\%$ of ITNs is achieved, it is expected to result in community-wide protection of non-users in most settings (14, 15). They are most effective where the mosquitoes bite mostly at night and people can be under their nets (13). Between 2000 and 2015, ITNs are thought to be responsible for an estimated 68% of the malaria cases averted (16). Published recommendations by the WHO in 2017 suggested that mass free ITN distribution should take place through multiple initiatives including antenatal care clinics and the Expanded Programme on Immunization (EPI). Mass campaigns should distribute one ITN for every 2 people at risk of malaria every three years, with continuous distribution through health clinics, faith and community-based networks and workplace programmes, supplementing the gaps (17). In 2020, 65% of households in sub-Saharan Africa had at least one ITN (1).

1.3.2 Indoor residual spraying (IRS)

This involves applying insecticide to potential mosquito resting surfaces including internal walls, eaves and ceilings of houses and structures. IRS, using a product that has been prequalified by WHO is recommended in all malaria endemic settings (13). There have been few RCTs performed on IRS with some studies suggesting significant benefit in reducing malaria transmission while others did not. This also depended on factors such as use of pyrethroid-like IRS and concomitant use of ITNs (18). In 2020, less than 6% of the population in each WHO region were protected by IRS (1).

1.3.3 Seasonal malaria chemoprevention (SMC)

SMC involves monthly administration of sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) to children under 6 years of age in areas with highly seasonal malaria transmission. This

should be given at the start of the malaria transmission season, with a maximum of four doses given during the same season (19). Data from seven studies conducted in highly seasonal malaria areas suggested that the use of SP+AQ monthly for up to four months per season prevented approximately 75% of all malaria episodes, 75% of severe malaria episodes and likely reduced the incidence of moderately severe anaemia (19). In 2019, the number of countries that implemented SMC in the Sahel were 13. Within these countries, approximately 31.2 million children were targeted in 2020 (1).

1.3.4 Intermittent preventive treatment in pregnancy (IPTp)

WHO recommends a number of interventions to help prevent malaria during pregnancy including use of ITNs, prompt and effective treatment for malaria case management and use of SP in areas of moderate to high transmission of *P. falciparum* (12). Dosing with SP in pregnancy (IPTp-SP) should start in the second trimester, provide at least three doses, at least one month apart (20). Three or more doses has been associated with reduced maternal parasitaemia, increased mean birth weight and decreased numbers of low birth weight infants (21). By 2020, estimated coverage was 32% receiving three doses. This is well below the target of $\geq 80\%$ (1, 2).

1.3.5 Rapid diagnostic testing (RDT)

RDTs detect parasite-specific antigens from a finger-prick blood sample. They do not require electricity or special equipment to be used but should be supported by a quality assurance programme. They can aid parasitological testing and can be used in areas where specialist skills or microscopy is limited, or as a quick diagnostic test (20). As antimalarial treatment should be for positive malaria cases only, RDTs can prompt reassessment for other causes of fever and avoid misuse of artemisinin-based combination therapy (ACT), potentially reducing treatment resistance (20). In 2020, 419 million RDTs were sold by manufacturers and 275 million disseminated by national malaria programmes (NMPs), mostly in sub-Saharan Africa (1).

1.3.6 Artemisinin-based combination therapies (ACT)

Suspected malaria should be confirmed by a parasitological test where available. ACT 3-day regimes are recommended by WHO for the treatment of *P. falciparum* in malaria endemic countries. ACTs have been assessed as being safe and have contributed significantly to reducing malaria morbidity and mortality globally. Combination therapy is advocated to help prevent or delay resistance and the 3-day course is thought to provide adequate efficacy, while promoting good adherence to reduce risks of drug resistance arising from incomplete treatment (20). Resistance to artemisinin's has increased recently in South-East Asia although several new combinations are in development (20). Between 2015 – 2019, household surveys showed that a median estimate of 81% of children under 5 received treatment with ACTs when care was sought. However, only 37.8% of children for whom care was sought, had a diagnosis of malaria confirmed by finger or heel prick test. This would suggest that many children were given ACTs without a parasitological diagnosis (2).

1.4 Further strategies in development for tackling malaria

1.4.1 Monoclonal antibodies (mAbs)

This involves the passive immunisation with functional antibodies as prophylactic, therapeutic or both. In the case of malaria, they can be used prophylactically (22). Prophylactic antibody-based vaccines have achieved high levels of protection through blocking infection in CHMI studies in malaria naïve volunteers (23) but this has not been the case in endemic settings (24, 25). It is thought that this may be due to pre-existing malaria-specific immune modulation in previously infected individuals. A mAb would be helpful as it does not rely on the individual's immune system to produce antibodies. With this in mind, mAbs could provide short-term protection from malaria to populations at high risk of clinical malaria or those who are less able to develop immune responses.

Advantages to mAbs include their good safety profile, effective dose delivery in a single dose and that they are effective very soon after administration. Several monoclonal antibodies are currently in clinical development targeting each stage of the malaria life cycle, however, cost

may be a barrier to further development. A single mAb targeting a single epitope is not thought to achieve sufficient efficacy and there are concerns over resistance with a single antigen approach. Therefore, it is thought that combinations of mAbs that target multiple epitopes and proteins are needed. These are difficult to produce, and produce at large-scale, and funding to contribute to progressing this process is limited (22).

The use of mAbs may be a future consideration for reducing malaria morbidity and mortality in adults. Their role could be in specific 'at risk' groups including pregnant or lactating women (1). Clinical development is ongoing for a number of mAb candidates.

1.4.2 Genetically modified mosquitoes (GMM)

The development of GMM are in progress to prevent the transmission of malaria. GMMs are regarded as a new, strong and cost-effective tool to combat malaria. Technologies are based on mosquito 'population suppression' or 'population replacement'. A WHO guidance document has recommended a phased testing pathway and progress through the phases will be dependent on regulatory and ethical approvals, efficacy and safety endpoints and social acceptance (26).

Advantages to GMM technologies include providing area-wide protection that is not dependent on socioeconomic level or access to medical facilities, no additional change in an individual's behaviour required for effectiveness. They can be developed for both urban and rural environments and can be highly specific unlike broad-spectrum insecticides, therefore, reducing potential environmental and health hazards (26). However, disadvantages have been identified including concerns over ecosystem interactions causing harm to animal or human health and water quality, stability and predictability of effect, limited possibilities for control if adverse effects are noted, and the development of resistance over time (26).

Several research forums have been working on developing genetic control tools including gene-drives to suppress mosquito populations transmitting malaria. A stepwise approach for field testing includes carefully planned field studies, informed by laboratory, insectary,

modelling and biosafety studies. The first field release of GMMs was conducted in 2019 in Burkina Faso. These were non-gene drive and a genetically modified sterile male strain of *Anopheles coluzzi* with the aim to estimate the daily survival rate of sterile male mosquitoes, their ability to participate in swarming activities, estimate the target population at the time of release and assess their dispersal in the release area. Recapturing commenced within two hours of mosquito release and collections at specified time points continued over seven months. Modelling data showed that the genetically modified male strain was less mobile than their wild type siblings and had lower survival (27). As this was the first field trial, this was a key milestone in the development of GMMs to help understanding of requirements, develop knowledge and skills to enable such trials.

While much work is still to be done, the aim would be to develop and release a GMM with gene drive technology. This would enable control of malaria and persistence for generations (27, 28).

1.4.3 Other methods of vector control in development

Due to widespread use, pyrethroids are becoming less effective. A new ITN combining pyrethroid with a repurposed insecticide (chlorfenapyr) has a different mode of action compared with pyrethroids alone and the mixture of these two is helping combat insecticide resistance. Three further ingredients for ITNs are in late-stage research or progressed to full development (29).

Larviciding, eave tubes and targeted sugar baits are designed to attract and kill mosquitoes. Sugar bait aims to address the threat of outdoor biting and is expected to achieve prequalification in 2025 (29).

1.5 *P. falciparum* malaria vaccine development

1.5.1 Malaria vaccine candidates to protect against *P. falciparum*

Malaria vaccine candidates have been in development for many years. However, no candidate has been found as yet to achieve high-level efficacy and mass deployment in sub-Saharan

Africa (2). The development of a malaria vaccine is complex due to the parasite and the multiple stages of its life cycle (30, 31), it's extensive antigenic variation (32) and the difficulty in understanding parasite interaction with the human immune system (33). *P. falciparum* can induce immunity, mostly at the blood-stage of infection, and as demonstrated by findings in areas of high transmission, immunity to severe disease is acquired in childhood, followed by immunity to mild disease acquired in late adolescence (34, 35). Meanwhile in adults, immunity acquired over time can lead to protection against disease, but sterile immunity to blood-stage infection rarely develops (34, 36).

Research funding has also been limited for malaria vaccines and a year on year increase was suggested in order to make substantial progress towards the targets set out in the Malaria Vaccine Technology Roadmap (37).

The current main malaria vaccines in clinical development are discussed below according to the stage of the *P. falciparum* life cycle that they target (Figure 1B).

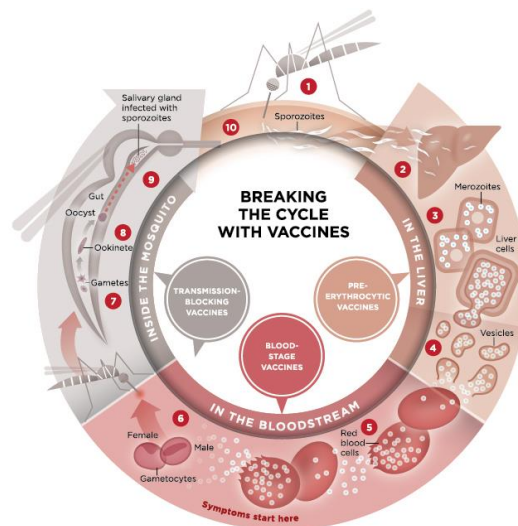


Figure 1B: Malaria vaccines explained according to stage of *P. falciparum* life cycle targeted (11).

1.5.1.1 Pre-erythrocytic vaccines

These vaccines target antigens in the sporozoite and liver stages of the *P. falciparum* life cycle and aim to clear parasites before release into the bloodstream and clinical disease. This is an

attractive target as parasite load is significantly lower compared to the number of merozoites following hepatic schizont rupture. These vaccines are designed to either induce antibodies against surface antigens that clear sporozoites or block them invading hepatocytes, or to induce T cell responses that attack infected hepatocytes (38).

The circumsporozoite protein (CSP) is a major focus of pre-erythrocytic vaccine development as it is key for sporozoite maturation and hepatocyte invasion in humans (39). RTS,S, the current leading malaria vaccine candidate, contains CSP comprising the central repeat and C-terminal regions fused with hepatitis B surface antigen (HBsAg), with a four-fold excess of HBsAg molecules that are not fused to any malaria sequence (40). RTS,S will be discussed in more detail later. It was formulated in AS01 adjuvant and was the first malaria vaccine candidate to complete phase III trials demonstrating efficacy against clinical malaria episodes. Highest efficacy noted was 36.3% in a four dose regime in 5-17 month olds (41). Due to concerns regarding the vaccine schedule (42) and potential safety signals (42-44), pilot implementation trials have been taking place in Malawi, Ghana and Kenya (45). There are other ongoing trials asking if seasonal vaccination with annual boosters improve efficacy in highly seasonal settings and if co-administration with SMC increases efficacy (46), as well as the potential of fractional dose regimes, which increased efficacy in malaria naïve adults against CHMI (47) but not in children in malaria endemic areas (48).

R21 has been developed at the Jenner Institute, University of Oxford (Figure 1C). This is similar to RTS,S but R21 particles are formed from a single CSP-HBsAg fusion protein resulting in a much higher proportion of CSP. In pre-clinical studies, R21 induced antibody responses at low doses and elicited sterile protection against sporozoite challenge when administered with adjuvants (49). This vaccine will be discussed later in further detail (section 1.7.1).

Thrombospondin-related adhesion protein (TRAP) is important for sporozoite motility and hepatocyte invasion in humans. This is fused to a multi-epitope (ME) string of known T-cell epitopes in the ME-TRAP vectored vaccine (50). Using the viral vector heterologous prime-

boost approach to induce antigen-specific T cells to target infected hepatocytes, ChAd63 ME-TRAP followed by MVA ME-TRAP eight weeks later was safe and immunogenic in malaria naïve adults as well as adults and children in malaria endemic countries (51-54). Modest protection was noted against sporozoite challenge with vaccine efficacy of 21% and delay to parasitaemia in a further 36%. ELISPOT response levels correlated with efficacy in CHMI trials. This sterile protection was associated with a peripheral CD8⁺ T cell response (55). In a small trial in adults in Kenya, this vaccine strategy significantly reduced the risk of infection by 67% over 8 weeks (56).

Pre-erythrocytic vaccines encompass strategies that induce humoral and cellular responses. While a CHMI study assessing the use of both strategies (administration of R21/Matrix-M and ChAd63-MVA ME TRAP) in combination did not improve on efficacy (57), this may be a future direction of work. Further antigens inducing T cells to target infected hepatocytes need to be identified to try and aid this strategy or it may be of value to combine antigens from different stages of the malaria life cycle to induce both antibodies and T cells. Equally, evidence has suggested that priming CD8⁺ T cells in peripheral tissue, followed by targeting them to the liver, via intravenous vaccination, resulted in a significant increase of antigen-specific CD8⁺ T cells locally in the liver leading to protection in mice (58). This prime-target strategy has been tested in phase I/IIa trials with modest efficacy demonstrated (unpublished data).

1.5.1.2 Whole sporozoite vaccines

Sanaria Inc., a biotechnology company working on malaria vaccine development, produced a whole sporozoite vaccine by harvesting sporozoites (PfSPZ) from the salivary glands of aseptic mosquitoes infected by cultured laboratory parasites. This was then followed by purification, vialling and cryopreservation in liquid nitrogen (59). PfSPZ are attenuated by different approaches to produce vaccines: radiation attenuated (PfSPZ vaccine), chemoattenuation involving concomitant administration of antimalarials in vivo (PfSPZ-CVac), and genetic attenuation involving deletion of genes to prevent liver-stage development (PfSPZ-GAP) (60).

Whole sporozoite vaccines express many malaria antigens and are thought to induce extensive immune responses, including CD4⁺ and CD8⁺ T cells as well as antibody responses (61).

The PfSPZ vaccine requires direct venous inoculation; dose dependent sterile protection against challenge with sporozoites to high levels has been noted in malaria naïve adults (62). In the malaria endemic setting of Mali, in adults, five doses of PfSPZ vaccine (2.7×10^5 PfSPZ) reduced the risk of new *P. falciparum* infections by 52% in a time-to-event analysis over 6 months following the last dose (63). More recently, another trial in Mali assessed a three-dose regime of PfSPZ vaccine in adults and efficacy was similar at 51% over the same time course (64). A three-dose regime was also tested in adults in Burkina Faso and efficacy at six months was 46% (65). However, it should be noted that artesunate (and amodiaquine in Mali) were administered prior to first and last vaccinations to clear pre-existing parasitaemia (64, 65). No significant vaccine efficacy at 6 months was noted in infants in western Kenya when administered three doses of PfSPZ vaccine (66). Results are awaited for additional field trials assessing three dose regimes in infants for the PfSPZ vaccine.

In a CHMI trial in malaria-naïve adults assessing PfSPZ-CVac, vaccine efficacy of 77% was noted when three vaccines and three doses of chloroquine were given over the course of one month (67). However, in a field trial in Mali, PfSPZ-CVac, when participants were given three doses over 2 months with chloroquine prior to first vaccination, weekly during vaccinations and seven daily doses of artesunate after the third vaccination, they were not significantly protected against malaria infection over six months (68). PfSPZ-GAP vaccines are still undergoing early phase trials in malaria naïve adults.

It has been encouraging that the PfSPZ vaccination strategy has evolved with a shorter dosing schedule. The main challenges with these vaccines are the logistics and cost of the cold chain which currently requires liquid nitrogen, the route of administration being intravenous unlike other EPI vaccines, and the need and cost of large-scale manufacturing. At the same time, drugs to clear parasitaemia have been used where vaccine efficacy has been noted and this

also needs to be thought about from a cost and logistical point of view when administering to larger populations.

1.5.1.3 Blood-stage vaccines

These vaccines target the stage of development where asexual parasites are undergoing repeated multiplication cycles in erythrocytes and clinical disease and symptoms are seen. This stage of the life cycle was an appealing target as previously in African children, parasitaemia was cleared through passive transfer of IgG purified from semi-immune African adults (69, 70).

Previous vaccine candidates targeted the antigens MSP1, AMA1, EBA-175, GLURP and MSP3 (38). Trials with these antigens aimed to induce high antibody titres against merozoite surface antigens to impair parasite invasion or, in the case of MSP3, to mediate antibody cellular inhibition (38). However, while antibodies against merozoite surface antigens were induced, this did not correlate with or lead to significant protection (71-73).

While AMA1 demonstrated poor efficacy, when complexed with RON2, it generates more potent anti-invasion antibodies. In monkeys, compared with AMA1 alone, significantly higher protection was noted against heterologous blood-stage challenge (74). Due to extensive sequence variation, future studies will need to assess the number of alleles required to confer effective immunity (38).

The candidate PfRH5 binds the essential red cell receptor basigin on the erythrocyte surface during invasion. PfRH5 has induced neutralizing antibodies in preclinical studies and when combined with viral vectors or protein adjuvants, was associated with protection against heterologous challenge (75, 76). In humans, when PfRH5.1 recombinant protein vaccine was combined with AS01_B, it was safe, and produced significantly increased antibody duration in adults when a third, fractional dose was administered. Following CHMI with PfRH5.1/AS01_B, a significant reduction in parasite growth rate was noted (77). Human monoclonal antibodies generated from individuals vaccinated with PfRH5 have been used to identify epitopes targeted

by antibodies (78), and it is hoped this will inform the design of new Rh5 immunogens that focus antibody response even more on neutralising and potentiating epitopes (38, 78).

Aside from merozoite targets, other blood-stage vaccine antigens have been investigated. These have included the variant surface antigen family PfEMP1 which is thought to mediate parasite sequestration and is a target of naturally acquired protective antibody (79). Due to its size, highly polymorphic sequence and structure, it has not been developed further as yet. An erythrocyte surface protein called PfGARP has also been described as a target of protective antibodies and in pre-clinical studies, PfGARP vaccines conferred partial protection against *P. falciparum* challenge (80).

Challenges in developing vaccines targeting this stage of the life cycle include the large number of parasites that need to be targeted, the fact that there is a very brief period of seconds when merozoites are accessible to antibodies between passing between erythrocytes, antigenic polymorphism, and redundant invasion pathways (38).

Chemically attenuated blood-stage parasites (CAP) have induced homologous and heterologous immunity in mice (81) and in humans CAP were well-tolerated in malaria-naïve individuals, where they induced cellular responses (82). Similar to the whole sporozoite vaccines, as well as safety, logistical and financial challenges will need to be addressed if this strategy is to progress.

1.5.1.4 Placental malaria vaccines

Placental vaccines target chondroitin sulfate A (CSA) binding parasites. These exclusively sequester in the placenta and these natural antibodies are associated with placental malaria protection (83). Placental parasites express VAR2CSA that binds CSA (84) and there are currently two vaccine candidates based on this model that have completed first-in-human trials. These include PAMVAC and PRIMVAC which have both been found to be safe, immunogenic and induce functional antibodies against homologous parasites (85, 86). However, the latter saw limited cross-reactivity against heterologous VAR2CSA variants but it is hypothesised that

different vaccine schedules, doses and combination with other VAR2CSA-based vaccines may improve this (38, 86).

1.5.1.5 Transmission-blocking vaccines

These vaccine candidates target the sexual stages of the *P. falciparum* life cycle, aiming to induce antibodies that stop sporozoite development and interrupt transmission through the mosquito vector (87). The four leading candidates are Pfs230, Pfs48/45, Pfs25 and Pfs28. These first two are gametocyte surface proteins and the latter two are ookinete surface proteins (38).

Due to equal or higher transmission-blocking activity compared with the other antigens, Pfs25 has been focused on, and Pfs25-EPA formulated with Alhydrogel® was safe in humans and induced antibodies blocking transmission to mosquitoes in membrane feeding assays. This did require four doses in humans and antibody titres and activity waned quickly (88). However, now Pfs230 and its comparison and combination with Pfs25 is in ongoing clinical trials (ClinicalTrials.gov ID NCT02334462). The use of different adjuvants is also being assessed and results are awaited (ClinicalTrials.gov ID NCT02942277, NCT03917654). It is expected that Pfs48/45 will enter clinical development in humans soon (38).

The main challenges currently with these and other malaria vaccines is that very high antibody responses are required to achieve any protection, and it has been even more difficult to maintain high levels of antibodies and to achieve herd immunity. Additionally, they must have an excellent safety profile as they do not prevent infection or clinical symptoms in the host, and therefore, are not of direct benefit to the individual (38).

1.5.2 Evaluating malaria vaccine candidates

I have outlined above, just a small number of the malaria vaccine candidates that have been identified, the progress, and the challenges associated with them. Difficulty also arises with the clinical development of these vaccines as they are usually developed pre-clinically in non-endemic malaria areas and begin early-phase trials in these places. The Controlled Human

Malaria Infection (CHMI) is a recognised valuable tool in the field of malaria vaccine and drug development for evaluating these products (89).

CHMI involves deliberate infection with malaria parasites by inoculation of sporozoites by mosquito bite, or by direct injection of sporozoites or parasitized erythrocytes. Liver and blood-stage infection can ensue with the inoculation of sporozoites while only blood-stage infection can develop with parasitized erythrocytes (89). Deliberate malaria infection with malaria parasites in humans was initially used in the treatment of neurosyphilis (90) and as a potential treatment for HIV (91). It was then used as a tool to evaluate antimalarial drugs and has become increasingly recognised to provide a safe model for *in vivo* assessment of the efficacy of malaria vaccine candidates (89).

Following phase I studies assessing safety and immunogenicity, CHMI can be used in phase IIa studies, in non-endemic malaria areas to try and establish proof-of-concept efficacy data, prior to embarking on costly phase IIb field efficacy trials in malaria endemic regions. This model involves study participants receiving bites of *Plasmodium*-infected insectary-raised mosquitoes. This method has been standardised over many years with infection achieved in malaria-naïve individuals through five laboratory-reared mosquitoes (92). Challenges with this model for clinical trial teams include the requirement for secure transportation of infected mosquitoes, access to an insectary, entomological expertise and precise timing of mosquito rearing and infection to coincide with vaccination schedules (92). Also, while the number of sporozoites can be measured in each salivary gland, this is a poor predictor of the number actually injected during a bite as this is highly variable (93). Another requirement is access to malaria parasites with a known sensitivity pattern to enable adequate antimalarial treatment to be initiated (89).

Aseptic, purified, cryopreserved *P. falciparum* sporozoites manufactured by Sanaria Inc., are infective *in vivo* when injected, and following optimisation of methodology, have been used for the CHMI model also (94). This results in a consistent sporozoite inoculum and aids direct

comparison of CHMI sporozoite challenge studies (95). As mentioned above, it is also a vaccination strategy being assessed.

Challenges pertaining to the CHMI model include the fact only a limited number of defined *P. falciparum* strains are available for use to assess vaccine candidates (96), development of the model is limited with other *Plasmodium* species and that CHMI models need to be established in malaria endemic areas. The latter would not only encourage capacity building, but trial participants would have similar genetic profiles to the target population, and prior malaria exposure and immunity and its effects on vaccine efficacy can be investigated (89).

CHMI is a useful clinical tool that can be used for evaluating new vaccine candidates, as well as an immunisation strategy and to gain a better understanding of human immune responses to malaria parasites. However, there is still the need to develop this model further by addressing the challenges and capacity for it to be used in multiple centres globally to help improve vaccine candidates (89).

1.5.3 Next-generation malaria vaccine candidates

To date, the pre-erythrocytic vaccine candidates have demonstrated the most success. There are lessons to be learned from what has been achieved so far in malaria vaccine development. Next-generation vaccines which improve on existing strategies, or develop new strategies, are required. This includes enhancing immunity by combining antigens from various stages of the life-cycle for a multi-stage vaccine. Aside from this, strategies to induce more durable and functional antibody responses, and likewise more durable and effective liver tissue-resident memory (TRM) T cells need to be considered. Alterations to immunogen design, delivery platform, methods of administration and vaccine schedules have the potential to provide more robust responses in future vaccine candidates (97).

1.5.4 Future aims of a malaria vaccine

Strategic goals to achieve by 2030, outlined in the Malaria Vaccine Technology Roadmap, included the development of a malaria vaccine protecting against *P. falciparum* clinical malaria

with efficacy of $\geq 75\%$, suitable for administration in malaria endemic areas. Protection must be demonstrated across ≥ 2 years and booster doses should be administered no more frequently than annually. Vaccines should also be developed to substantially reduce transmission which are then suitable for administration through mass vaccination campaigns (98).

Malaria vaccines should complement the existing malaria interventions (99). In order to ensure that malaria vaccines are introduced and implemented as quickly as possible, novel regulatory strategies need to be developed to expedite approval while maintaining quality and safety. In line with this, strategies should be developed to address post-approval pharmacovigilance and effectiveness in malaria endemic regions (98). It is important that there is access to low-cost manufacturing under Good Manufacturing Practice (GMP) for late-stage development and commercial production of malaria vaccines, as well as support for malaria vaccine development and implementation through investment strategies (98), so that vaccines are not only able to be manufactured at large-scale but also are affordable for the target population, mainly based in low and middle income countries.

EPI aims for universal access to childhood vaccines (100). This infrastructure provides contact with healthcare at certain time points in a child's life and it would be of benefit for a malaria vaccine to be administered at similar time points within the programme. Equally, EPI vaccines are transported at a $\sim 4^{\circ}\text{C}$ cold chain and administered orally, intramuscularly or subcutaneously (101); therefore, a malaria vaccine should aim to also meet these requirements.

1.6 The most advanced malaria vaccine candidate - RTS,S/AS01 phase III clinical trial and progress

1.6.1 Overview of trial

Between March 2009 and February 2011, young infants, aged 6-12 weeks, and children, aged 5-17 months, were enrolled across 11 sites in seven countries in sub-Saharan Africa for the phase III trial assessing the efficacy and safety of RTS,S/AS01 malaria vaccine. Participants

were randomised to receive four doses of RTS,S/AS01 at 0, 1, 2 months and a booster dose at 20 months, three doses of RTS,S/AS01 at 0, 1, 2 months and a booster dose of a control vaccine (Menjugate for infants and VeroRab for children) at 20 months, or the control vaccine at 0, 1, 2 months with a control booster dose at 20 months. Cases of clinical and severe malaria were recorded through passive case detection. SAEs were recorded through the trial, and local and systemic reactogenicity within seven days after vaccination in the first 200 participants enrolled at each centre (41, 102, 103).

1.6.2 Safety and efficacy results

The modified intention-to-treat analyses included 8922 children and 6537 young infants. Baseline characteristics were similar across the three study groups but varied according to site. Children were followed up over median of 48 months and young infants a median of 38 months. Vaccine efficacy (VE) was highest in the groups that received 3 doses and a booster of RTS,S/AS01 when assessing the primary case definition of a fever $\geq 37.5^{\circ}\text{C}$ and 5000 parasites/ μl . In children VE was 36.3% (95% CI 31.8-40) and in the young infants, it was 25.9% (95% CI 19.9-31.5). VE against severe malaria with this vaccine regime was 32.2% (95% CI 13.7-46.9) in children and 17.3% (95% CI -9.4-37.5) in young infants (41).

Overall RTS,S/AS01 was more reactogenic than the control vaccine but grade 3, severe adverse events, were uncommon. Pain and fever were the most common symptoms reported with RTS,S/AS01. The frequency of most SAEs was balanced between the groups. Meningitis was reported as an SAE in 22 children, 21 of these being in the two malaria vaccine groups and one of these in the control group (41, 102).

Generalised convulsive seizures within seven days of vaccination were all associated with history of fever. In children, the incidence of these was 1.04 per 1000 doses (95% CI 0.62-1.64) in the RTS,S/AS01 group compared with 0.57 per 1000 doses (95% CI 0.19-1.34) which received the rabies vaccine giving a risk ratio of 1.8 (95% CI 0.6-4.9). In the young infants, the incidence was 0.16 per 1000 doses (95% CI 0.02-0.57) in the RTS,S/AS01 group compared

with 0.47 per 1000 doses (95% CI 0.10-1.37) which received the meningococcal vaccine giving a risk ratio of 0.3 (95% CI 0.1-2.0) (102).

1.6.3 Safety signals

Further to the results mentioned above, detailed post-hoc analyses were conducted on the RTS,S/AS01 safety and efficacy results (43).

The incidence of febrile convulsions within seven days of RTS,S/AS01 vaccination increased with higher dose numbers compared with the control group (102), and this trend was confirmed in the child age group, with the highest risk ratio observed for the three day period following vaccination (43).

The imbalance of meningitis cases reported more frequently in the RTS,S/AS01 groups showed heterogenous aetiologies, with no specific clusters when looking at time of onset following vaccination nor clustering according to location, suggesting an outbreak (43). This imbalance had also not been observed in other RTS,S/AS01 trials (104). It is therefore hypothesised that the meningitis signal may be a chance finding (42, 43).

There had been an increased risk of severe malaria noted in the children age group which received three doses of RTS,S/AS01 (41) and further analyses indicated a safety signal for cerebral malaria. Significantly more cases of severe malaria were observed in children in all groups which received RTS,S/AS01 compared with children in the control group. Again, no clustering when looking at time of onset was observed to indicate a direct effect of the vaccine (43). A potential hypothesis for the imbalance of meningitis and cerebral malaria cases in the child age group was that the rabies control vaccine potentially provided a non-specific protective effect on central nervous system (CNS) infections (105).

Klein et al. showed that vaccinations with RTS,S/AS01 in both infant and children groups were associated with a higher all-cause mortality in girls, with a mortality ratio of 1.91 (95% CI 1.30-2.79) but not in boys, mortality ratio 0.84 (95% CI 0.61-1.17). Between genders, this difference was significant ($p=0.0001$) resulting in concerns being raised (44). However, the WHO felt this

was mostly due to low female mortality in the control group and could be due to chance (42). Further analyses also suggested that as the mortality was evaluated by modelling known risk factors retrospectively, data were not always available. This was because the data were not collected per protocol. The female mortality rate in the control arm was lower than the mortality rate in all other study arms for both male and female (43). Equally data were unavailable on gender-specific parental care-seeking behaviours (106).

Seven-year follow up of a subset of participants in the RTS,S/AS01 phase II (Kenya and Tanzania participants) and III (Burkina Faso, Kenya and Tanzania participants) trials have shown that vaccine efficacy in the RTS,S/AS01 study groups (three and four dose groups) was lower than in the control group, suggesting that initial protection provided by the vaccine was offset by rebound after five years, in areas with high exposure to malaria parasites (24, 107). However, significant vaccine efficacy against severe malaria incidence was noted 6-7 years following RTS,S/AS01 vaccinations, therefore the increased risk of clinical malaria was not accompanied by an increased risk in severe or cerebral malaria (24).

1.6.4 Developing RTS,S/AS01 further

New vaccine distribution methods have been trialled with RTS,S/AS01, the most successful malaria vaccine to date. Administering the vaccine prior to the malaria season in areas of highly seasonal malaria transmission was noninferior to SMC in preventing clinical malaria. While SMC was more protective than RTS,S/AS01 in the malaria season for four months when it was administered, RTS,S/AS01 provided protection year-round. Interestingly, when RTS,S/AS01 was combined with SMC administration, lower incidence of clinical malaria, severe malaria and malaria deaths were noted when compared with either intervention alone (46).

1.6.5 Malaria Vaccine Implementation Programme (MVIP)

In July 2015, following the results of the phase III trial of RTS,S/AS01, the vaccine received a positive scientific opinion from the European Medicines Agency (EMA) under Article 58 (108).

In January 2016, WHO adopted the joint recommendation of the Strategic Advisory Group of Experts (SAGE) on Immunisation and the Malaria Policy Advisory Group (MPAG) for pilot implementation of the vaccine (42). The vaccine efficacy was found to be low in the infants (6-12 weeks) and therefore, use was not recommended in this age category (42, 103). Highest protection was noted in the 5-17-month-old children but this was in a four-dose schedule (42, 102). This would require new contacts and did not fit into the schedule of routine health systems. Therefore, further evaluation of this vaccine for feasibility of deployment was recommended in a series of pilot implementation programmes (42).

These programmes would also allow a number of other questions to be answered, including the impact of RTS,S/AS01 on all-cause mortality and gender-specific mortality and to further assess adverse events including potential links to meningitis and cerebral malaria. These trials also allow the impact of the vaccine to be assessed when added to existing malaria interventions and to see if there was any evidence of adverse effects of vaccine implementation on these other malaria interventions (42).

In 2019, the MVIP, coordinated by WHO, was launched in Ghana, Kenya and Malawi, in selected areas of moderate to high malaria transmission. Any children who presented to health facilities for vaccinations were eligible and were first offered RTS,S/AS01 at 5 months in Malawi and at 6 months in Ghana and Kenya. (45). By April 2021, more than 1.7 million doses had been administered across the three countries to more than 650,000 children (109). At this point, it was felt that sufficient data was available to evaluate safety concerns in a primary analysis. When eligible children living in the implementation areas were compared with those in the comparison areas, no evidence of an excess of meningitis, cerebral malaria or gender-specific mortality was found (110).

When looking at the effectiveness of RTS,S/AS01 in the MVIP at that time point, there was a 30% reduction in severe malaria and a 21% reduction in hospitalisation with malarial parasitaemia. Both these results were statistically significant (110, 111).

1.6.6 WHO recommendation of RTS,S/AS01 malaria vaccine

In October 2021, the WHO recommended the use of the RTS,S/AS01 vaccine at a minimum of four doses among children from 5 months of age living in areas of moderate to high *P. falciparum* malaria transmission. In addition, countries with highly seasonal malaria or perennial transmission with seasonal peaks can consider providing the vaccine seasonally with a five-dose strategy (110, 112). This was ground-breaking news as this was the first malaria vaccine to advance this far and it is anticipated that deployment will begin in 2023. In 2022, the WHO has also granted prequalification to RTS,S/AS01 (113).

However, it is estimated that over 25 million children are born each year in over 30 countries with moderate to high malaria transmission and initial vaccine supply will not be sufficient to meet this need. This is due to issues with the speed of production of the vaccine. Limited supply is expected during the first 4-6 years following expected introduction in 2023 and it is difficult to predict when constraints might ease. A framework was developed to ensure that until vaccine supply is sufficient, the mechanism of vaccine distribution is fair and equitable (114). However, until vaccine supply is in the tens of millions, it would be difficult to ensure a fair and equitable distribution. This reinforces the need for ongoing malaria vaccine development to continue.

1.7 R21/Matrix-M as a malaria vaccine candidate

1.7.1 Description of R21

R21 was produced at the Jenner Institute, University of Oxford (Figure 1C) and consists of a C-terminal portion of CSP from *P. falciparum* (strain NF 54) fused to the N-terminus of HBsAg. The CSP portion contains 19 NANP repeats from the central repeat region, known to be a protective B cell epitope and the C-terminal region contains T cell epitopes (49). It self-assembled into virus-like particles (VLPs) expressed in *P. pastoris*. In order to scale up manufacturing, it is currently manufactured by the Serum Institute of India Private Ltd. (SIPL) and is now expressed in *Hansenula polymorpha*. R21 is similar to RTS,S as they both include HBsAg fused to the C-terminal and central repeats of CSP. However, R21 contains only fusion

protein, whereas RTS,S contains 20% fusion protein and the remaining 80% is HBsAg monomers alone. It is for this reason R21 may be more immunogenic and efficacious as the majority of the surface is covered in CSP antigen (49).

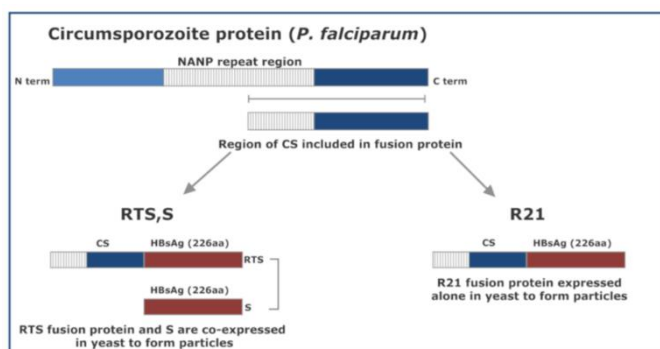


Figure 1C: Schematic representation of R21 and RTS,S (49).

1.7.2 Description of Matrix-M™

Matrix adjuvant technology involves the purification and characterisation of saponin fractions and the formulation of selected saponin fractions. The major source of the saponin is the bark of the tree *Quillaja Saponaria Molina*, which will grow only in Chile and global supply is finite. Matrix is a 40nm-sized complex consisting of this saponin, formulated cholesterol and phospholipid into nanoparticles. Matrix-M™ consists of two different formed matrix particles, the highly active Fraction-C saponin and the weaker Fraction-A saponin. It is thought that mixing the two improves adjuvant activity and reduces reactogenicity (115). This adjuvant is known to stimulate both B-cell and T-cell immunity (116). Matrix-M is manufactured by Novavax.

1.7.3 Pre-clinical data on R21/Matrix-M

R21 was tested pre-clinically with a number of adjuvants individually and in combination. Groups of BALB/c mice were vaccinated with three doses of vaccine formulated with adjuvant. All mice developed detectable NANP-specific IgG but the highest responses were noted with R21 and the Abisco-100 (an earlier name for Matrix-M) adjuvant, which was consistently more immunogenic and protective than the AddaVax adjuvant (Figure 1D). CSP-specific T cell

responses were measured and these were also induced in all groups, the highest being in the group given R21 with Abisco-100 or R21 with Abisco-100 and Addavax (49).

In these same groups, HBsAg-specific IgG was measured and minimal responses were noted, demonstrating that it is likely that HBsAg is inaccessible on the R21 surface. They were almost undetectable in the group that received R21 with Abisco-100 (49).

When R21 was formulated with Abisco-100 or Matrix-M (pre-clinical and clinical versions of the same adjuvant), sterile efficacy against transgenic sporozoite challenge (*P. berghei* parasites which express *P. falciparum* CSP in addition to the *P. berghei* CSP) was 100% and 87.5% respectively (Figure 1E). There was no significant difference in the level of NANP-specific IgG in the two groups. When R21 was formulated with AddaVax, no sterile protection was noted (49).

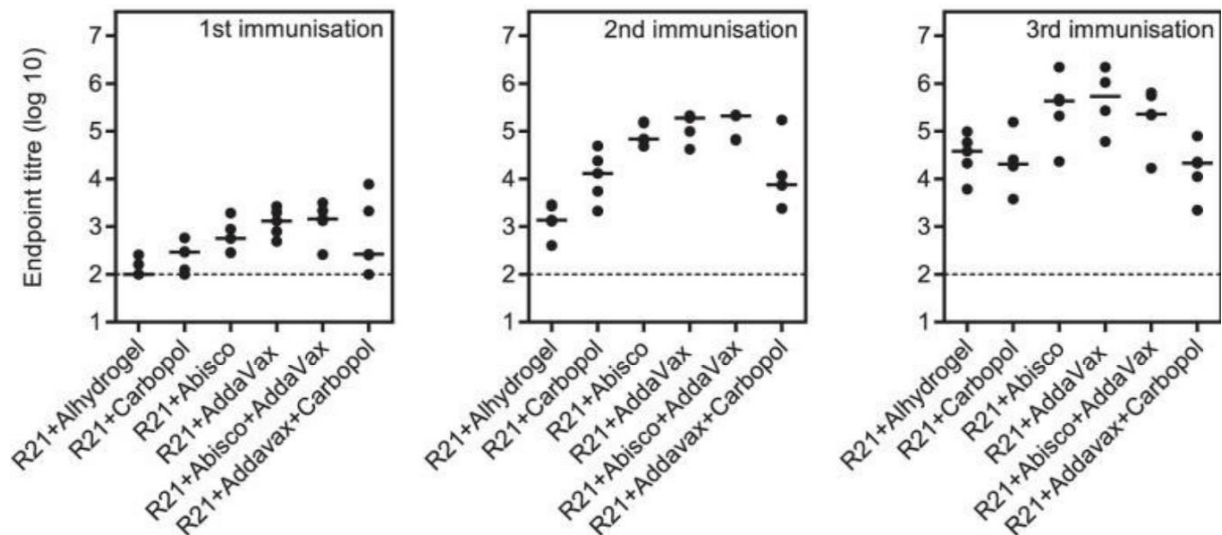


Figure 1D: Total NANP- specific IgG.

BALB/c mice were immunised intramuscularly with 0.5µg R21 formulated in adjuvant 3 times, 3 weeks apart, and assayed three weeks after each immunisation. Dotted line indicates the limit of detection (49).

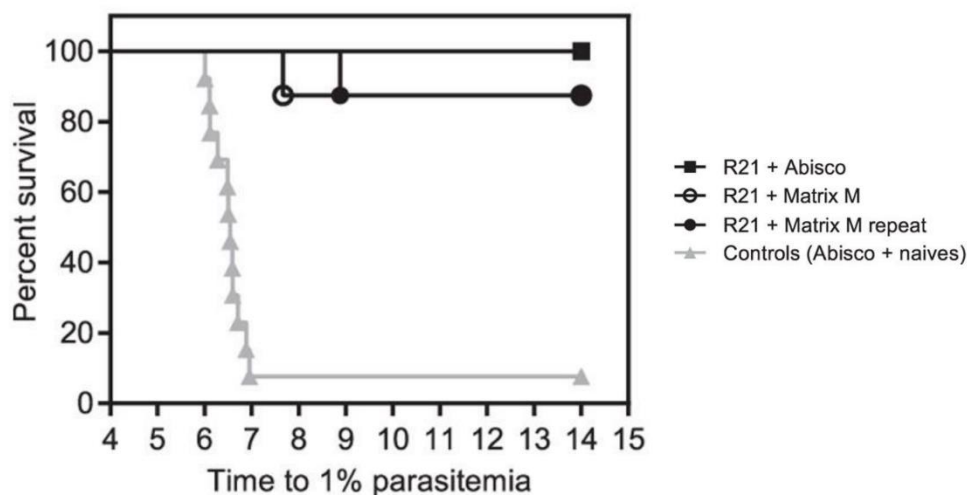


Figure 1E: Kaplan-Meier survival graphs displaying protective efficacy of R21 with adjuvants.

BALB/c mice were immunised intramuscularly with 0.5µg R21 and adjuvant twice, eight weeks apart. Mice were challenged 3 weeks after the final vaccination by IV injection of 1000 sporozoites (Tg Pb+PfCSP), along with eight naïve mice. Blood-stage parasitaemia was monitored from day 5 after challenge by thin film blood smear, and time to 1% parasitaemia was calculated using linear regression (49).

1.7.4 Clinical data on R21/Matrix-M

After the encouraging pre-clinical results with R21, early clinical development commenced. R21 was initially manufactured to GMP standard at the Clinical Biomanufacturing Facility (CBF) in Oxford, where a C-tag (E-P-E-A) was added for purification by immunoaffinity chromatography (R21c). A phase I study (VAC 053) assessing R21c administered with or without Matrix-M was conducted in healthy UK volunteers using different doses of antigen and adjuvant. Three vaccinations were administered four weeks apart and a good safety profile was noted. Antibody titres (Figure 1F) were significantly higher in the intermediate dose of R21 (10µg) compared with the high dose (50µg), and these titres were durable at six months (117).

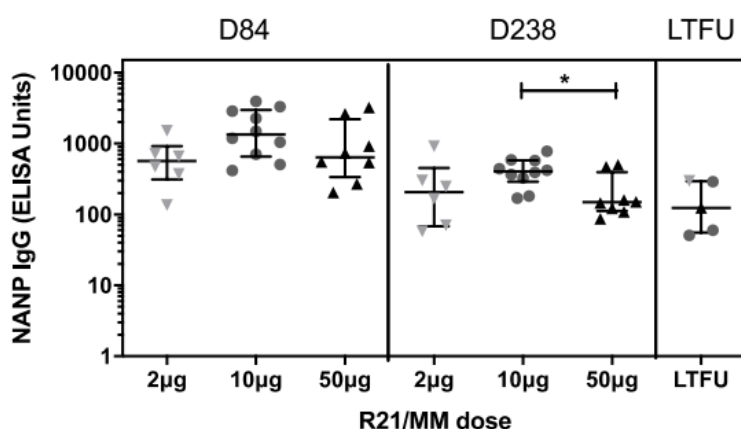


Figure 1F: Total NANP-specific IgG in healthy UK adults to R21/Matrix-M.

These were measured at 28 days following third vaccination (D84), 6 months following third vaccination (D238) and long-term follow up (LTFU) between 22 and 35 months after third vaccination. Mann-Whitney analyses between groups at D84, $p = 0.20$, and D238, $p = 0.02$ (117).

A phase IIa study (VAC 065) assessing the safety, immunogenicity and efficacy of R21c with Matrix-M and in combination with ChAd63-MVA ME-TRAP also showed the vaccine was safe and well-tolerated. Responses to NANP were noted in all participants vaccinated with R21c and Matrix-M and these were well maintained at 8.5 months after vaccination. High-level efficacy of 82% was observed when three doses of 10µg R21c were administered with Matrix-

M, 4 weeks apart (Figure 1G). The addition of viral-vectored vaccines or a fractional third dose did not improve efficacy. Durable efficacy (60%) was noted at 8.5 months following initial vaccinations. When a vaccine regime of only two vaccine doses was assessed, vaccine efficacy of 57% was observed, the highest level of efficacy reported for a two-dose malaria vaccine regime against sporozoite challenge (57).

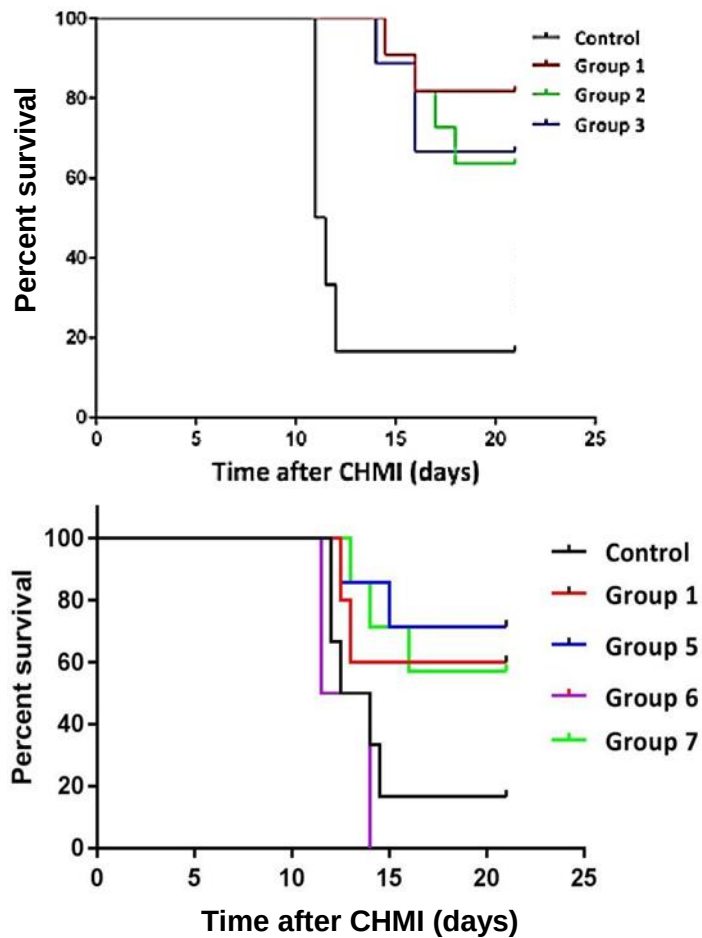


Figure 1G: Efficacy of R21c in VAC 065 clinical trial.

Efficacy measured by CHMI 28 days following third vaccination. First panel shows the first CHMI. Group 1 received R21c 10 µg, 10 µg, 10 µg with Matrix-M 50 µg, group 2 received R21c 50 µg, 50 µg, 10 µg with Matrix-M 50 µg and group 3 received R21c 10 µg, 10 µg, 10 µg with Matrix-M 50 µg and ChAd 63 MVA ME-TRAP. Second panel shows the second CHMI. Group 1 which were sterilely protected in first CHMI, were re-challenged at 8.5 months, group 5 received R21c 10 µg, 10 µg, 2 µg with Matrix-M 50 µg, group 6 received R21c 10 µg, 10 µg, 10 µg with Matrix-M 50 µg in the phase I R21 trial and were being challenged 20 months later, and group 7 received only two doses of R21c 10 µg, 10 µg with Matrix-M 50 µg.

Following these initial exciting results, it was necessary to first find a partner who could manufacture the R21 vaccine at large-scale, and low cost, and then to test this new vaccine again in a UK population for comparable safety, immunogenicity and efficacy. If this was successful, it would then be necessary to move clinical development to the target population in malaria endemic areas and to age de-escalate to children.

1.8 Vaccines and immunogenicity

Varied humoral and cellular responses to many licensed and candidate vaccines have been noted.

A recent extensive review concluded that vaccine responses were strongly influenced by intrinsic factors such as age, gender, genetics and comorbidities (118). Vaccine responses were found to be reduced in early life and in elderly people, who have rapid waning of antibodies. Gender-specific responses were noted with certain childhood vaccines, inducing higher antibody responses in either gender according to specific vaccine (119). Different ethnic groups had different responses (120) and durability (121), even when living in the same location, suggesting human genetics as a factor and studies looking at those with chronic diseases showed impaired immune responses (118). Factors specific to the vaccine including the type (118), if they contain adjuvants (122), and what schedule they are administered in (123, 124) also seemed to heavily influence immune responses.

Data on extrinsic factors (antibiotics, infections, microbiome), nutritional factors and perinatal factors (birth weight and feeding methods) affecting immune responses to vaccines were less convincing (118), although maternal antibodies did result in diminished responses to childhood vaccines (125). Further data from studies assessing vaccine responses and behavioural factors such as smoking, alcohol, exercise and psychological stress were inconsistent (118).

Differences in vaccine responses were noted according to geographical location but it is difficult to assess the true nature of these as there are many other factors that potentially play a role in this, including the ones mentioned such as genetics, nutrition, microbiome and pre-

existing immunity (118). It is necessary to gain further understanding of all factors and their potential confounders to improve future vaccine immunogenicity and efficacy.

1.8.1 Vaccine immunogenicity in LMICs (target population for a malaria vaccine)

Vaccine immunogenicity is frequently impaired for both oral and parenteral vaccines in individuals from low middle-income countries (LMICs) compared with those in high-income countries (HICs), but reasons for this are not fully understood. Poorer immunogenicity results in lower efficacy. Examples of impaired responses to oral vaccines include oral rotavirus vaccines (ORVs) (126), oral polio vaccines (OPV) (127), and oral vaccines against cholera (128). A meta-analysis of all RCTs analysing Rotarix and RotaTeq ORVs demonstrated that VE in HICs was 98% after 2 weeks and 94% after 1 year. However, in LMICs, it was 66% and 44% respectively (126). While some studies have shown that there is comparable immunogenicity between LMICs and HICs with parenteral vaccines (DTP-HB-Hib) (129), others have shown impaired immunogenicity. These outcomes were noted in a phase III efficacy trial of an inactivated quadrivalent influenza trial (130), three phase I Ebola candidate vaccine trials (131) and when measuring B cell responses, as well as CD8 T cell responses, to the yellow fever 17D vaccine (132).

1.8.2 Potential mechanisms for reduced vaccine immunogenicity and efficacy in LMICs

The potential mechanisms for reduced vaccine responses in LMICs remain poorly understood but there are some factors thought to be contributory. Malnutrition during gestation may be associated with a smaller thymus, affecting T cell development. This results in weaker T cell responses from a less diverse repertoire, potentially impairing immune responses to infection and vaccines (133).

Apart from the development of an efficacious vaccine, a healthy immune system is required to respond efficiently to a vaccine (134). Coinfection with HIV and malaria in pregnant women has been implicated in reduced immunogenicity. Also, malaria can cause restricted foetal

growth, preterm delivery, low birth weight, reduced transfer of maternal immunity and diminished potential immune responses to infectious diseases (133). Antibody titres to tetanus were 48% lower in new born infants where mothers had placental malaria (135). While there have been direct or indirect mechanisms attributed to causing immunosuppression specifically by malaria, these are still unclear and a number of aspects have been suggested. These include the direct effect of the *P.falciparum* parasite, a prolonged effect on the immune system following acute infection, immunosuppression developing in the host during parasite growth or through continuous and repetitive infections, and that malaria infection suppresses antibody production as well as dampening antigen-specific T cell responses and T cell proliferation (134, 136, 137). Immunosuppression in other ways related to *Plasmodium* species has been suggested through a lower incidence of rheumatoid arthritis and other autoimmune conditions in people exposed to malaria compared with those of a similar genetic background (138). A similar hypothesis of reduced immune responses was suggested regarding less severe COVID-19 infection in those with malaria exposure, discussed later in the chapter (139).

Apart from malaria, other parasitic infections have also been implicated in the placental transfer of parasites. This is thought to stimulate an immune response in the foetus and cause strong immunomodulatory effects. Exposure to helminths in utero has been shown to decrease the efficacy of BCG as a result of decreased IFN- γ , and Hib vaccine efficacy as a result of lower antigen-specific IgG (133).

Environmental enteropathy, associated with continuous contact with many enteric pathogens, nutritional stresses (140) and lack of basic sanitary facilities (141) leads to blunted epithelial villi in the small bowel, increased inflammation, bacterial overgrowth and malabsorption of nutrients. These imbalances may accompany a change in the composition of the gut microbiome and these microbial communities may directly or indirectly affect vaccine efficacy and effective or durable immune responses (140).

In order to improve infant immunity through vaccination, further research is required investigating potential mechanisms and factors contributing to reduced vaccine

immunogenicity. However, it should be noted that in most studies, vaccine responses are quantified using antibodies but other responses are also important when thinking about efficacy such as innate, cellular and cytokine responses. As such, antibody levels do not always represent correlates of protection.

1.9 The gut microbiome and immune responses

As mentioned above, vaccine immunogenicity is reduced in infants when compared with healthy adults (133) and also dependent on geographical location (118). Studies and data in this field suggested there may be causal links between vaccine immunogenicity and the gut microbiome.

1.9.1 Factors affecting the gut microbiome

The composition of the gut microbiome is very variable between individuals. In early infancy, there are low levels of diversity and the gut microbiome is unstable. The diversity increases and is similar to that of adults by two to three years of age (142). The gut microbiome of adults is more diverse, and in the absence of external factors causing dysbiosis, is generally stable. Diversity and stability of the gut microbiome then declines as people age (143).

Factors affecting the microbiome composition that may influence immune responses or efficacy of vaccines include intrinsic host factors, perinatal host factors, the environment, nutrition and behavioural factors (118).

1.9.2 Current evidence regarding the gut microbiome and vaccine responses

There have been studies in infants assessing responses to oral rotavirus vaccines (ORVs) and significant associations with the gut microbiota have been found. In Ghana, increased relative abundance of *Streptococcus bovis* was positively correlated with vaccine responses and *Bacteroides* and *Prevotella* were negatively correlated (144). In Pakistan, increased relative abundance of *Preoteobacteria* was positively correlated with vaccine response (145). An increased ratio of *Enterobacteriaceae: Bacteroides* was noted in both countries in vaccine responders and the investigators questioned if vaccine responses were improved by

lipopolysaccharide (LPS) produced by gram negative Enterobacteriaceae (144). Overall sample numbers were small, with 78 Ghanaian infants being analysed and 10 infants in Pakistan. In India and Nicaragua, no significant differences were found in the gut microbiome between responders and non-responders to ORVs (146, 147).

When looking at responses to OPV, a study in China found that increased abundance of *Bifidobacteria* was positively correlated with poliovirus-specific IgA responses (148) but a similar study in India did not confirm these findings (149). However, both studies showed that lower immune responses were associated with more diverse microbiomes. *Bifidobacteria* has also been positively associated with CD4 T-cell responses, enhancing immunological memory to BCG, tetanus toxoid and hepatitis B vaccines (parenteral vaccines) in early infancy. This association also correlated with T cell responses as well as antibody responses at 2 years, suggesting that the microbiome may also be linked to durability of vaccine responses (150). In adults, composition of the gut microbiome has been correlated with parenteral HIV vaccine immunogenicity (151).

1.9.3 Interventions that may affect the gut microbiome and vaccine responses

Interventional studies have been performed in adults, assessing if antibiotics and their impact on the gut microbiome can influence responses to influenza vaccines or ORVs. Antibiotics can potentially influence responses to influenza vaccination and ORVs, but they did not affect influenza vaccine responses in those with high levels of pre-existing immunity or ORV responses in those with high levels of baseline seropositivity (152, 153). An increased ratio of *Enterobacteriaceae: Bacteroides* was noted in vaccinated adults with increased IgA levels with OPV, similar to what was seen in infants (152). It has been proposed that these studies suggest the microbiome has more of an impact on primary responses to vaccination rather than secondary (154).

Similarly, a study was conducted in mice assessing antibody responses and antibiotic exposure in early life. Antigen-specific IgG responses to BCG, meningococcal serogroup B vaccine (Men B), NeisVac-C meningococcal serogroup C vaccine (MenC), INFANRIX Hexa

combination vaccine and 13-valent pneumococcal conjugate vaccine (PCV13) were all impaired in antibiotic-treated mice. This was despite boosting, designed to mimic childhood routine vaccinations (155). However, when adult mice exposed to the same dose of antibiotics were given the BCG or PCV13 vaccine, they did not have impaired IgG responses. This further adds to the data that dysbiosis caused by antibiotics, may specifically impair antibody responses in early life (155). When translating this to infants, one study found that responses to DTP, Hep B, HiB, OPV and PCV13 were not significantly different if antibiotics were administered in the first week of life (119). This is further being investigated in an ongoing study investigating routine childhood vaccination responses and microbiome dysbiosis in infants exposed to antibiotics in the intrapartum or neonatal period (ACTRN12617000856314). Interventions such as prebiotics, probiotics and faecal microbiota transplantation are being used to target the microbiome, assessing the effects on vaccine responses in a number of studies and contexts (154). Conflicting evidence currently exists as to the conclusions to draw from these trials with limitations prohibiting comparisons between studies.

1.9.4 Potential mechanisms of how the gut microbiome may affect vaccine responses

A number of reasons have been proposed for why the microbiome may modulate immune responses to vaccination. The gut microbiome may be providing a natural adjuvant to vaccinations. An example to demonstrate this is the seasonal influenza vaccine which induces expression of Toll-like receptor 5 (TLR5), a pattern recognition receptor for flagellin, in PBMCs in the week following vaccination. In antibiotic-treated mice and germ-free mice, there was significant reduction to antigen-specific IgM and IgG antibody responses. These responses were restored though in these mice by an oral reconstitution with a flagellated strain of *Escherichia coli* but not a non-flagellated strain (156). This would suggest that flagellin is the natural adjuvant for the influenza vaccine by signalling through TLR5 (157). This hypothesis was also tested with a number of other vaccines to check this was not specific to the influenza vaccine. The only response that appeared to be dependent on the microbiome was the

inactivated polio vaccine, the only non-adjuvanted vaccine tested, suggesting again that the microbiome acts as an adjuvant to those vaccines that don't have exogenous adjuvants (157).

Other factors are likely to influence the gut microbiome providing natural adjuvants and it is likely there is a complex interplay of all these factors on immune responses. As discussed earlier, LPS was thought to be a contributory factor to increased ORV responses (144) and is thought to act as an adjuvant to increase antibody responses (158). LPS may influence vaccine response; increased levels of LPS may be seen in the gut or periphery when the gut is inflamed or following antibiotic exposure (159). Parenteral vaccine responses influenced by the gut microbiome may be dependent on gut barrier integrity (154). When this is compromised by factors such as malnutrition, gut inflammation or antibiotic exposure, immunomodulatory products produced by the gut microbiome that can alter systemic immune responses are found in the periphery (160). Abundance of specific taxa may also alter the integrity of the gut barrier and therefore, also the natural adjuvants produced by the microbiome in the periphery (154).

The microbiome has also been suggested to potentially modulate dendritic cell (DC) function. DCs play a vital role in the immune response to vaccines as antigen-presenting cells and by secreting cytokines. They are subdivided into plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs). pDCs produce high levels of type I interferons (IFN-I) and cDCs induce T cell responses (161). The composition of the gut microbiota is thought to have an impact on the number of gut pDCs (162) and thereby the gut microbiome may control the production of IFN-I by pDCs. Further to this, the IFN-I receptor then instructs a specific metabolic state in cDCs resulting in increased priming of T cells (161).

The microbiome produces many metabolites which can alter immune responses. An example of these are short-chain fatty acids (SCFAs) which have been associated with B cell activity, supporting antibody production as well as enhancing gene expression for plasma cell differentiation and class switching (163). Recently though, SCFAs have been found to inhibit antibody responses (164). Metabolites produced by the microbiome are also thought to aid

gut barrier integrity (165), therefore, potentially regulating vaccine immune responses by avoiding escape of potential microbes that improve parenteral vaccine responses (154).

1.9.5 Thinking about the future: assessing the gut microbiome and vaccine responses

Findings to date with regards to composition of the gut microbiome and vaccine responses have been inconsistent, reported in small sample sizes, and mainly involving oral vaccines. Equally, understanding of mechanisms in which the gut microbiome may influence vaccine responses are poorly understood. Further studies in larger sample sizes, multiple contexts, and assessing intramuscular and subcutaneous vaccine responses will help to increase understanding of possible mechanisms, and potential significant differences in microbiome composition.

1.10 COVID-19 and malaria

1.10.1 Impact

SARS-CoV-2 had spread to all malaria endemic countries by April 2020. Two years after the start of the COVID-19 pandemic, malaria endemic countries had reported more than 101 million cases and 2.4 million deaths due to COVID-19 (1). The COVID-19 pandemic and its related restrictions caused disruption to malaria services and early messaging aiming to reduce COVID-19 transmission suggested the public stay at home if they had fever. However, this disrupted treatment of many other febrile illnesses including malaria (2). The latest WHO World Malaria Report states that there were an estimated 14 million more malaria cases and 47, 000 more deaths in 2020 compared with 2019, due to disruptions to services during the pandemic. Overall, moderate levels of disruption to malaria services were experienced in malaria endemic countries (1).

1.10.2 Existing campaigns

Several stakeholders worked to try and ensure that malaria control programmes continued as far as possible. Countries with planned SMC campaigns were on track to complete them,

despite moderate delays. By the third week of November 2020, 105 million of 222 million long-lasting insecticide-treated nets (LLINS) that were planned for distribution has been distributed. Twenty three out of 47 countries had completed their IRS campaigns, 13 were on track to complete and 11 at risk of not completing them (2). In reality, 18 out of 31 countries that had planned ITN campaigns in 2020, completed their campaigns by the end of 2020 and 72% of the ITNs had been distributed (1).

1.10.3 Malaria disease modelling in the pandemic

In April 2020, modelling was performed to assess the impact of malaria during the COVID-19 pandemic. Initially, this did not factor in the potential disruption to SMC or IRS, and also did not account for potential disruptions to global supply chains of preventative measures, diagnostics and treatment. The modelling did account for ITN coverage and showed that even with this at expected levels, there would be severe disruption in management of malaria. If there were no ITN campaigns but continuous ITN distribution and access to effective malaria treatment reduced by 50%, this would result in a 10% increase in malaria cases and a 53% increase in malaria deaths (166).

Further modelling concluded that by the end of 2020, even if all prevention campaigns completed in sub-Saharan Africa but there were reductions in access to effective antimalarial treatment by 10%, 15%, 25% and 50%, there could be an additional 19,000, 28,000, 46,000 or 100,000 deaths respectively (2). Robust data is currently unavailable to confirm or refute this modelling.

1.10.4 COVID-19 and malaria co-infection

A cohort study was conducted in Uganda looking at co-infection and previous *P. falciparum* exposure. The prevalence of malaria in the 597 participants (PCR confirmed COVID-19) analysed was 11.7%, compared with the national average of 9% and significant malaria burden was noted in patients >60 years of age. Symptoms of vomiting and confusion were noted more frequently in those with malaria co-infection and mean platelet counts and serum albumin

levels were lower. Low previous *P. falciparum* exposure was associated with more severe COVID-19, and a trend of a lower probability of unfavourable outcomes was noted in those with COVID-19 and *P. falciparum* infection (139).

This study also found that the severity of COVID-19 was associated with an increase in cytokines and chemokines, with IL-6 positively correlating with mortality. *P. falciparum* infection did not significantly affect cytokine levels except for TNF- α , which was higher in these co-infected patients. However, those patients with previous high exposure to *P. falciparum* had normal cytokine profiles. Achan et al. suggested these findings may be due to blunting of cytokine and chemokine responses to malaria (139), similar to what has been shown in older children and adult in malaria endemic areas as a result of high malaria exposure (167). The authors also suggested that the imbalance in pro- and anti-inflammatory cytokines may have explained the clinical signs noted in the co-infected patients (139).

This study was a small sample size and conducted in just one country. Another study in India also looked at 27 patients who had co-infection of COVID-19 with malaria. Co-infection significantly reduced the number of days for viral clearance and also shortened recovery time (168). It will be important to further investigate the associations found in these studies in other countries, and larger populations, to understand the impacts of two of the infectious diseases contributing to some of the world's highest morbidity and mortality.

1.11 Aims of this thesis

I have described the significant burden of malaria and current strategies to help control and potentially eliminate malaria. The most recent World Malaria report suggests that progress has stalled (2). New measures are needed to meet the goals of the GTS (8).

It is only in the last year that the WHO has recommended a malaria vaccine for use for the first time. This is following many years of work on malaria vaccines (169), assessing many vaccine candidates. RTS,S/AS01 showed efficacy of 36.3% in the target population following four doses. Given this efficacy, and vaccine supply issues, it is important that other malaria vaccine

candidates are developed and assessed. I have discussed the various malaria vaccines that target different stages of the life cycle. R21 is a malaria vaccine candidate that has been developed at the Jenner Institute, University of Oxford (49). Following preliminary clinical trials and encouraging results (117), the manufacturing and up-scaling of R21 was transferred to SIIPL. R21 is mixed with Matrix-M adjuvant (produced by Novavax) prior to administration and both are packaged at SIIPL.

I aimed to take a new malaria vaccine candidate (R21/Matrix-M) through the clinical development process. With many collaborators (please see acknowledgements), I assessed safety, immunogenicity and efficacy in a malaria naïve population, prior to proceeding to a malaria endemic population. As the target population for this malaria vaccine is children, following age de-escalation, I assessed the safety and immunogenicity in this cohort followed by field-efficacy in a high transmission area. Further, to try and understand why malaria vaccine immune responses and efficacy may be different in adults and children, and malaria naïve and endemic populations, or why more malaria episodes may be experienced by certain participants, I analysed the composition of the gut microbiome of African infants.

Chapter 3 describes the safety, immunogenicity and efficacy using sporozoite challenge of R21/Matrix-M in healthy adults in the UK (Phase I/IIa).

Chapter 4 describes the safety and immunogenicity of R21/Matrix-M in healthy adults, young children and infants in Kenya (Phase Ib).

Chapter 5 describes the safety, immunogenicity and field-efficacy of R21/Matrix-M in infants in Burkina Faso (Phase IIb).

Chapter 6 describes the composition of the gut microbiome in Burkinabe infants and its relationship to age, vaccine immune responses and efficacy.

2 Materials and methods

2.1 Vaccines

2.1.1 R21

R21 was developed at the Jenner Institute, University of Oxford. It is produced by using recombinant HBsAg particles expressing the central repeat and the C-terminus of the circumsporozoite protein (CSP). R21 was originally GMP manufactured at the Clinical Biomanufacturing Facility (CBF) in Oxford in *Pichia pastoris* and is now being manufactured at the Serum Institute of India Private Ltd. (SIPL) in *Hansenula polymorpha*. R21 is similar to RTS,S, which also targets the pre-erythrocytic circumsporozoite protein, the major functional protein in sporozoite development and hepatocyte invasion. R21 is 14 amino acids smaller than the RTS fusion protein at the C-terminus of the CSP sequence and lacks the four-fold excess of HBsAg contained in RTS,S.

R21 vaccine was in formulation buffer and the drug product was filled into 4ml glass vials with a 13mm grey bromobutyl rubber freeze-dry stopper and a 13mm complete tear, clear lacquered aluminium seal. The containers and closures were tested for compliance with defined specifications.

R21 was stored long-term as a frozen -80°C (nominal temperature) formulation or refrigerated at +2 to +8°C.

2.1.2 Matrix-M

Matrix-M was manufactured in compliance with GMP by Novavax AB, Sweden. It was supplied at a concentration of 1mg/ml and fill-finished at 200µg/ml in siliconized glass vials by SIPL. It is formulated in formulation buffer (2.7mM KCl, 8mM Na₂HOP₄, 2mM KH₂PO₄ and 137mM NaCl, pH 7.2).

Matrix-M is stored refrigerated at +2 to +8°C and protected from light.

2.1.3 R21/Matrix-M vaccinations

Eligibility of each participant to have vaccinations was assessed at screening but also prior to each vaccination, and vaccination deferral criteria are described later in the chapter. Urinary β HCG was performed prior to all vaccinations in female adult participants.

A mixture of R21 with Matrix-M was administered to all participants according to the Jenner Institute trial-specific SOPs designed for each site according to dose of antigen and adjuvant required. R21 was mixed with Matrix-M at the bedside, immediately prior to administration. R21 was administered intramuscularly within one hour of thawing or 30 minutes if previously stored in the refrigerator.

Following administration of vaccinations, all participants were observed at the study clinic for one hour.

R21 was given at a dose of 2 μ g, 5 μ g, 10 μ g or 50 μ g and Matrix-M at a dose of either 25 μ g or 50 μ g. Three doses were given initially 4 weeks apart and this was considered the primary series of vaccinations. Subsequent vaccinations given after the primary series of vaccinations were boosters.

2.1.4 Rabivax-S

This is a licensed lyophilized vaccine, manufactured by SIIPL, for prevention of rabies and was used as the control vaccine in VAC 076.

2.2 Study preparation

I prepared all the study documents for each trial (e.g. trial protocol, participant information sheet, consent forms and GP letters) that were initially reviewed by the Sponsor (Clinical Trials Research Governance-CTRG, University of Oxford) prior to submission to local ethics committees (EC) and regulatory authorities (RA). Additionally, I prepared the documents to obtain approval from the local Research and Development departments at the local collaborating NHS sites in the UK for VAC 072, and from the Oxford Tropical Research Ethics Committee (OxTREC) for the international trials.

I performed training at all study sites to delegated individuals on the protocol, SOPs and vaccine preparation. The Chief Investigator (CI) for all trials was Professor Adrian Hill and I was the delegated lead clinician responsible for the trial conduct and clinical work, data analysis and presentation of results. Each site had a local Principal Investigator (PI) who was responsible for activities and delegation of trial tasks at their site.

All trials were independently monitored and conducted according to the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonisation Good Clinical Practice guidelines.

Trial-specific details are described in the chapters relating to each individual study.

2.3 Study design

VAC 072 was a phase I/IIa trial assessing the safety, immunogenicity and efficacy using sporozoite challenge of R21/Matrix-M in healthy adults in the UK. VAC 073 was a phase Ib trial assessing the safety and immunogenicity of R21/Matrix-M in healthy adults, young children and infants in Kenya. VAC 076 was a phase IIb trial assessing the safety, immunogenicity and field-efficacy of R21/Matrix-M in infants in Burkina Faso. The study design for each trial is described in detail in the respective chapters.

During the COVID-19 pandemic, personal protective equipment (PPE) and social distancing measures were implemented in all study clinics with local country guidelines followed. In the cases where participants were not allowed to travel to attend clinic appointments, these study visits were done virtually, as far as possible, to ensure adequate safety follow-up.

2.4 Recruitment of participants

2.4.1 Recruitment

2.4.1.1 VAC 072

Healthy participants aged between 18 and 45 years were recruited using advertisements and registration forms approved by the local EC through various written media, public place advertising and social media.

2.4.1.2 VAC 073

In the adult age group, participants aged between 18 and 45 years were recruited. In the young children group, participants were aged between 1 and 5 years and the infants were 5 - <12 months. Community meetings took place initially with local administrative and community leaders along with healthcare workers. After this, public meetings were held in the villages where the study was recruiting to inform and engage the community about the study. These meetings involved discussing current malaria strategies, status of vaccine development, study procedures, and risks and benefits of being part of the study. Following these meetings, field workers identified potential participants/parents who were interested in their child taking part, and the participant information sheets (PIS) were distributed in their local language. Thereafter, the field workers had individual meetings with potential participants and parents to ensure full understanding of the information sheet and the consent form. If, after these steps, the participant or parent wanted to be part of the study, they were invited to the study centre, where written informed consent was taken and the screening visit conducted.

2.4.1.3 VAC 076

Participants aged between 5 and 17 months were recruited. Community sensitisation took place to inform and engage the community about the study. This involved informing the local community leaders followed by the local social science team holding local community meetings. At these meetings, the current status of malaria burden, vaccine development, study processes, as well as risks and benefits of taking part in the trial were discussed and it was emphasised that participation was voluntary. After these meetings, based on a list of appropriately aged children provided by the local Health and Demographic Surveillance System (HDSS) and if parents/guardians were interested to participate, invitations for screening visits were arranged.

2.4.2 Informed Consent and screening

2.4.2.1 VAC 072

All participants were provided with a written PIS explaining the trial at least 24 hours prior to the screening visit. At the screening visit, they were informed verbally of all aspects of the trial, the potential risks and their requirements in order to be a part of the trial. If the participant was happy to proceed, they were then asked to sign and date an informed consent form prior to any study-specific procedures being performed. If a participant was taking part in the CHMI part of the trial, they were also asked to complete a questionnaire to test their understanding of what was required for their involvement, obligations and risks involved. They were expected to answer all questions correctly with a maximum of two attempts in order to be eligible to take part in the CHMI. Following this, a full medical and travel history was taken, and a physical examination and blood tests (haematology, biochemistry, liver function, HLA typing, HIV/HCV/HBsAg and β HCG where applicable) were performed. Medical history was also sought from GPs (with participant's consent) in order to corroborate their medical history and registration on the TOPS database confirming that there was no participation in other ongoing trials which may affect data interpretation. Following results of all of these investigations, each participant was assessed for eligibility using trial-specific inclusion and exclusion criteria that can be found in the supplementary appendix, section 8.1. For the CHMI, all participants underwent additional safety checks including an ECG, a serum β HCG (for female participants) on the day prior to challenge, along with routine follow-up bloods and a PCR swab during the COVID-19 pandemic.

2.4.2.2 VAC 073

As discussed earlier, the PIS was provided at the community meetings and discussed at the individual meetings with the field workers. At the screening visit, informed consent was taken if appropriate. Subsequently, a full medical history, physical examination, ECG (for adults), saturation monitoring (for infants for cyanotic congenital heart disease) and blood sampling (haematology, biochemistry, liver function, HLA typing, HIV/HCV/HBsAg, screening for

haemoglobinopathies and β HCG where applicable) was performed to assess for eligibility, using the trial-specific inclusion and exclusion criteria that can be found in the Supplementary appendix, section 8.2.

2.4.2.3 VAC 076

At the screening visit, all the details of the PIS were clearly explained to parents/guardians during individual meetings. If the parents/guardians appeared to not understand very well, they were considered unable to give consent. If the investigator determined that the parent/guardian was able to give free and informed consent for their child to participate, they were asked to complete the consent form. In the case of illiteracy, the consent form could be thumb printed. A literate, impartial witness was present for all screening procedures and countersigned the consent form if the parents/guardians were illiterate.

Following informed consent, medical history, physical examination and blood sampling (haematology bilirubin, alanine aminotransferase (ALT), creatinine, blood films) were performed in order for eligibility to take part in the trial to be assessed. This was done using trial-specific inclusion and exclusion criteria that can be found in the Supplementary appendix, section 8.3.

2.5 Randomisation and blinding

2.5.1 VAC 072

This was an open-label, non-randomised trial.

2.5.2 VAC 073

This was an open-label, non-randomised trial.

2.5.3 VAC 076

This was a double-blind, randomised controlled trial.

Further details of this are described in the relevant chapter.

2.6 Compensation for trial participants

2.6.1 VAC 072

Participants were compensated for their time, travel expenses and inconvenience caused by blood-taking procedures. Additionally, if part of the CHMI, illness compensation was provided for potential days they may not be able to attend work. This was calculated according to the standard costs used across all clinical trials at the Jenner Institute, University of Oxford.

2.6.2 VAC 073

Participants (and their parents/guardians where applicable) were compensated according to local KEMRI guidelines. Financial compensation for transport and time for clinic study visits was provided. Refreshments were provided where a study visit exceeded four hours. Female adult participants who were not on adequate contraception were referred to their local dispensary where this was issued for free, with costs being covered by the study.

2.6.3 VAC 076

Participants and their parents/guardians were compensated for travel expenses and provided with refreshments at study clinic visits.

2.7 Data handling

All participant data were anonymised and entered into the OpenClinica database. Depending on context, this was either done in real-time or retrospectively with data initially being recorded in a paper CRF if the latter. Double-data entry was performed to ensure accuracy of data if not entered in real-time. All data was cleaned prior to database lock and extraction for analysis.

2.8 Assessment of vaccine safety

2.8.1 Definitions

2.8.1.1 Adverse Event (AE)

An AE is any untoward medical occurrence in a participant, including a dosing error, which may occur during or after administration of an IMP and does not necessarily have a causal relationship with the intervention. An AE can therefore be any unfavourable or unintended sign

(including an abnormal laboratory finding), symptom, or disease temporally associated with the study intervention, whether or not considered related to the study intervention.

2.8.1.2 Adverse Reaction (AR)

An AR is any untoward or unintended response to an IMP. This means that a causal relationship between the IMP and an AE is at least a reasonable possibility, i.e., the relationship cannot be ruled out. All cases judged by either the reporting medical investigator or the sponsors as having a reasonable suspected causal relationship to an IMP (i.e. possibly, probably or definitely related to an IMP) will qualify as adverse reactions.

2.8.1.3 Unexpected Adverse Reaction

An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g. Investigator's Brochure for an unapproved investigational medicinal product) is considered an unexpected adverse drug reaction.

2.8.1.4 Serious Adverse Event (SAE)

An SAE is an AE that results in any of the following outcomes, whether or not considered related to the study intervention.

- Death (i.e. results in death from any cause at any time)
- Life-threatening event (i.e. the participant was, in the view of the investigator, at immediate risk of death from the event that occurred). This does not include an AE that, if it occurred in a more serious form, might have caused death.
- Persistent or significant disability or incapacity (i.e. substantial disruption of one's ability to carry out normal life functions).
- Hospitalisation or prolongation of hospitalisation, regardless of length of stay, even if it is a precautionary measure for continued observation. Hospitalisation (including inpatient or outpatient hospitalization for an elective procedure) for a pre-existing condition that has not worsened unexpectedly does not constitute a serious AE.
- An important medical event (that may not cause death, be life threatening, or require

hospitalization) that may, based upon appropriate medical judgment, jeopardize the participant and/or require medical or surgical intervention to prevent one of the outcomes listed above.

- Congenital anomaly or birth defect.

2.8.1.5 Serious Adverse Reaction (SAR)

An adverse event (expected or unexpected) that is both serious and, in the opinion of the reporting investigator or sponsors, believed to be possibly, probably or definitely due to an IMP or any other study treatments, based on the information provided.

2.8.1.6 Suspected Unexpected Serious Adverse Reactions (SUSARs)

A SUSAR is a SAE that is unexpected and thought to be possibly, probably or definitely related to an IMP.

2.8.2 Solicited adverse events

These were collected over a 7-day follow-up period (day of vaccination and 6 days thereafter). Solicited adverse events are listed below.

2.8.2.1 VAC 072

Local solicited AEs included pain, redness, warmth and itch at the injection site. Systemic solicited AEs included fever, feverishness, arthralgia, myalgia, fatigue, headache, nausea and malaise. Participants were asked to record their temperature every day and to record this along with any of the above-mentioned AEs in an electronic diary. All solicited AEs were graded 1-3 according to severity using standardised methods and can be found in supplementary appendix table 1 - supplementary appendix table 2.

2.8.2.2 VAC 073

2.8.2.2.1 Adults

Local solicited AEs included pain, redness and swelling at the injection site. Systemic solicited AEs included fever, feverishness, arthralgia, myalgia, fatigue, headache and malaise. Participants were visited at home daily by field workers for the recording of these AEs in a

paper diary. These were graded 1-3 according to severity using standardised methods and can be found in supplementary appendix table 1 - supplementary appendix table 2.

2.8.2.2.2 Young children and infants

Local solicited AEs included pain and swelling at the injection site. Systemic solicited AEs included fever, irritability, drowsiness and loss of appetite. Participants were visited at home daily by field workers for the recording of these AEs in a paper diary. These were graded 1-3 according to severity using standardised methods and can be found in supplementary appendix table 3 - supplementary appendix table 4.

Booster vaccination visits took place during the COVID-19 pandemic and field worker visits were not always possible. During this time, parents were issued with a thermometer, ruler, copy of the diary card and a pen, and taught how to complete the reactogenicity assessment in the diary. They also were advised to contact the study team as soon as possible if they had any medical concerns about their child and a clinical review would be arranged.

2.8.2.3 VAC 076

Local solicited AEs included pain, swelling and redness/discolouration at the injection site. Systemic solicited AEs included fever, irritability, drowsiness and loss of appetite. Participants were visited at home, daily, by field workers for the recording of these AEs in a paper diary. These were graded 1-3 according to severity using standardised methods and can be found in supplementary appendix table 5 - supplementary appendix table 6.

2.8.3 Unsolicited adverse events

For all trials, these were any AEs that occurred following vaccinations (apart from those listed as solicited AEs that occurred within 7 days of vaccination). For the analyses, these were all AEs reported within a 28-day follow-up period of each vaccination. All of these AEs were coded according to the preferred term (PT) classification of diagnoses from the Medical Dictionary for Regulatory Activities (MedDRA). The relationship of the AE to the investigational medicinal product (IMP) was assessed as detailed in section 2.8.6.

2.8.4 Serious adverse events

For all trials, these were or are being collected for the duration of the trial. These were also coded according to the MedDRA PT classification.

2.8.5 Laboratory adverse events

2.8.5.1 VAC 072

Blood samples were taken for haematology, biochemistry and liver function tests at specific time points to aid safety assessment of vaccine and, where applicable, malaria course following CHMI. Tables detailing this can be found in the supplementary appendix table 7 - supplementary appendix table 10. AEs were graded according to the local NHS laboratory's reference ranges where the sample was being processed. Site-specific grading tables were based on FDA guidance relative to the local reference ranges and can be found in the site local SOPs.

2.8.5.2 VAC 073

Blood samples were taken for haematology, biochemistry and ALT across all age categories of participants. The schedule of sampling can be found in the supplementary appendix table 11. AEs were assessed for a change from baseline and graded using the most up to date Division of AIDS (DAIDS) Table for grading the severity of adult and paediatric adverse events (v2.1, March 2017). Blood volumes were calculated according to age and local guidelines but for safety bloods, the volume withdrawn was 2-5ml.

2.8.5.3 VAC 076

Blood samples were taken for haematology and creatinine, ALT, aspartate aminotransferase (AST), and bilirubin at 28 days to assess the safety of the vaccination, and the schedule for this can be found in the supplementary appendix table 12. AEs were assessed for a change from baseline and graded using the most up to date DAIDS Table, similar to VAC 073. The volume of blood taken at any donation was 1ml/kg, up to a maximum of 8ml (inclusive of immunology bloods).

2.8.6 Causality of adverse events

Across all the clinical trials in this DPhil thesis, an assessment of the relationship of an unsolicited AE to the IMP was undertaken by myself or clinicians delegated by the CI or PI at coordinating sites. This relationship was categorised as no relationship, unlikely, possibly, probably or definitely related to the IMP (Table 2a). Clinical judgment of the delegated clinician determined this relationship. These assessments took place at planned safety reviews, interim analyses and at the final endpoint analyses except for SAEs, which were assigned by the reporting investigator in real-time. For final analyses in the UK trial, causality assessments were blindly peer-reviewed and any discrepancies discussed prior to final causality assignment.

Table 2a: Guidelines for assessing the relationship of vaccine administration to an AE.

0	No Relationship	No temporal relationship to study product; and Alternate aetiology (clinical state, environmental or other interventions); and Does not follow known pattern of response to study product
1	Unlikely	Unlikely temporal relationship to study product; and Alternate aetiology likely (clinical state, environmental or other interventions); and Does not follow known typical or plausible pattern of response to study product.
2	Possible	Reasonable temporal relationship to study product; or Event not readily produced by clinical state, environmental or other interventions; or Similar pattern of response to that seen with other vaccines
3	Probable	Reasonable temporal relationship to study product; and Event not readily produced by clinical state, environment, or other interventions; or Known pattern of response seen with other vaccines
4	Definite	Reasonable temporal relationship to study product; and Event not readily produced by clinical state, environment, or other interventions; and Known pattern of response seen with other vaccines

2.8.7 Holding rules

Holding rules were applicable for the VAC 072 phase I/IIa UK trial due to minimal experience with administration of R21 in healthy human participants. These applied to all participants receiving vaccinations.

The holding rules are detailed below.

2.8.7.1 Solicited local adverse events

If more than 25% of doses of the vaccine at a given time point (e.g. day 0, day 28) in a study group were followed by the same grade 3 solicited local AE beginning within 2 days after vaccination (day of vaccination and one subsequent day) and persisting at grade 3 for >48 hours.

2.8.7.2 Solicited systemic adverse events

If more than 25% of doses of the vaccine at a given time point (e.g. day 0, day 28) in a study group were followed by the same grade 3 solicited systemic AE beginning within 2 days after

vaccination (day of vaccination and one subsequent day) and persisting at grade 3 for >48 hours.

2.8.7.3 Unsolicited adverse events

If more than 25% of participants at a given time point (e.g. day 0, day 28) in a study group developed the same grade 3 unsolicited AE (including the same laboratory adverse event) that persists at grade 3 for > 48 hours.

2.8.7.4 Activation of holding rules

If a holding rule was activated, then further vaccinations in any group would not have occurred until an internal safety review by the Local Safety Monitor (LSM), study sponsor and the CI was conducted and it was deemed appropriate to restart dosing. The regulatory authority would be informed and a request to restart dosing with pertinent data would be submitted as a request for a substantial amendment. The internal safety review would consider:

- Relationship of the AE or SAE to the vaccine
- Relationship of the AE or SAE to the vaccine dose, or other possible causes of the event
- If appropriate, additional screening or laboratory testing would have been provided to other participants to identify participants who may develop similar symptoms, and alterations to the current Participant Information Sheet (PIS) would be discussed
- New, relevant safety information from ongoing research programmes on the vaccines

All ethical committee(s), LSMs and Novavax would be notified if a holding rule was activated or released. If the clinical trial halted and was unable to resume, a formal letter would be sent to the regulatory authorities by the sponsor explaining the reasons for the cessation of the study.

2.8.8 Stopping rules

In addition to the holding rules above for VAC 072, stopping rules for individual participants applied for VAC 072 and VAC 073. Participants who presented with at least one of the following stopping rules were withdrawn from further vaccinations in the study.

The stopping rules are detailed below according to trial and category.

2.8.8.1 VAC 072

2.8.8.1.1 Local reactions

Any participant who experienced injection site ulceration, abscess or necrosis.

2.8.8.1.2 Laboratory AEs

If the participant developed a grade 3 laboratory AE considered possibly, probably or definitely related within 7 days after vaccination and it persisted continuously at grade 3 for > 72 hours.

2.8.8.1.3 Systemic solicited AEs

If the participant developed a grade 3 systemic solicited AE considered possibly, probably or definitely related within 2 days after vaccination (day of vaccination and one subsequent day) and it persisted continuously at grade 3 for > 72 hours.

2.8.8.1.4 Unsolicited AEs

- If the participant had any grade 3 AE considered possibly, probably or definitely related to vaccination, persisting continuously at grade 3 for >72 hours.
- If the participant had an SAE considered possibly, probably or definitely related to vaccination.
- If the participant had an acute allergic reaction or anaphylactic shock following the administration of vaccine investigational product.

2.8.8.2 VAC 073

2.8.8.2.1 SAEs

If any participant experienced any SAE related to the study product (possibly, probably or definitely related).

2.8.8.2.2 SUSARs

If any participant experienced a SUSAR.

2.8.9 Contraindications to subsequent vaccinations

2.8.9.1 Deferral of vaccination

These rules applied to all the clinical trials. If a participant had an acute illness (moderate or severe illness with or without fever) or a fever (oral temperature greater than or equal to 37.5°C) at the scheduled time of administration of the IMP, they were not vaccinated. The vaccine was administered to that participant at a later date within the time window specified in the protocol or withdrawn from the study at the discretion of the investigator.

If a participant was an infant who received a licensed vaccination (such as part of the Expanded Programme of Immunisation, EPI), a minimum of a one-week interval was applied.

2.8.9.2 Absolute contraindications to further vaccination and withdrawal of participant

These rules applied to all the clinical trials. If a participant experienced any of the following, in line with the stopping rules mentioned above, they were withdrawn from the trial:

- Injection site ulceration, abscess, or necrosis associated with product administration.
- An acute allergic reaction, significant IgE-mediated event or anaphylactic shock following the administration of the IMP.
- Any significant disease, disorder or situation which, in the opinion of the investigator, CI, LSM, study Sponsor or EC, would put the participants at risk because of participation in the trial, or may influence the result of the trial, or the participant's ability to participate in the trial.

All vaccinated participants were followed for safety until resolution or stabilization (if determined to be chronic sequelae) of their AEs if they were deemed possibly, probably or definitely related to the IMP.

2.8.10 Local Safety Monitor (LSM)

LSMs were appointed for all studies to provide real-time safety oversight. These were clinicians resident locally to the study sites but independent of the study team. They reviewed all SAEs and were available for clinical advice if any of the study teams felt this was required.

They were also able to recommend withdrawal of participants to the sponsor, if deemed clinically necessary.

In VAC 072, the LSM was provided with safety reports on participants' adverse events following first vaccinations according to a specific schedule detailed in the relevant chapter. They made the decision based on these data to proceed with dose escalation and the rest of the study. In VAC 076, in case of an urgent safety issue or on the advice of the DSMB, the LSM was able to unblind participants without unblinding the local study team.

2.8.11 Data and Safety Monitoring Board (DSMB)

There was the same DSMB for both the VAC 073 and VAC 076 studies and this was coordinated by the Multi-stage Malaria Vaccine Consortium (MMVC). It included multiple members and a requirement was at least one clinician and one statistician and a quorum of four members was required for specific decisions to be made. They convened prior to the studies starting to review the protocol and their responsibilities, and were provided with all relevant data on R21/Matrix-M. They reviewed all SAEs when they occurred and were provided with interim safety reports as and when data were available for review. Based on any of the data provided to them, they could request withdrawals or unblinding of participants (in the case of VAC 076).

In VAC 073, prior to each age de-escalation and dose escalation, the DSMB received a report on safety data to date in the trial and met to discuss any recommendations and next steps. Written approval from the DSMB was required for progression to the next dose or age group.

In VAC 076, interim blinded safety data were presented after the first 30 vaccinations, following the primary series of vaccinations and following the booster vaccinations.

All published work was sent to the DSMB.

2.9 Assessment of vaccine immunogenicity

2.9.1 Total NANP- specific IgG responses

Enzyme-linked immunosorbent assays (ELISA) measuring total human IgG responses to the NANP repeat region of CSP were performed according to SOP ML036: NANP6C CS Standardised ELISA at the Jenner Institute, University of Oxford.

Nunc 96-well ELISA plates were coated with a synthetic peptide based on the PfCSP repeat region (NANP) 6, diluted to 0.2µg/mL in 100µL of Dulbecco's phosphate-buffered saline (dPBS) per well and incubated overnight at room temperature (RT). Plates were washed six times with PBS containing 0.5% Tween-20 (PBS/T) and blocked with casein for one hour at RT. After this, plates were washed again and the serum samples were diluted in casein at 1:100, 1:500, 1:1000 or 1:5000, and kept for two hours at RT. After washing again, secondary antibody (goat anti-human IgG conjugated to alkaline phosphatase, Sigma) was added at 1:1000 in casein for one hour at RT. Plates were washed a final time and developed using 4-nitrophenyl phosphate in diethanolamine buffer (Pierce).

Optical density (OD) was read at 405nm using an ELx800 microplate reader.

A reference pool of positive serum formed a standard curve on each plate and was used to calculate ELISA Units for each. An internal control was included on each plate to standardise between assays.

Table 2b: ELISA plate layout

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
Blank	St 1:100	St 1:200	St 1:400	St 1:800	St 1:1600	St 1:3200	St 1:6400	St 1:12800	St 1:25600	St 1:51200	Blank
Blank	St 1:100	St 1:200	St 1:400	St 1:800	St 1:1600	St 1:3200	St 1:6400	St 1:12800	St 1:25600	St 1:51200	Blank
Sample 13	Sample 14	Sample 15	Sample 16	Internal control	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
Sample 13	Sample 14	Sample 15	Sample 16	Internal control	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
Sample 13	Sample 14	Sample 15	Sample 16	Internal control	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23

2.10 Assessment of vaccine efficacy

2.10.1 Controlled Human Malaria Infection (CHMI)

In VAC 072, the UK trial, CHMI was used to assess efficacy of R21/Matrix-M. CHMI took place in a designated CL3 suite in the Sir Alexander Fleming building, Imperial College, London. This involved administration of five infectious mosquito bites, performed according to the Jenner Institute SOP MC011: Mosquito Bite Malaria Sporozoite Challenge.

2.10.1.1 Preparation

The mosquitoes were provided by the Walter Reed Army Institute of Research (WRAIR), USA, which were infected via a membrane feeder containing fully sensitive 3D7 *Plasmodium falciparum* plus fresh O+ red cells and A+ human serum. Blood products were purchased commercially by WRAIR but have been screened according to the US Red Cross guidelines for *Trypanosoma cruzi*, HIV 1/2, HBV, HCV, HTLV, West Nile virus and syphilis.

A representative of WRAIR directly supplied the infected mosquitoes to an appropriate researcher at Imperial College and they were transferred to a secure room within the insectary. Each pot of mosquitoes was stored in strong, transparent plastic boxes or a secure Perspex glovebox and manipulation of mosquitoes took place in the glovebox cabinet.

Immediately prior to the challenge, an appropriately trained researcher prepared the mosquitoes, transferring them into a pot according to SAF Level 6 COP SOP 7: Use of Glovebox to Remove Unfed Mosquitoes. The mosquitoes are transferred in groups of 5, 4, 3, 2, and 1 into small capture cups. These pots are put in a second cup to minimise escape and covered with netting.

2.10.1.2 CHMI procedure for participants

CHMI participants were in a secure room in the malaria culture facility with appropriate safety measures in place in the event of a participant becoming unwell or suffering anaphylaxis. Initially, participants placed a cup with five infectious mosquitoes on their forearm for approximately five minutes. Following this, fed mosquitoes (evident by the blood meal in their

abdomen) were individually dissected by staff from WRAIR according to WRAIR SOP UWF-B-M-204 Dissection and Evaluation of Infected Salivary Glands. The number of sporozoites per pair of infected salivary glands was estimated using a scale mentioned in the SOP (0 to +4; a gland rating of +2 or more, representing 10 or more observed sporozoites, qualified as infectious). By this method, if the participant had been inoculated by less than five infected mosquitoes, further mosquitoes fed on the participant until a total of five appropriately infected mosquitoes had fed. Once this had occurred, the participant was able to leave the secure room and return home.

2.10.1.3 Follow-up of participants following CHMI

Participants were called daily for the first five days to check they were well and contactable. From the evening of day 6 following malaria challenge (C+6.5), they attended the clinic for clinical review and a blood test twice daily until the evening of day 14 (C+14.5) (supplementary appendix table 9 and supplementary appendix table 10). If participants reached day 15 without being diagnosed, they were reviewed once daily until day 21. If they reached this point (day 21) without diagnosis, they were considered protected but were still treated appropriately for malaria according to local and national guidelines. Any participant diagnosed with malaria was treated immediately. Once treated for malaria, regardless of diagnosis, participants had two further blood tests at 24 and 48 hours post treatment to ensure resolution or no infection.

Due to the COVID-19 pandemic restrictions, and the need to reduce clinic appointments as well as facilitate social distancing and exposure risk, participants did not have the once daily follow-up between day 15 and day 21 for the repeat CHMI for groups 2 and 3, and the initial CHMI of groups 4 and 7 (VAC 072B). Participants were considered protected if they were not diagnosed by day 15. This was considered appropriate as in the first malaria challenge (VAC 072A), all participants who were not protected had a positive quantitative PCR (qPCR) result of >20 parasites/ml by day 13 (C+13). These participants all went on to meet the initial efficacy endpoint and were diagnosed with malaria. In the control group, the mean time to positive qPCR result was day 8 (C+8) and for vaccinated participants, it was 10 days post challenge.

All participants, regardless of recruitment site, were reviewed at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM) in Oxford and were required to stay in Oxford for the duration of follow-up and treatment. All participants had access to an on-call service 24 hours/day where an appropriately trial-delegated clinician could advise or review the participant in case of acute illness.

2.10.1.4 Diagnosis of malaria in CHMI participants

Venous blood samples were taken at each study visit during the post-CHMI follow-up period and processed within 6 hours of collection. A diagnosis of malaria infection was made and treatment with anti-malarial treatment initiated immediately for any participant who satisfied the following criteria discussed below.

2.10.1.4.1 VAC 072A (first malaria challenge)

1. The presence of symptoms suggestive of malaria infection (in the absence of a clear alternate aetiology) in addition to:

Any available qPCR result $\geq 1,000$ parasites/ml

2. Asymptomatic participant with any available qPCR result $\geq 10,000$ parasites/ml.
3. Any participant deemed to have symptoms suggestive of malaria infection in the absence of a clear alternate aetiology if they developed the following:

Any one of (strong indicators):

- Fever with temperature > 38.0 (documented in clinic or on diary card)
- Rigors

OR

A pattern of other clinical indicators which at the investigator's judgement was deemed to be highly likely to represent malaria infection with evident impact on daily activities (for example - any symptom at grade 3; multiple symptoms at grade 2):

- Fever 37.5-37.9°C; Feverishness; chills; sweats; headache; nausea; vomiting; diarrhoea; myalgia; arthralgia; low back pain, fatigue, malaise
- Physical findings, tachycardia, hypotension.

2.10.1.4.2 VAC 072B (repeat challenge for groups 2 and 3 and first challenge for groups 4 and 7)

1. Any available qPCR result ≥ 20 parasites/mL on 2 separate occasions.
2. A pattern of other clinical indicators which the investigator deems likely to represent malaria infection, with evident impact on daily activities (e.g. a single grade 3 symptom or multiple grade 2 symptoms as discussed above).

2.10.1.4.3 Other guidelines applicable to both malaria challenges

The investigators were able to treat any participant for malaria regardless of the qPCR result if they were clinically concerned (and discussed the case with the CI), or if the participant wanted to withdraw from the study. However, this did not occur in the VAC 072 study.

When a case of malaria was diagnosed, each participant had a clinical evaluation by one of the investigators (a clinician) with appropriate history and physical examination where deemed to be necessary. If required, the case could be discussed with the infectious diseases team. If required, there was the possibility for the participant to be admitted under their care at the Oxford University Hospital NHS Foundation Trust for observation and further medical management.

2.10.2 Parasite Quantitative PCR (qPCR)

P. falciparum qPCR was performed as previously described (170), with the following modifications. Blood was collected at baseline and at clinical protocol defined time points following CHMI for qPCR in 2.0ml tubes containing ethylenediaminetetraacetic acid (EDTA). The method for testing by qPCR can be found in SOP ML008: Malaria qPCR and was performed at the Jenner Institute, University of Oxford.

DNA was extracted from 0.4mL EDTA whole blood using a Qiasymphony SP robot, utilising the Qiagen DSP Blood Midi Kit and the pre-loaded Blood 400 v6 extraction protocol, with a 100µl elution in ATE (elution) buffer selected. Five microlitres of each extraction were used per assay well and were run in triplicate for qPCR (equivalent to 60µL blood directly assessed). Parasites per mL (p/mL) equivalent mean values were generated by a standard Taqman absolute quantitation, against a defined standard curve of diluted *P. falciparum* 3D7 DNA, qualified against DNA from counted parasites in whole blood, previously extracted by the same method. qPCR was conducted on an ABI StepOne Plus machine with v2.3 software, using default Universal qPCR and QC settings, apart from the use of 45 cycles and 25µL reaction volume.

This process has been formally validated as suitable for diagnostic purposes and in-house qPCR detection methods are regularly externally assessed by participation in the UKNEQAS Malaria (Molecular) scheme.

2.10.3 Field efficacy

This was assessed in VAC 076 in the phase IIb trial in Burkina Faso. Active and passive case detection was used for the first 6 months following the primary series of vaccinations. That meant that all participants presenting to health facilities were evaluated for clinical malaria and participants were also visited by field workers every 30 days until 6 months following vaccination. If they were febrile at the visit, or there was a history of fever in the preceding 24 hours, they were referred to the study centre for a blood film to be taken to assess for malaria or for other sources of infection to be investigated. After 6 months following the primary series of vaccinations, all malaria case detection was passive, with parents advised to seek medical attention in the case of their child being unwell. Episodes of clinical malaria were measured as per protocol at specific time points: 6 and 12 months following the primary series of vaccinations, and 6 and 12 months following the booster vaccinations.

Blood films were taken at these specific time points also to evaluate for asymptomatic malaria.

2.10.3.1 Primary case definition of clinical malaria

Presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ AND *P. falciparum* parasites density > 5000 asexual forms/ μL .

2.10.3.2 Secondary case definition of clinical malaria

Presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ and/ or history of fever within the last 24 hours AND *P. falciparum* parasites density > 0 .

2.10.3.3 Primary case definition of asymptomatic malaria

Presence of axillary temperature $< 37.5^{\circ}\text{C}$ and absence of history of fever within the last 24 hours AND *P. falciparum* parasites density > 0 asexual forms/ μL .

2.10.3.4 Primary case definition of severe malaria

Presence of *P. falciparum* parasites density > 5000 asexual forms/ μL AND one of more of the following criteria of disease severity:

- Prostration/respiratory distress
- Blantyre coma score ≤ 3
- Seizures: 2 or more
- Hypoglycaemia $< 2.2\text{mmol/L}$
- Acidosis BE $\leq -8.0\text{mmol/L}$
- Lactate $\geq 5.0\text{mmol/L}$
- Anaemia $< 5.0\text{g/dL}$
- Acute kidney injury/pulmonary oedema
- Significant bleeding/shock (systolic BP $< 70\text{mmHg}$);

AND without any of the following criteria of co-morbidity:

- Pneumonia (confirmed by X-ray)
- Meningitis (confirmed by CSF examination)
- Sepsis (with positive blood culture)

- Gastroenteritis with dehydration

2.10.3.5 Secondary case definitions of severe malaria

- a) Presence of *P. falciparum* parasites density > 5000 asexual forms/μL AND one of more of the following criteria of disease severity:

- Prostration/respiratory distress
- Blantyre coma score ≤ 3
- Seizures: 2 or more
- Hypoglycaemia < 2.2mmol/L
- Acidosis BE ≤ -8.0 mmol/L
- Lactate ≥ 5.0 mmol/L
- Anaemia < 5.0g/dL
- Acute kidney injury
- Pulmonary oedema
- Significant bleeding
- Shock (systolic BP <70mmHg);

And with any of the following criteria of co-morbidity:

- Pneumonia (confirmed by X-ray)
- Meningitis (confirmed by CSF examination)
- Sepsis (with positive blood culture)
- Gastroenteritis with dehydration

- b) Presence of *P. falciparum* parasites density > 0 asexual forms/μL AND one of more of the following criteria of disease severity:

- Prostration/respiratory distress
- Blantyre coma score ≤ 3

- Seizures: 2 or more
- Hypoglycaemia < 2.2mmol/L
- Acidosis BE $\leq$ -8.0 mmol/L
- Lactate $\geq$ 5.0 mmol/L
- Anaemia < 5.0g/dL
- Acute kidney injury
- Pulmonary oedema
- Significant bleeding
- Shock (systolic BP <70mmHg);

AND without any of the following criteria of co-morbidity:

- Pneumonia (confirmed by X-ray)
- Meningitis (confirmed by CSF examination)
- Sepsis (with positive blood culture)
- Gastroenteritis with dehydration

2.10.4 Thick blood film preparation and interpretation

These procedures were performed according to local site SOPs at the Nanoro site in Burkina Faso based on the WHO SOPs for 'Giemsa staining of blood films', 'Microscopy examination of thick and thin blood films for examination for identification of malaria parasites' and 'Malaria parasite counting'. Two independent microscopists reviewed each blood film to assess species and calculated the parasite density for asexual parasites and gametocytes. These results were checked by the laboratory co-ordinator (or delegated individual) and if they were not concordant in terms of species, or the error margin allowed for parasite density for asexual parasites or gametocytes, a third, more experienced microscopist was required to review the blood film. The two results that agreed formed the final interpretation.

2.11 Assessment of gut microbiome

2.11.1 Gut microbiome sampling

All participants in the VAC 076 study had a rectal swab prior to first vaccination. Rectal swabs used were the Norgen Fecal swab collection and preservation system.

2.11.2 DNA extraction from samples

This took place at the Jenner Institute, University of Oxford. The QIAamp 96 PowerFecal QIAcube HT kit with the QiaCube HT was used to perform DNA extractions according to the kit protocol. Briefly, 300µl of rectal swab fluid samples (clarified by centrifugation to remove particles) were added to a deep-well plate, followed by 20µl Proteinase K. These were then placed on the QiaCube HT for the extractions to be performed according to the pre-loaded protocol for the PowerFecal kit. Following this, DNA extracts were quantified using a Thermo Fisher NanoDrop One instrument for initial quality assessment of sample DNA. Microbial Community standards (ZymoBIOMICS, USA, D6300) and Microbial DNA standards (ZymoBIOMICS, USA, D6306) were used as positive extraction and sequencing controls, respectively. A negative extraction control was also included using Phosphate-buffered saline (PBS) instead of a sample.

2.11.3 16S rRNA amplicon sequencing

Sequencing was performed at the Oxford Genomics Centre (OGC). For bacterial 16S rRNA gene amplicon sequencing, the variable V3 and V4 regions of the 16S rRNA gene were amplified from genomic DNA using the primers (standard IUPAC nucleotide nomenclature):

Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC

WGCAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG,

Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG
TATCTAATCC.

The amplicons were then attached with indices and Illumina sequencing adapters using the Nextera XT index kit. The 16S amplicon libraries were pooled and sequenced on two Illumina MiSeq v3 flowcell as 300 paired-end reads. Sequences from two flowcells were then combined for subsequent sequence processing. Read depth averaged at 50 000 reads/sample.

2.11.4 Sequence processing

This was performed by the Oxford Centre for Microbiome Studies (OCMS). Quality of raw sequencing data was assessed using FastQC (v0.11.07). Following quality assessment, downstream processing was performed using dada2 (PMID:27214047) which was implemented in the OCMS 16S pipeline from the OCMS 16S repository (https://github.com/OxfordCMS/OCMS_16S). Host sequences were aligned and removed using the MetaSequencing pipeline (<https://github.com/microbialman/MetaSequencing>) which used Bowtie2 (<http://bowtie-bio.sourceforge.net/index.shtml>) and its default parameters to align amplicon sequences against the human genome (hg38). The filterAndTrim function in dada2 was used to truncate the reads to 200bp. Retained primer sequences were removed from reads (17bp) which resulted in final read lengths of 183bp. Error learning, de-replication and sample inference were performed using dada2 with default parameters. Taxonomy was assigned to amplicon sequence variants (ASVs) using the assign taxonomy function in dada2 and the NCBI RefSeq training data (https://zenodo.org/record/2541239/files/RefSeq-RDP16S_v2_May2018.fa.gz).

2.11.5 Data Processing

All positive and negative controls were analysed using OCMS Standards pipeline (https://github.com/OxfordCMS/OCMS_zymoBIOMICS). Batch effects from sample DNA extraction were detected with the adonis function from the vegan R package, applied to Bray-Curtis distances at family level relative abundances. Batch effects were corrected using the ComBat_seq function from the sva R package. Batch corrected sequences were then aggregated at each taxonomic level for subsequent analyses. Aggregated sequences that were in low abundance and low prevalence were removed. Aggregated sequences were

filtered using 5 read counts and 3 sample prevalence as thresholds. Filtered, aggregated counts were used for all subsequent analyses.

2.12 Statistics

2.12.1 Safety

All adverse events were tabulated or figures were produced to display these, AEs were described in numbers and percentages according to group, frequency and severity. Where applicable, chi-square, Fisher's exact test or logistic regression models were used to compare data between groups.

These analyses were performed using GraphPad PRISM v9.0 and Stata v16.1.

2.12.2 Immunogenicity

Across all the trials, descriptive summaries and plots over the time course of individual participant groups were presented. Where appropriate, highly skewed data were log-transformed and presented as geometric means (GMT) with 95% confidence intervals (CI). Significance testing between groups was performed using Mann-Whitney and Dunn's multiple comparisons test. A p value of < 0.05 was considered significant.

Spearman's rank correlation between number of malaria episodes and NANP-specific IgG responses was performed for each malaria vaccine group (1 and 2) and for these groups combined to assess for an immune correlate of protection. A linear regression model was used to estimate the difference in mean log (ELISA) between those participants who had at least one episode of malaria and those participants who did not. Hazard ratios (HR) for log (ELISA) were calculated when included in the Cox model for time to first episode of malaria for each year. A reverse cumulative distribution of antibody titres was then plotted to enable calculation of a threshold of anti-NANP antibodies required for the efficacy observed in the malaria vaccine groups.

These analyses were performed using GraphPad PRISM v9.0 and Stata v16.1.

2.12.3 Efficacy

2.12.3.1 VAC 072

The number of completely protected individuals was presented for each vaccine group and the controls. Completely protected individuals were those who following sporozoite challenge, did not develop blood stage infection as defined in section 2.10.1.4 by day 21 in the first challenge and day 14 in the second challenge. Due to the number of participants that can safely undergo CHMI at one time, numbers for CHMI were limited to 42 which is common across CHMI studies worldwide. Therefore, this meant the study was likely underpowered to detect a difference between vaccine groups. Kaplan-Meier analyses were performed for each vaccine regime and controls. A statistically significant difference between a vaccine regime and control group by log-rank analysis of the Kaplan-Meier curves was considered as an indicator of likely VE compared with the control group. A p value of < 0.05 was considered significant.

These statistical analyses were performed using GraphPad PRISM v9.0.

2.12.3.2 VAC 076

The study was powered to provide an initial point estimate of VE in either of the malaria vaccine groups, assuming VE over 6 months was $>50\%$. Cox regression models were used to analyse the first episode of clinical malaria from 14 days after the third vaccination in the primary series to 6 months and 1 year following primary series of vaccinations. The same occurred for the first booster vaccination. Participants without clinical malaria were censored at their date of withdrawal or the date of their 6- or 12-month blood sample. The primary comparisons were prespecified as comparisons between groups 1 and 3, groups 2 and 3 (i.e. malaria vaccine groups versus control group) (171).

A secondary analysis adjusted for confounding factors including gender, age category at randomisation (5-9 months, 10-12 months and >12 months), adequate bed net use and rounds of SMC. VE was calculated as $1 - HR$ (171).

Primary analyses of VE were based on a modified intention-to-treat population. The secondary outcomes of asymptomatic malaria and multiple malaria episodes were analysed at 6 and 12 months using a log-binomial model, including a randomised group as a covariate. Adjustment for confounders was again performed (171).

Negative binomial regression models were used to analyse multiple episodes of the primary efficacy case definition and adjustment for confounders performed. In order to try and avoid double-counting of malaria episodes which were associated with more than one healthcare contact, attendances at clinic within 7 days of a previous episode were not counted. No adjustment was made to the person-time at risk after a disease episode. A modified ordinary least squares regression approach was used (172) to calculate the incidence rate differences (IRD) per 1000 child-years at risk between the study groups. This allowed an estimate to be made of potential cases averted by vaccination (173).

These statistical analyses were performed using Stata v16.1.

2.12.3.3 Gut microbiome

Details of the statistical analysis used for analysing the gut microbiome will be described in detail in chapter 6.

- 3 A Phase I/IIa sporozoite challenge study to assess the safety, immunogenicity and protective efficacy of adjuvanted R21, administered in different dose schedules in healthy UK volunteers**

3.1 Introduction

The success of the VAC 065 clinical trial conducted at the Jenner Institute, assessing the safety, immunogenicity and efficacy of R21c/Matrix-M, prompted the transfer of manufacturing of this vaccine to Serum Institute of India Private Ltd. (SIPL). At the same time, a commercial licence was agreed between Oxford University Innovation and SIPL. This was to ensure large-scale manufacturing was possible and at a lower cost. This resulted in a new batch of R21 vaccine being produced: the C-tag (4 amino acids: E-P-E-A) was no longer required for purification due to updated manufacturing methods, and expression in a different yeast (*Hansenula polymorpha*) to increase vaccine yield. Given these changes, the vaccine was considered a new vaccine by the UK regulatory authority (MHRA) and therefore, required to go through phase I testing again.

R21/Matrix-M required this evaluation and progress in clinical development as it is potentially a more efficacious, less expensive vaccine that can be more readily deployed at scale than the current leading malaria vaccine, RTS,S/AS01, for three reasons: 1) the antigen dose of R21 is approximately five-fold lower and due to the lack of unfused HBsAg molecules in the virus-like particle (VLP), there is a higher proportion of antigen available in the vaccine, 2) Matrix-M is a less complex and less expensive adjuvant that can be manufactured easily at very large scale and 3) a commitment from SIPL for large-scale supply at an affordable price to sub-Saharan Africa, where mortality from malaria is highest.

In this chapter, I report on the safety, immunogenicity and efficacy (using the sporozoite challenge model) of R21/Matrix-M in different dosing schedules, in a phase I/IIa trial in healthy malaria-naïve adults in the UK.

3.2 Methods

Detailed methods of assessment of safety data, assays used for immunology data collection, efficacy data collection and case definitions can be found in chapter 2.

3.3 Study design

3.3.1 Overview

This was a phase I/IIa, open-label, non-randomised sporozoite challenge study to assess the safety, immunogenicity and efficacy of R21 adjuvanted with Matrix-M, when administered in different dose schedules in healthy UK volunteers.

3.3.2 Study sites and population

All participants were healthy, malaria naïve adults aged between 18 and 45 years.

Recruitment and vaccinations were conducted at four sites in the UK: Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), University of Oxford; NIHR Wellcome Trust Clinical Research facility, University of Southampton, NIHR Imperial Clinical Research Facility, Imperial College; and NIHR Guy's and St Thomas' Clinical Research Facility.

All participants who underwent controlled human malaria infection (CHMI) travelled to Imperial College, London, to undergo CHMI by mosquito bites. From day 6.5 following CHMI until completion of a curative course of anti-malarial therapy, all participants stayed in Oxford and attended follow-up visits at CCVTM.

3.3.3 Vaccines

All participants received three intramuscular vaccinations for the primary series of vaccinations. The doses of vaccine tested included 10µg R21/50µg Matrix-M, 50µg R21/50µg Matrix-M and 2µg R21/50µg Matrix-M (Table 3a).

3.3.4 Study groups

3.3.4.1 Group 1

Groups 1a and 1b received three doses of 10µg R21/50µg Matrix-M 4 weeks apart. Group 1a was the safety lead-in group, which will be discussed in more detail in section 3.3.6.

3.3.4.2 Group 2

Groups 2a and 2b received two doses of 10µg R21/50µg Matrix-M 4 weeks apart, and a third dose approximately 24 weeks later. Following the third dose of vaccination, group 2a underwent CHMI with sporozoite challenge (mosquito bite) 4 weeks later.

3.3.4.3 Group 3

Groups 3a and 3b received three doses of 10µg R21/50µg Matrix-M 4 weeks apart in a schedule allowing for sporozoite challenge at the same time as group 2. This was 4 weeks following the third dose of vaccine.

3.3.4.4 Group 4

Groups 4a and 4b received two doses of 50µg R21/50µg Matrix-M 4 weeks apart and a 3rd dose of 10µg R21/50µg Matrix-M at approximately 24 weeks. Group 4a was the safety lead-in group, which will be discussed in more detail in section 3.3.6.

3.3.4.5 Group 5

These participants received two doses of 10µg R21/50µg Matrix-M 4 weeks apart and a third dose of 2µg R21/50µg Matrix-M at approximately 24 weeks.

3.3.4.6 Groups 6 & 7

These participants received no vaccinations as they were infectivity control volunteers for the sporozoite challenge procedures.

3.3.4.7 Re-challenge groups

Participants in groups 2a and 3a who demonstrated sterile protection in the first sporozoite challenge were invited back 6-9 months later for an optional booster vaccination and repeat sporozoite challenge.

3.3.5 Study design amendment

3.3.5.1 CHMI

An amendment was submitted in March 2020 to enable participants in groups 4 and 5 to undergo malaria challenge four weeks following their last vaccination. This was optional to participants.

3.3.5.2 Booster vaccinations

An amendment was submitted in March 2020 for participants in groups 1, 2b and 3b to receive an optional booster vaccination approximately 12 months following the third vaccination. An optional booster vaccination was also available for those sterilely protected following the first malaria challenge in groups 2a and 3a. This booster vaccination would be 4 weeks prior to the malaria challenge.

3.3.5.3 COVID-19 pandemic

An amendment was submitted in August 2020 for the re-challenge of sterilely protected participants in groups 2a and 3a to take place later than originally planned as a result of the COVID-19 pandemic. Follow-up time following malaria challenge was also changed. Additionally, windows on booster vaccinations for groups 1-3 and third vaccinations in groups 4-5 were extended.

Table 3a: VAC 072 trial design.

CHMI = Controlled Human Malaria Infection. Groups 2a, 3a and 6 timelines converged at first CHMI. Sterilely protected participants from the first CHMI in groups 2a and 3a could optionally attend for repeat CHMI and timelines converged with group 7. Groups 2a and 3a which were sterilely protected had the option to receive a booster vaccination approximately 6-8 months following their third vaccination. CHMI was optional for groups 4a, 4b and 5.

Week	0	4	8	60
Group 1a (n=3)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M
Group 1b (n=7)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M

Week	0	4	24	28	56-80	60-84
Group 2a (n=16)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	CHMI	10µg R21/50µg Matrix-M	Repeat CHMI
Group 2b (n=2)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M			
Week	0	4	8	12	40-64	44-68
Group 3a (n=16)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	CHMI	10µg R21/50µg Matrix-M	Repeat CHMI
Group 3b (n=2)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M			
Week				0		0
Group 6 (n=6-8)				CHMI		
Group 7 (n=6-8)						CHMI
Week	0	4	24			28
Group 4a (n=3)	50µg R21/50µg Matrix-M	50µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M			CHMI
Group 4b (n=5-7)	50µg R21/50µg Matrix-M	50µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M			
Week	0	4	24			28
Group 5 (n=8-10)	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	2µg R21/50µg Matrix-M			CHMI

3.3.6 Local safety monitor (LSM)

An independent Local Safety monitor (LSM) was appointed to provide real-time safety oversight. As this was the first time R21 manufactured at SIIPL was being administered to humans, it was necessary to stagger the vaccinations to allow sufficient time to assess safety and reactogenicity, and to have suitable safety reviews conducted by an LSM. They also reviewed all SAEs.

After the first vaccinations for the safety lead-ins, as well as dose escalation, the LSM was consulted and provided with a review of safety data and adverse events (AEs) before proceeding to the next vaccine dose. The first participant in group 1a was vaccinated alone and then reviewed 72 hours following vaccination. As there were no safety concerns with this first vaccination, another two group 1a participants were vaccinated, at least one hour apart, and reviewed 72 hours later. As there were no safety concerns, the remaining participants in groups 2, 3 and 5 were vaccinated. The same safety procedure occurred for groups 4a and 4b, as this group was receiving a higher dose of R21.

3.4 Study design rationale

In the VAC 065 clinical trial previously conducted at the Jenner Institute, high-level efficacy (78%) was noted with R21c/Matrix-M. Furthermore, durable efficacy and antibody responses were maintained at 8.5 months after the third vaccine dose (57). At the time of this study starting, prior to the COVID-19 pandemic, Matrix-M was a relatively new adjuvant that had demonstrated good safety and immunogenicity profiles in a number of trials (116, 174-176).

In view of these encouraging results, it was deemed important to progress clinical evaluation of this new malaria vaccine in populations that might benefit most from it, particularly African infants and children. In order for this to occur, a manufacturing and development partner was required to enable large-scale vaccine supply, at an affordable cost, as discussed previously. Therefore, SIIPL began producing R21. As the manufacturing process was different, this vaccine needed safety, immunogenicity and efficacy evaluation in a malaria-naïve population initially, which is the trial being described in this chapter (VAC 072). Soon after this trial

commenced and preliminary results were available, the phase Ia study in Kenya (VAC 073) started in adults followed by children and infants (discussed in chapter 4).

The standard dosing schedule of malaria vaccines and R21c to date has been three vaccine doses, four weeks apart, and safety and immunogenicity of this newly manufactured R21 with the same regime required assessment. This was assessed in group 1 of this trial. The actual dosage regimes were informed from the previous phase I/IIa trials assessing R21c/Matrix-M. For these trials, various dose schedules were tried based on the doses used with RTS,S/AS01 as this was a similar vaccine. R21 contains recombinant particles expressing the central repeat and C-terminus of *circumsporozoite* protein (CSP) fused to Hepatitis B surface antigen (HBsAg). RTS,S has a four-fold excess of unfused HBsAg resulting in a large proportion of the antibody response towards HBsAg. Therefore, a 50µg dose of R21 contains approximately 25µg of CSP. A 50µg dose of RTS,S contains approximately 10µg of CSP (117). As this dose demonstrated a favourable safety profile and efficacy with RTS,S, but also, given the higher proportion of CSP, and the expected targeted antibody response to CSP rather than HBsAg, lower doses of R21 were also included in the trial design,

Data from other trials have suggested that different vaccine doses and schedules may enhance immunogenicity and efficacy (47, 77, 177). In malaria naïve adults, a delayed fractional dose of RTS,S/AS01, was found to improve immunogenicity and protection against infection (47). Therefore, in this trial, the other aim was to assess if this was the case with R21/Matrix-M, but equally, if the delay in administering the third dose alone may be the reason for the increased immunogenicity and efficacy. The delayed, fractional dosing schedule was assessed in groups 4 and 5 of this trial, while the evaluation of delaying the third dose alone in comparison with the standard dosing regime was assessed in groups 2 and 3. In VAC 065, a fractional third dose was tested in the standard dose regime and this showed no significant difference in immunogenicity or efficacy at 28 days following third vaccination (117).

Booster vaccinations were added to this study design following encouraging initial safety and immunogenicity assessments in this trial. This was because with the RTS,S/AS01 phase III

trial, efficacy was noted to be higher following a fourth booster dose approximately 18 months following the third vaccine dose (41).

This trial was the start of the clinical development programme of R21/Matrix-M, providing adequate safety, immunogenicity and efficacy data, through phase I-III clinical trials in order to progress to an application for vaccine licensure (pending adequate results).

3.5 Ethics and regulatory approval

The trial was approved by the UK National Research Ethics Service, Committee South Central–Oxford A (Ref: 18/SC/0679), the Medicines and Healthcare Products Regulatory Agency (Ref: 21584/0413/001-0001), and the Oxford University Clinical Trials and Research Governance team, who independently and externally monitored compliance with Good Clinical Practice (GCP) guidelines. The trial was registered with ClinicalTrials.gov (Ref: NCT03970993) and an independent local safety monitor provided safety oversight.

3.6 Objectives

3.6.1 Primary objectives

3.6.1.1 Assessment of safety

To assess the safety and tolerability of adjuvanted R21 using different immunisation schedules in healthy malaria-naïve volunteers.

3.6.1.2 Assessment of efficacy

To assess the efficacy (prevention of occurrence of *P. falciparum* parasitaemia, assessed by qPCR) of adjuvanted R21 against malaria sporozoite challenge, in healthy malaria-naïve volunteers using two immunisation regimes.

3.6.2 Secondary objectives

3.6.2.1 Assessment of immunogenicity

To assess humoral immunogenicity generated in malaria-naïve individuals by adjuvanted R21 using different immunisation schedules in healthy malaria-naïve volunteers.

3.6.2.2 Assessment of safety following booster vaccinations

To assess the safety and tolerability of adjuvanted R21 using different immunisation schedules in healthy malaria-naïve volunteers, and the efficacy (prevention of occurrence of *P. falciparum* parasitaemia, assessed by PCR) of adjuvanted R21 against malaria sporozoite challenge, in healthy malaria-naïve volunteers following a booster vaccination.

3.6.2.3 Assessment comparing R21 with R21c

To assess the efficacy, measured as time to *P. falciparum* parasitaemia, assessed by PCR of adjuvanted R21 in two different vaccination regimes and compared to a previous trial of R21c against malaria sporozoite challenge, in healthy malaria-naïve volunteers.

3.7 Assessment of endpoints

3.8 Study participants

All participants were required to sign and date a trial-specific informed consent form prior to any study procedures being performed. The participant information sheet (PIS) was sent to the participant at least 24 hours prior to the screening visit. Participants who wanted to be part of the malaria challenge were required to fill in a questionnaire assessing their understanding of the trial. Following this, participants were assessed and required to satisfy all inclusion criteria and not meet any exclusion criteria (supplementary appendix section 8.1).

3.9 Study interventions

3.9.1 Vaccine manufacture

3.9.1.1 R21

R21 was manufactured under Good Manufacturing Practice (GMP) conditions by the Serum Institute of India Pvt. Ltd. It was supplied in sterile 2ml glass vials and allowed for extraction of a single dose from each vial. It was stored frozen at a nominal temperature of -80°C. Accountability, storage, shipment and handling of R21 were in accordance with relevant local SOPs and forms.

3.9.1.2 Matrix-M

Matrix-M was manufactured under GMP conditions by Novavax AB, Sweden. It was supplied in sterile 2ml glass vials at a concentration of 0.375mg/ml, allowing for more than one dose to be extracted from each vial. It was stored refrigerated at a temperature between +2 - +8°C and required protection from light. Accountability, storage, shipment and handling of Matrix-M were in accordance with relevant local SOPs and forms.

3.9.2 Vaccine preparation

Vaccine preparation was undertaken under aseptic conditions by a delegated clinical research nurse and myself (or another delegated clinician) according to the Jenner Institute SOPs 'VAC 072 Vaccine Dilutions' (supplementary appendix, section 8.6-8.8). On the day of vaccination, R21 was allowed to thaw to room temperature. Following this, it was mixed with Matrix-M. R21 was administered within one hour of removal from the freezer.

3.9.3 Vaccine administration

Vaccines were administered into the non-dominant arm of the participants. During administration of the study vaccines, advanced life support drugs and resuscitation equipment were immediately available for the management of anaphylaxis. Administration of vaccines was performed according to local trial site SOPs.

3.9.4 Assessment of vaccine safety

Participants were observed for sixty minutes following each vaccination and asked to record their temperature and any solicited AEs in the 7-day follow-up period, and unsolicited AEs in the 28-day follow-up period in an electronic diary. A review of solicited, unsolicited and serious AEs occurred at each relevant follow-up visit. Local AEs included pain, redness, warmth and itch. Systemic AEs included fever, arthralgia, myalgia, feverishness, headache, fatigue, nausea and malaise.

Safety bloods included a full blood count, renal and liver function, which were performed at visits on days of each vaccination and 7 and 28 days following each vaccination in the primary

series. Safety bloods following the booster vaccination were taken 28 days later. For those taking part in the CHMI, safety bloods were performed on the day before challenge, 7 days after the challenge, day of diagnosis and 35 and 90 days following the challenge (supplementary appendix table 7 - supplementary appendix table 10).

As a result of the COVID-19 pandemic, the safety assessments were adjusted to facilitate local public health restrictions. Participant safety was paramount at all times and therefore, follow-up visits were conducted virtually where possible.

During the malaria challenge, as detailed in chapter 2, according to time point, participants received once daily phone calls or once or twice daily physical reviews in clinic.

During the vaccine trial and malaria challenge follow-up period, participants had access to a 24 hour on-call phone service for medical advice. This phone was held by myself or a trial-delegated clinician.

3.9.5 Assessment of vaccine efficacy

Efficacy was assessed using CHMI. CHMI took place approximately 28 days following the third vaccination dose or booster dose where applicable. The procedure, follow-up schedule and method of diagnosing and treating malaria are detailed in chapter 2 and the supplementary appendix. *P. falciparum* qPCR was performed by extracting DNA from 0.4mL EDTA whole blood using a Qiasymphony SP robot. Parasites per ml (p/ml) equivalent mean values were generated by a standard Taqman absolute quantitation, against a defined standard curve of diluted *P. falciparum* 3D7 DNA, qualified against DNA from counted parasites in whole blood, which were previously extracted by the same method. qPCR was conducted on an ABI StepOne Plus machine with v2.3 software (see chapter 2). Diagnosis of malaria was based on *P. falciparum* qPCR results and/or clinical signs and symptoms according to the following criteria:

3.9.5.1 VAC 072A (first malaria challenge)

1. The presence of symptoms suggestive of malaria infection (in the absence of a clear alternate aetiology) in addition to:

Any available qPCR result $\geq 1,000$ parasites/ml

2. Asymptomatic participant with any available qPCR result $\geq 10,000$ parasites/ml.
3. Any participant deemed to have symptoms suggestive of malaria infection in the absence of a clear alternate aetiology if they developed the following:

Any one of (strong indicators):

- Fever with temperature > 38.0 (documented in clinic or on diary card)
- Rigors

OR

A pattern of other clinical indicators which at the investigator's judgement was deemed to be highly likely to represent malaria infection with evident impact on daily activities (for example - any symptom at grade 3; multiple symptoms at grade 2):

- Fever $37.5-37.9^{\circ}\text{C}$, feverishness, chills, sweats, headache, nausea, vomiting, diarrhoea, myalgia, arthralgia, low back pain, fatigue, malaise
- Physical examination findings, tachycardia, hypotension.

3.9.5.2 VAC 072B (second malaria challenge)

1. Any available qPCR result ≥ 20 parasites/ml on 2 separate occasions.
2. A pattern of other clinical indicators which the investigator deems likely to represent malaria infection, with evident impact on daily activities (e.g. a single grade 3 symptom or multiple grade 2 symptoms as discussed above).

3.9.6 Assessment of vaccine immunogenicity

NANP-specific IgG were measured by enzyme-linked immunosorbent assay (ELISA) as described in chapter 2. NANP is the central repeat of *P. falciparum* CS protein. Samples were obtained for immunogenicity on the day of first vaccination, 28 days following all vaccinations, and four, six or twelve months following final vaccination according to which group participants were in, and if they took part in the CHMI.

3.10 Results

3.10.1 Participant flow

All participants recruited were healthy, malaria naïve adults aged between 18 and 45 years. Participant recruitment per group is detailed in Figure 3A. Due to time constraints, groups 2a/2b were not fully enrolled. In groups 4a/4b, a participant withdrew after the first vaccination and therefore, was replaced.

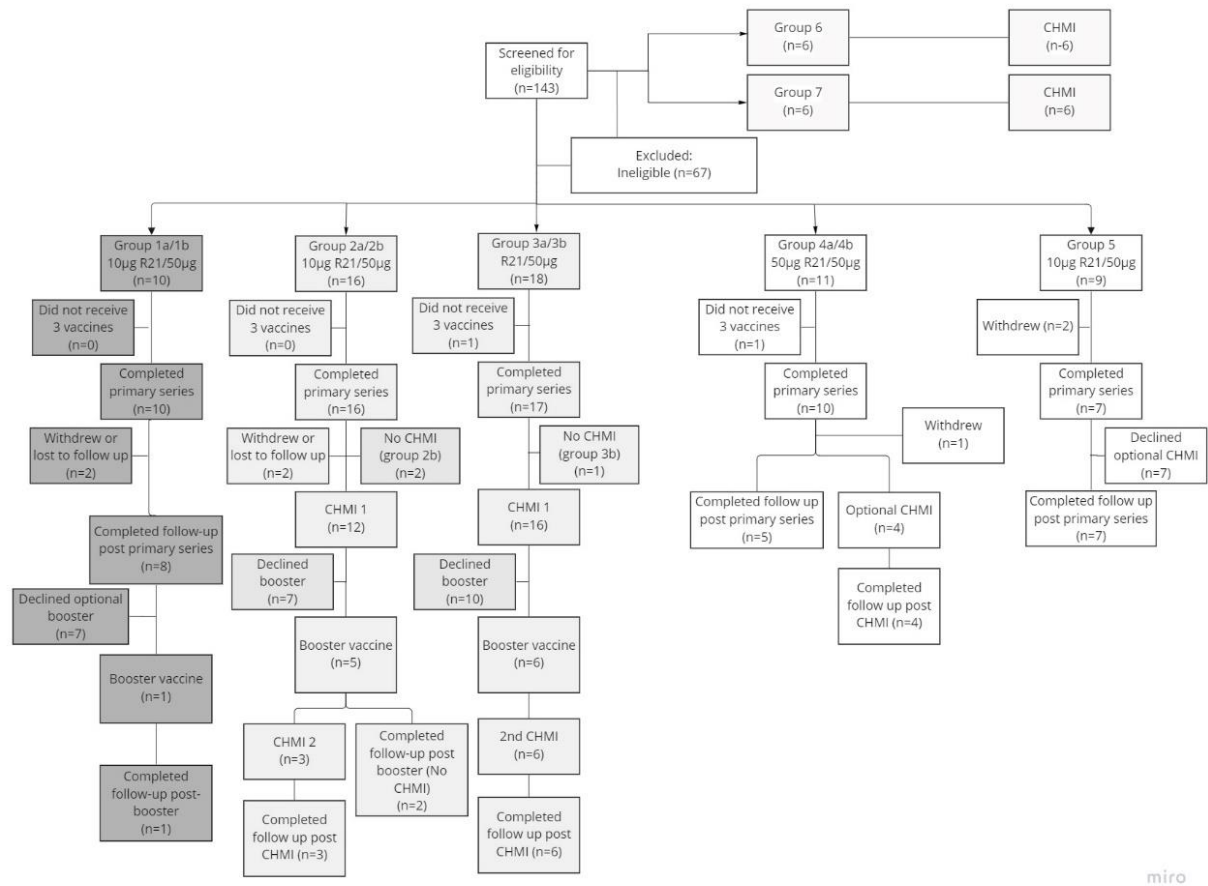


Figure 3A : VAC 072 flow chart of study design and participant recruitment.

A total of 143 volunteers were screened and 76 were enrolled in total. CHMI - controlled human malaria infection.

3.10.2 Participant demographics

Participants were recruited across Oxford, London and Southampton. Their baseline characteristics at the screening visit can be found in Table 3b.

Table 3b: Baseline demographics of vaccinated participants in VAC 072.
Numbers are number of participants (%) unless stated otherwise.

	Group 1 n = 10	Group 2 n = 16	Group 3 n = 18	Group 4 n = 11	Group 5 n = 9
Age in years					
Mean (SD)	26.8 (7.1)	31.0 (9.9)	35.6 (7.0)	30.7 (7.8)	27.3 (5.4)
Gender					
Male	7	9	9	4	2
Female	3	7	9	7	7
BMI					
Mean (SD)	24.8 (3.4)	26.0 (6.1)	26.3 (2.9)	28.2 (6.4)	24.9 (5.7)

3.10.3 R21/Matrix-M safety

3.10.3.1 Serious Adverse Events (SAEs)

There has been one SAE in this trial which was assessed as not related to the investigational medicinal product (IMP). This was a left wrist fracture that occurred in a participant two months following their third vaccination after a sports injury and required surgical intervention.

3.10.3.2 Solicited adverse events (AEs)

Solicited AEs are reported according to dose of vaccine received.

3.10.3.2.1 10µg R21/50µg Matrix-M

The majority of AEs at this dose were mild (Figure 3B and Figure 3C). Five participants suffered a severe AE over the course of vaccinations and most of these improved within 24 hours. One of these AEs was a fever but all AEs improved or resolved within 24-48 hours. Across four doses, eight participants in total had a fever with half of these participants experiencing fever after the third dose. A constellation of systemic AEs were experienced by participants, which were mostly mild flu-like symptoms. The most common local AE experienced was pain at the injection site and the majority of participants experienced this following every dose of vaccination. Typically, the pain resolved within 48-72 hours of vaccination.

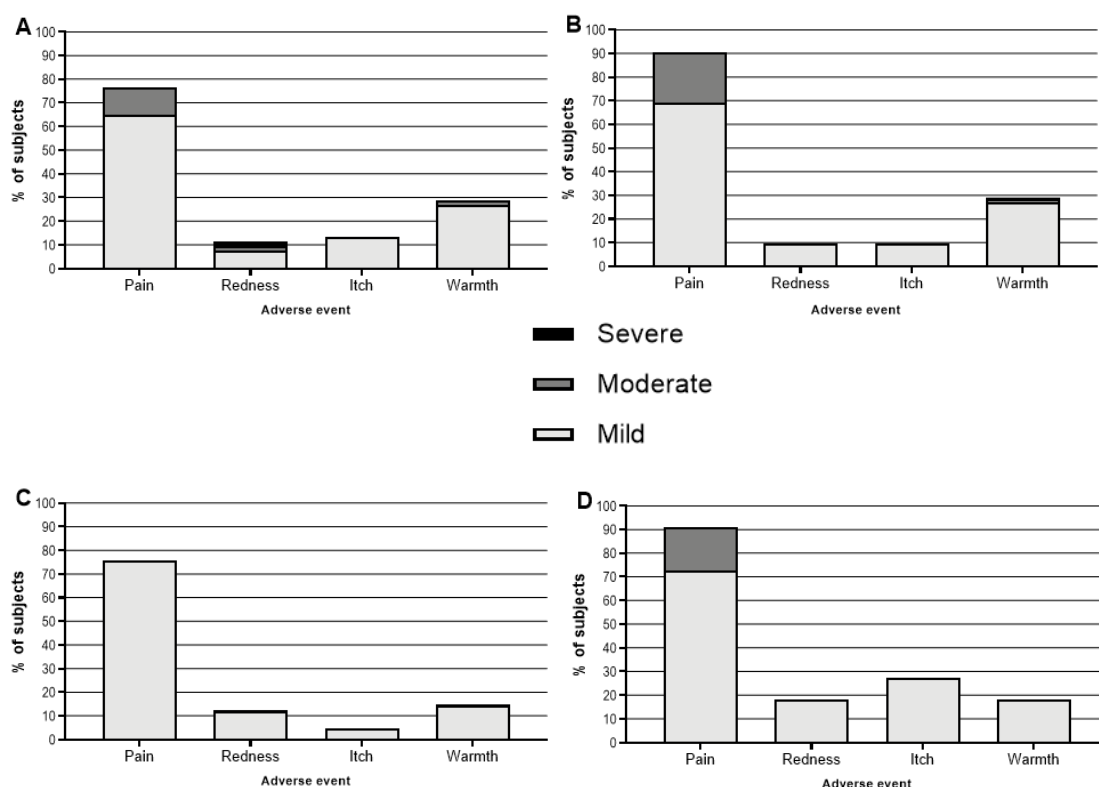
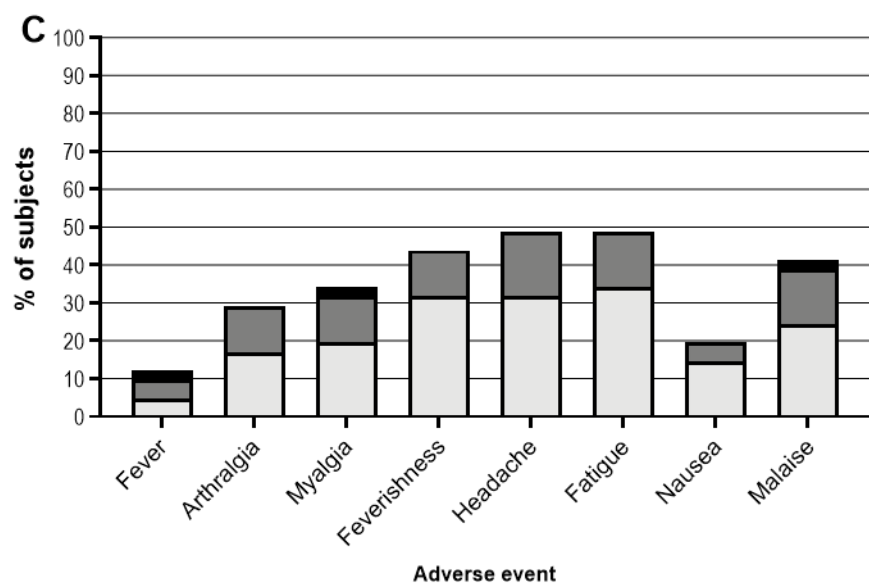
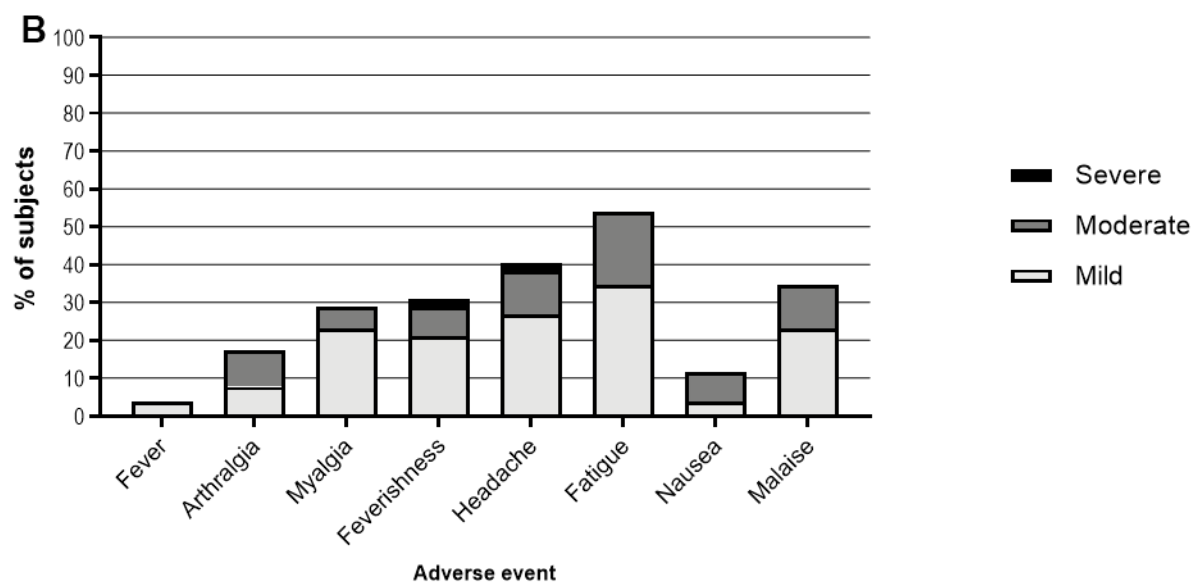
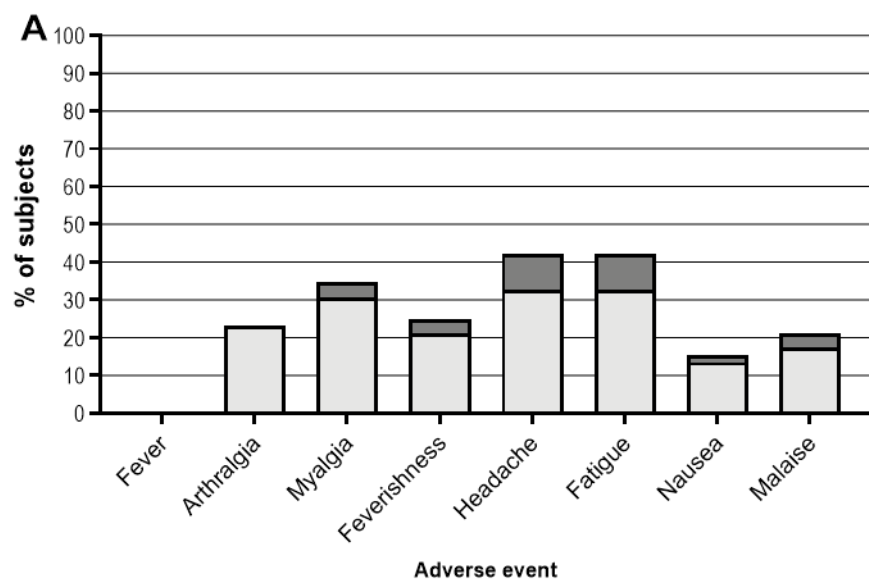


Figure 3B : Local solicited adverse events reported in the first seven days in groups that received 10µg R21/ 50µg Matrix-M.

All participants in groups 1 and 3 received three vaccinations four weeks apart. Participants in group 2 received a delayed third dose and participants in group 5 only received two vaccinations at this dose 4 weeks apart. Eleven participants, in groups 2 and 3, received a fourth booster dose eight months after the third vaccination. Panel A corresponds to the first dose of vaccine (n = 52), panel B corresponds to the second dose of vaccine (n = 52), panel C corresponds to the third dose of vaccine (n = 41) and panel D corresponds to the booster dose of vaccine (n = 11). After each dose, each adverse event per participant is counted once at the highest reported grade.



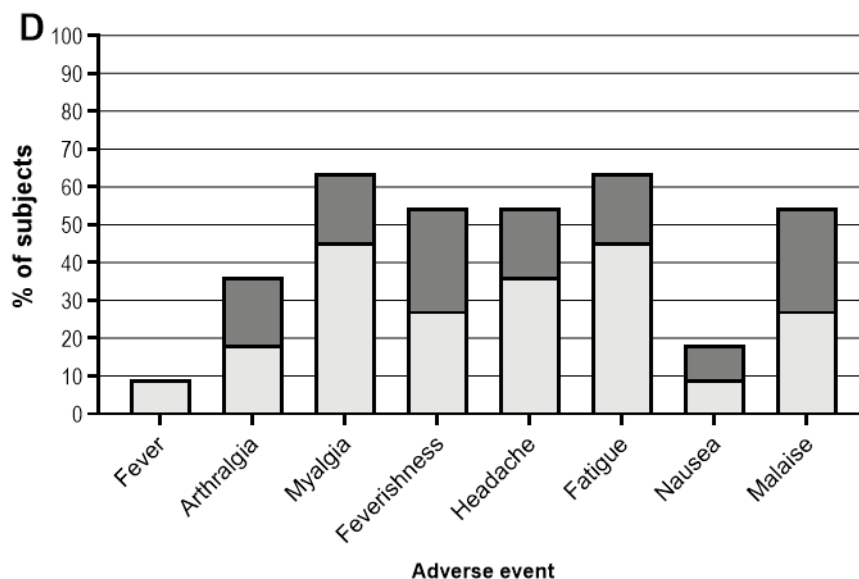


Figure 3C: Systemic solicited adverse events reported in the first seven days in groups that received 10µg R21/ 50µg Matrix-M.

All participants in groups 1 and 3 received three vaccinations four weeks apart. Participants in group 2 received a delayed third dose and participants in group 5 only received two vaccinations at this dose 4 weeks apart. Eleven participants, in groups 2 and 3, received a fourth booster dose eight months after third vaccination. Panel A corresponds to the first dose of vaccine (n = 52), panel B corresponds to the second dose of vaccine (n = 52), panel C corresponds to the third dose of vaccine (n = 41) and panel D corresponds to the booster dose of vaccine (n = 11). After each dose, each adverse event per participant is counted once at the highest reported grade.

3.10.3.2.2 50µg R21/50µg Matrix-M

The majority of AEs at this dose were also mild (Figure 3D and Figure 3E). Two participants experienced severe AEs and these were after the second dose of vaccine. These were pain at the injection site and myalgia for one participant and pain at the injection site and nausea for the other. These did improve within 24-48 hours. No participants experienced fever at this dose, and similar to the 10µg R21/50µg Matrix-M dose, pain at the injection site was the most common local AE. Every participant experienced pain at the injection site following the second dose of vaccine but the majority of these AEs were mild.

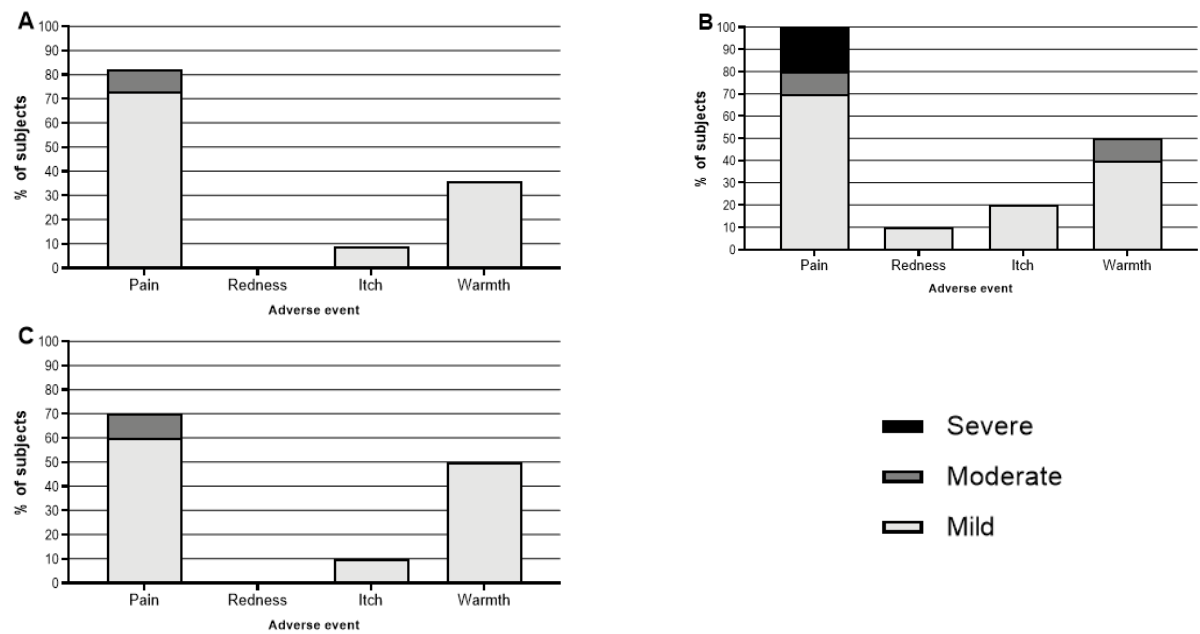


Figure 3D: Local solicited adverse events reported in the first seven days in groups that received 50µg R21/ 50µg Matrix-M and 10µg R21/ 50µg Matrix-M in group 4.

All participants received 50µg R21/ 50µg Matrix-M for the first two doses four weeks apart. A third, delayed dose of 10µg R21/ 50µg Matrix-M was administered approximately six months later. Panel A corresponds to the first dose of vaccine (n = 11), panel B corresponds to the second dose of vaccine (n = 10) and panel C corresponds to the third dose of vaccine (n = 10). After each dose, each adverse event per participant is counted once at the highest reported grade.

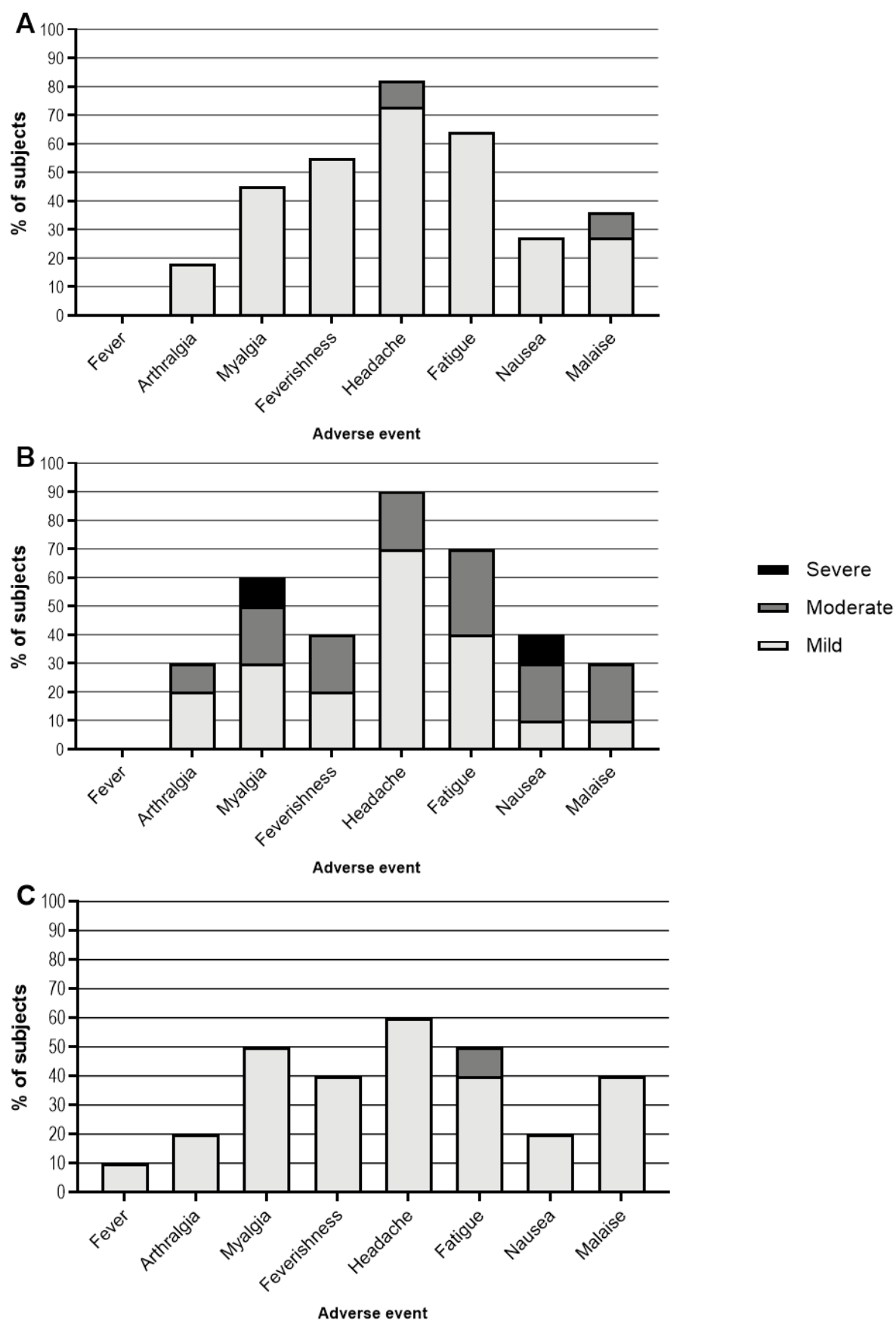


Figure 3E: Systemic solicited adverse events reported in the first seven days in groups that received 50µg R21/ 50µg Matrix-M and 10µg R21/ 50µg Matrix-M in group 4.

(Figure 3E continued) All participants received 50µg R21/ 50µg Matrix-M for the first two doses four weeks apart. A third, delayed dose of 10µg R21/ 50µg Matrix-M was administered approximately six months later. Panel A corresponds to the first dose of vaccine (n = 11), panel B corresponds to the second dose of vaccine (n = 10) and panel C corresponds to the third dose of vaccine (n = 10). After each dose, each adverse event per participant is counted once at the highest reported grade.

3.10.3.2.3 2µg R21/50µg Matrix-M

Few AEs were experienced at this dose and these were almost all mild in severity. No fever was experienced (Figure 3F).

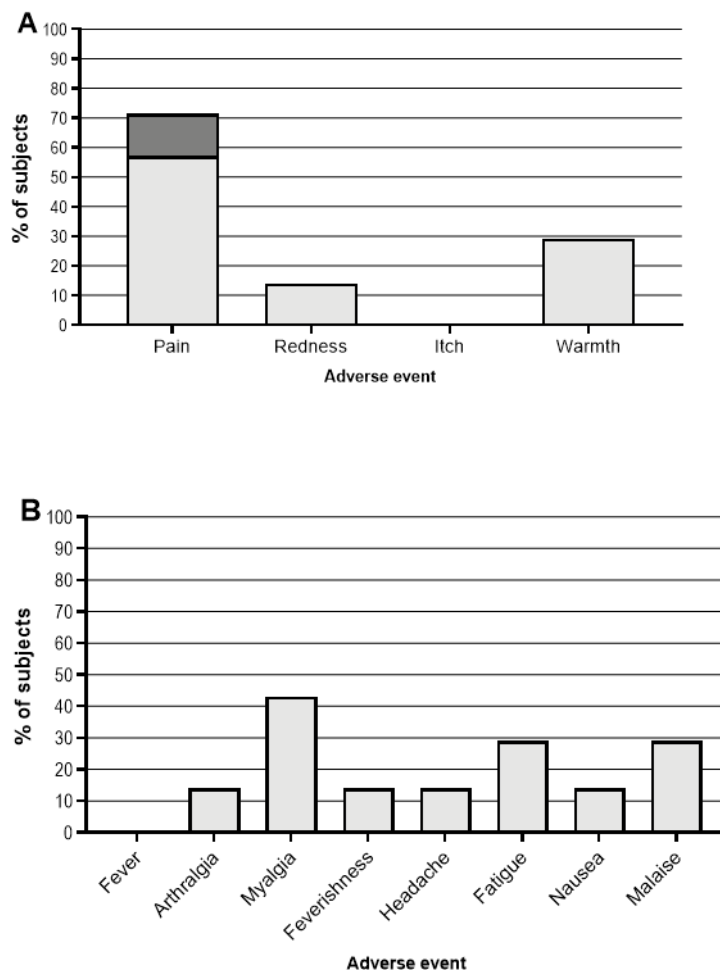


Figure 3F: Local and systemic solicited adverse events reported in the first seven days in groups that received 2µg R21/ 50µg Matrix-M in group 5.

All participants in this group received 10µg R21/ 50µg Matrix-M for the first two doses four weeks apart. A third, delayed dose of 2µg R21/ 50µg Matrix-M was administered approximately six months later. Panel A corresponds to local solicited adverse events (n = 7) and panel B corresponds to systemic solicited adverse events (n = 7). After each dose, each adverse event per participant is counted once at the highest reported grade.

3.10.3.3 Unsolicited adverse events

There were no specific trends across the groups noted with unsolicited AEs. Oropharyngeal pain and rhinorrhoea were the most common experienced unsolicited AEs and for two participants, local AEs of warmth and itch were after the period of solicited AEs. Details of these can be found in Table 3c.

Table 3c: Unsolicited AEs reported up to 28 days follow-up following each vaccination.

All AEs are coded according to MedDRA Preferred Term (PT). AEs displayed were those assessed as possibly, probably or definitely related to vaccination. Each AE is counted once following each vaccination, at the highest reported grade. Grading and causality assessed as per tables in the Supplementary appendix.

Group	MedDRA PT	Severity	Causality	Time following vaccine	point a
3	Abdominal pain	3	Possible	2-3	
1	Abdominal pain upper	1	Possible	0-2	
3		1	Possible	2-3	
2	Back pain	1	Possible	0-1, 4-7	
2	Cough	1	Possible	0-8	
		2	Possible	1-3	
3		1	Possible	4	
4	Diarrhoea	1	Possible	1-3	
		2	Possible	2	
1	Disturbance in attention	1	Possible	0-1	
1	Dizziness	1	Possible	0-1	
4		2	Possible	0	
2	Ear congestion	1	Possible	3-4	
		1	Possible	0-24	
3	Hypertension	3	Possible	0-1	
3	Influenza like illness	2	Possible	0-6	
2	Lymphadenopathy	1	Probable	1-4	
		1	Probable	1-5	
1	Malaise	2	Possible	9-11	
2	Menstrual disorder	1	Possible	25-26	
3	Migraine	2	Possible	1-2	
2	Muscle spasms	1	Possible	3-5	
4	Muscular weakness	3	Probable	0-4	
2	Nasal congestion	1	Possible	0-1	
3		1	Possible	2-3	
1	Nausea	2	Probable	0-1	
3	Oral herpes	2	Possible	5-14	
2	Oropharyngeal pain	1	Possible	0-2	
		1	Possible	5-6	
3		1	Possible	4-6	
		1	Possible	7-10	
		1	Possible	2-4	
		1	Possible	1-3	
4		1	Possible	0-2	

Group	MedDRA PT	Severity	Causality	Time following vaccine point a
5		1	Possible	6-8
1	Papule	2	Possible	4-7
3	Poor quality sleep	2	Possible	1-3
2	Pruritus	1	Possible	0-1
3	Rash	1	Possible	4-5
2	Rash pruritic	1	Possible	3-6, 16
		1	Possible	2-6, 10-11
4		1	Probable	1-2
3	Restlessness	1	Possible	1-2
1	Rhinitis allergic	1	Possible	1-2
1	Rhinorrhoea	1	Possible	6-7
2		1	Possible	0-5
		1	Possible	0-1
3		1	Possible	6-7
		1	Possible	0-6
		1	Possible	1-2
5		1	Possible	0-3
1	Sneezing	1	Possible	0-1
3	Sinus headache	1	Possible	1-6
3	Syncope	3	Possible	2-3
1	Throat tightness	1	Possible	0-2
3	Tonsillar inflammation	1	Possible	2-6
3	Vaccination site discoloration	1	Definite	1-3
2	Vaccination site hypoaesthesia	1	Probable	6
4	Vaccination site pruritus	3	Probable	7-8
2	Vaccination site swelling	1	Definite	0-1
		1	Definite	0-1
3		1	Definite	1-3
4		1	Definite	2-6
4	Vaccination site warmth	1	Probable	7-8
3	Vomiting	3	Possible	2-3

3.10.3.4 Laboratory adverse events

There were no specific laboratory abnormalities following any of the doses of vaccines.

3.10.4 R21/Matrix-M efficacy

3.10.4.1 VAC 072A

This was the first CHMI assessing R21/Matrix-M vaccine efficacy (VE) after three doses in groups 2 and 3. There was also a control group. In group 2, the group which received the delayed third dose, 9/12 (75%) participants were sterilely protected against malaria and 10/16

(62.5%) in group 3 (Figure 3G). All participants in the control group (group 6), were diagnosed with malaria. When comparing VE between groups 2 and 3, there was no significant difference ($p = 0.52$) but when comparing group 2 and 3 to the control group, there was a significant difference ($p < 0.0001$). The mean time to meet one of the diagnostic endpoints in group 2 was 13.8 days, in group 3 it was 14.2 days and in the control group, it was 12.1 days. The mean time to diagnosis based on two blood samples with detectable levels of parasitaemia (>20 parasites/ml) was also calculated; in group 2, it was 10.7 days, in group 3 it was 11.1 days and in the control group, it was 8.2 days (Figure 3G). All control participants, 1/3 participants in group 2 and 3/6 participants in group 3 were diagnosed using the criteria of clinical malaria symptoms and any available qPCR result $\geq 1,000$ parasites/ml. The remaining five participants were asymptomatic and diagnosed when their qPCR result was $\geq 10,000$ parasites/ml.

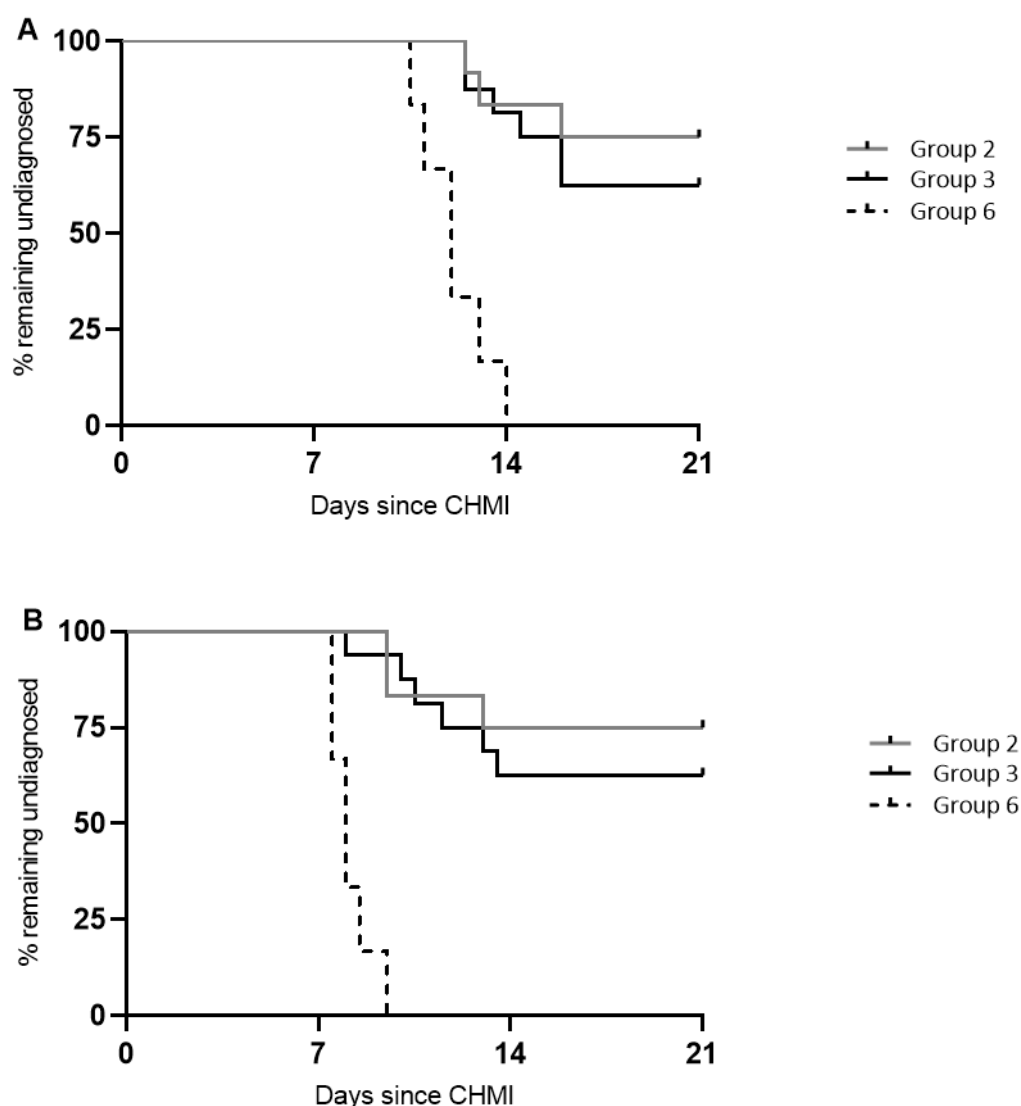


Figure 3G: VAC 072A malaria challenge results. Efficacy of R21/Matrix-M administered in different dose schedules.

Panel A shows Kaplan-Meier survival analysis of time to treatment according to pre-specified diagnostic endpoints and panel B shows time to participant having >20 parasites/ml detectable on two blood samples. Group 2 received 2 doses of 10µg R21/ 50µg Matrix-M 4 weeks apart and a delayed dose 6 months later. Group 3 received 3 doses of 10µg R21/ 50µg Matrix-M 4 weeks apart. Group 6 was the control group and received no vaccinations. CHMI was approximately 28 days following third vaccination. Log-rank test was used to calculate p values.

3.10.4.2 VAC 072B

This was the second CHMI that took place assessing re-challenge of protected participants from the first challenge in groups 2 and 3 following a booster vaccination. Additionally, participants in group 4 (regime: 50µg R21, 50µg R21, delayed 10µg R21) were also assessed and a control group (group 7). In group 2, 2/3 participants were sterilely protected against

malaria and in group 3, all six participants were protected. In group 4, 2/4 participants were protected and in the control group, 5/6 participants were diagnosed with malaria. In view of one of the control group not being diagnosed with malaria, VE was calculated accordingly, resulting in VE of 60%, 100% and 40% in groups 2, 3 and 4 respectively (Figure 3H). No significant difference in survival between group 2 and the control group ($p = 0.151$) or group 4 and the control group ($p = 0.164$) was noted. There was only a significant difference in survival between group 3 and the control group ($p = 0.004$). The mean time to meet one of the diagnostic endpoints in group 4 was 12.3 days and in the control group, it was 8.8 days. In group 2, the participant was diagnosed at day 12.5. All diagnosed participants were asymptomatic apart from one control participant who had an intercurrent viral illness.

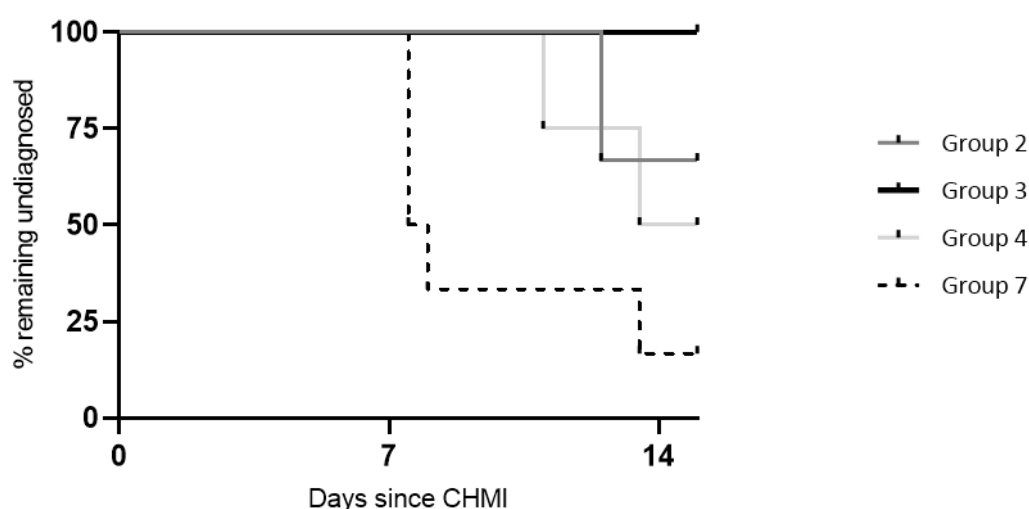


Figure 3H: VAC 072B malaria challenge results. Efficacy of R21/Matrix-M administered in different dose schedules.

Kaplan-Meier survival analysis of time to treatment according to pre-specified diagnostic endpoints for this challenge presented. This was a re-challenge for sterilely protected participants from groups 2 and 3. Group 2 received 2 doses of 10µg R21/ 50µg Matrix-M 4 weeks apart, a delayed dose 6 months later and a booster a further 8 months later. Group 3 received 3 doses of 10µg R21/ 50µg Matrix-M 4 weeks apart and a booster 8 months later. Group 4 received 2 doses of 50µg R21/ 50µg Matrix-M 4 weeks apart and a delayed, fractional dose 6 months later. Group 7 was the control group and received no vaccinations. CHMI was approximately 28 days following third vaccination for group 4 and also 28 days following booster vaccinations for groups 2 and 3. Log-rank test used to calculate p values.

3.10.4.3 Comparison of efficacy between R21c and R21

Participants in group 3 of this trial were administered the same vaccine dose and had the same schedule regime as participants in group 1 in the previous clinical trial, VAC 065, who received R21c. In that trial, 9/11 (82%) participants were sterilely protected against sporozoite

challenge (Figure 3I). Direct comparison is not possible as these are slightly different vaccines (manufactured by different companies), assessed in CHMI trials three years apart, and with different control participants. Indirect comparison shows there was no significant difference in efficacy between the two groups ($p = 0.260$).

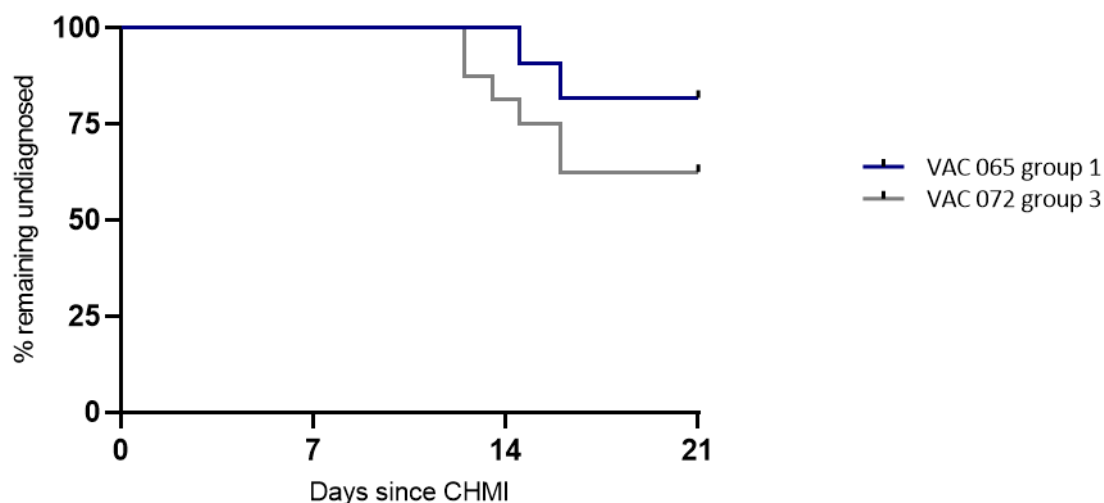


Figure 3I: Efficacy of R21 and R21c adjuvanted with Matrix-M.

VAC 065 group 1 received 3 doses of 10µg R21c/ 50µg Matrix-M 4 weeks apart. VAC 072 group 3 received 3 doses of 10µg R21/ 50µg Matrix-M 4 weeks apart. Participants underwent CHMI approximately 28 days following 3rd vaccination. Log-rank test used to calculate p values.

3.10.5 R21/Matrix-M immunogenicity

3.10.5.1 Humoral responses before and after the primary series of vaccinations

Total NANP-specific IgG was measured at baseline (day of first vaccination) and 28 days following each vaccination. NANP-specific IgG was also measured at 4 to 8 months following third vaccination according to group. Antibody responses were induced following vaccination in all participants and gradually waned over time (Figure 3J). Peak geometric mean titres (GMTs) were noted at 28 days following third vaccinations in all vaccine groups after the primary series of vaccinations.

In groups 1 and 3, which received the standard regime of three vaccinations, 4 weeks apart, the GMT at 28 days following the third vaccination was 1,389 EU (95% CI 1,031-1,871), while in group 2, which received the same dose of vaccinations but with a delayed third dose, GMT was 2,661 EU (95% CI 1,771-3,998). In group 4, where participants received the higher dose

of R21 (50µg) for the first 2 doses and a delayed fractional dose, GMT was 1,009 EU (95% CI 316-3,221). In group 5, which received the standard dose of R21 (10µg) for the first 2 doses and a delayed fractional dose, GMT was 3,980 EU (95% CI 2,500-6,337).

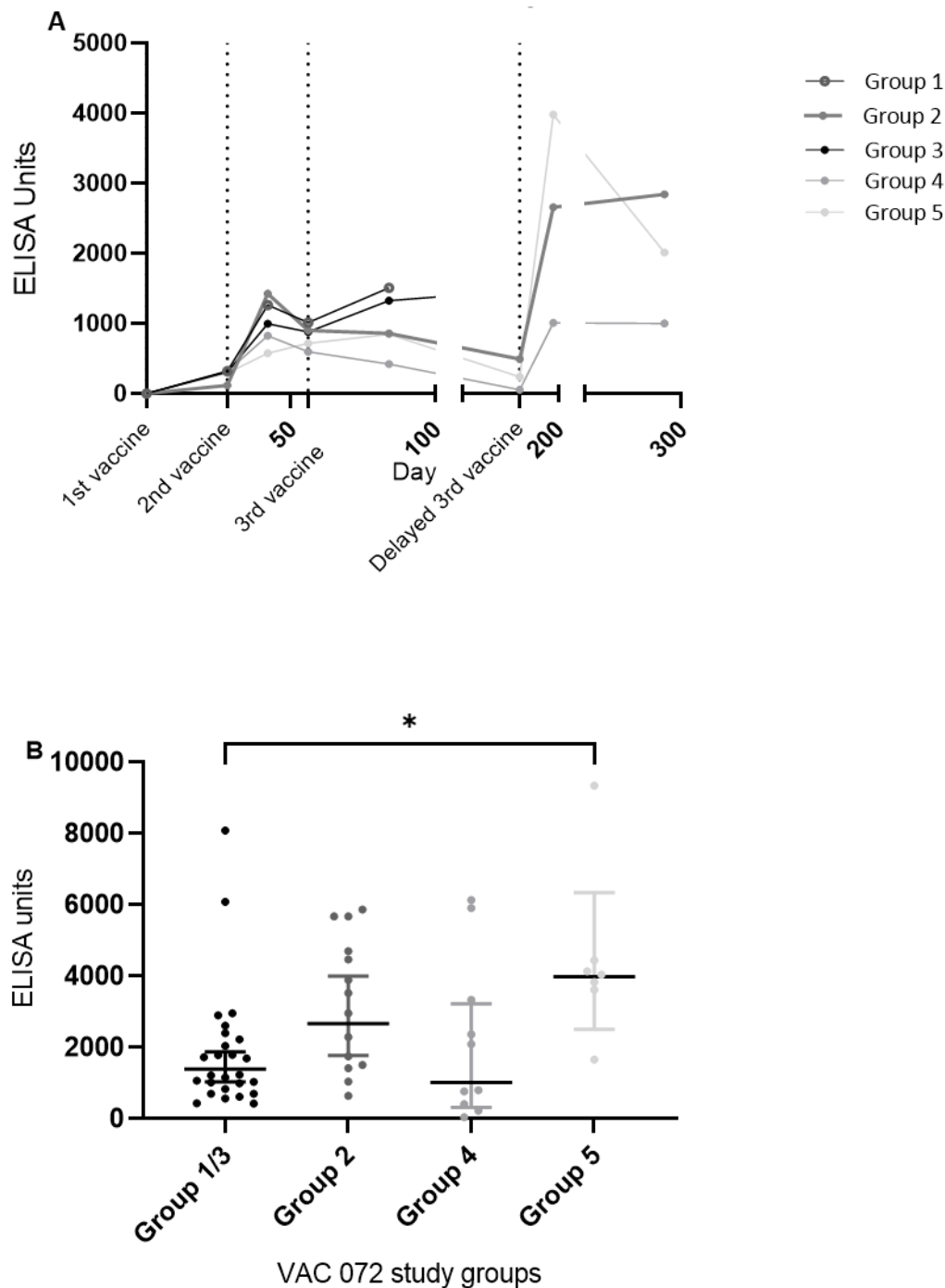


Figure 3J: Total NANP IgG as measured by ELISA.

Panel A shows GMT responses across groups following each vaccination and up to 3 months following the third dose. Participants in groups 2 and 3 underwent CHMI at 28 days following third vaccine. Panel B shows antibody responses for each participant at 28 days following third vaccination. GMT and 95% CI are shown. Groups 1 and 3 received 3 doses of 10µg R21/50µg Matrix-M 4 weeks apart, group 2 received the same dose with a delayed third dose. Group 4 received two doses of 50µg R21/50µg

Matrix-M 4 weeks apart and a delayed fractional third dose of 10µg R21/50µg Matrix-M. Group 5 received two doses of 10µg R21/50µg Matrix-M 4 weeks apart and a delayed fractional third dose of 2µg R21/50µg Matrix-M. Dunn's multiple comparisons test was used to calculate p values.

3.10.5.2 Impact of delaying the third dose on antibody responses

Group 2 (16 participants) received the same dose of R21/Matrix-M as groups 1 and 3 (21 participants), although the third dose in group 2 was administered approximately 6 months after the first vaccination. These were the first three groups to be vaccinated in the trial. When comparing responses between these two vaccine schedules 28 days following the third vaccination, and prior to the first malaria challenge, VAC 072A, NANP-specific IgG was significantly higher in the group with the delayed third dose ($p = 0.012$, Figure 3K). However, once all results at this time point were available across all the groups, and Dunn's multiple comparisons test was used, no significant difference was noted between groups 1/3 and 2 ($p = 0.120$, Figure 3J, supplementary appendix table 13 and supplementary appendix table 14).

3.10.5.3 Impact of giving a third delayed fractional dose

Twenty-one participants in groups 1 and 3 who received three doses of 10µg R21/50µg Matrix-M four weeks apart had antibody titres measured at 28 days following third vaccination. Seven participants in group 5 also had antibody titres measured at the same time point when receiving two doses of 10µg R21/50µg Matrix-M four weeks apart and a third dose of 2µg R21/50µg Matrix-M at six months. Antibody titres at this time point were significantly higher ($p = 0.025$) when the third dose was delayed and fractional (Figure 3J, supplementary appendix table 13 and supplementary appendix table 14).

3.10.5.4 Impact of different doses in the delayed fractional dose regime

Ten participants in group 4 and seven participants in group 5 had antibody titres measured at 28 days following their third, delayed, fractional dose. At all vaccinations, group 4 received five times the dose of group 5, but there was no significant difference in antibody titres between groups ($p = 0.112$). When assessing durability, there was no significant difference in antibody titres in group 4 between 28 days following their third vaccine and four months following the

third vaccination ($p = 0.72$). However, in group 5, titres had significantly dropped from 28 days following third vaccination ($p = 0.007$, Figure 3J, supplementary appendix table 13 and supplementary appendix table 14). Despite this reduction in group 5 titres at four months following the third vaccine, there was no significant difference between groups 4 and 5 at this time point.

3.10.5.5 Impact of giving a booster vaccination

Three participants in group 2 and six participants in group 3 received a booster vaccine (fourth dose) approximately eight months after their third vaccination, and seven months after taking part in the first CHMI (VAC 072A). Antibody titres were boosted at 28 days following this booster vaccination/the day prior to re-challenge. At this time point, the GMT in group 2 was 7,705 EU (95% CI 1614-36791) and in group 3 the GMT was 2375 EU (95% CI 997-5657). There was no significant difference in antibody titres between the groups following booster vaccinations ($p = 0.095$). When comparing antibody titres at 28 days following third vaccination and 28 days following booster vaccination, antibody titres were significantly higher following the booster in group 2 ($p = 0.032$) but not in group 3 ($p = 0.201$, Figure 3K and supplementary appendix table 15).

It should be noted when interpreting the immunology results detailed in sections 3.10.5.4 and 3.10.5.5, it is difficult to make comparisons between the various groups using antibody titres. The reasons for this are that participant numbers were low, particularly in the delayed, fractional dose groups and the groups that received a booster vaccination. Additionally, there were large variations in immune responses between the participants leading to wide confidence intervals.

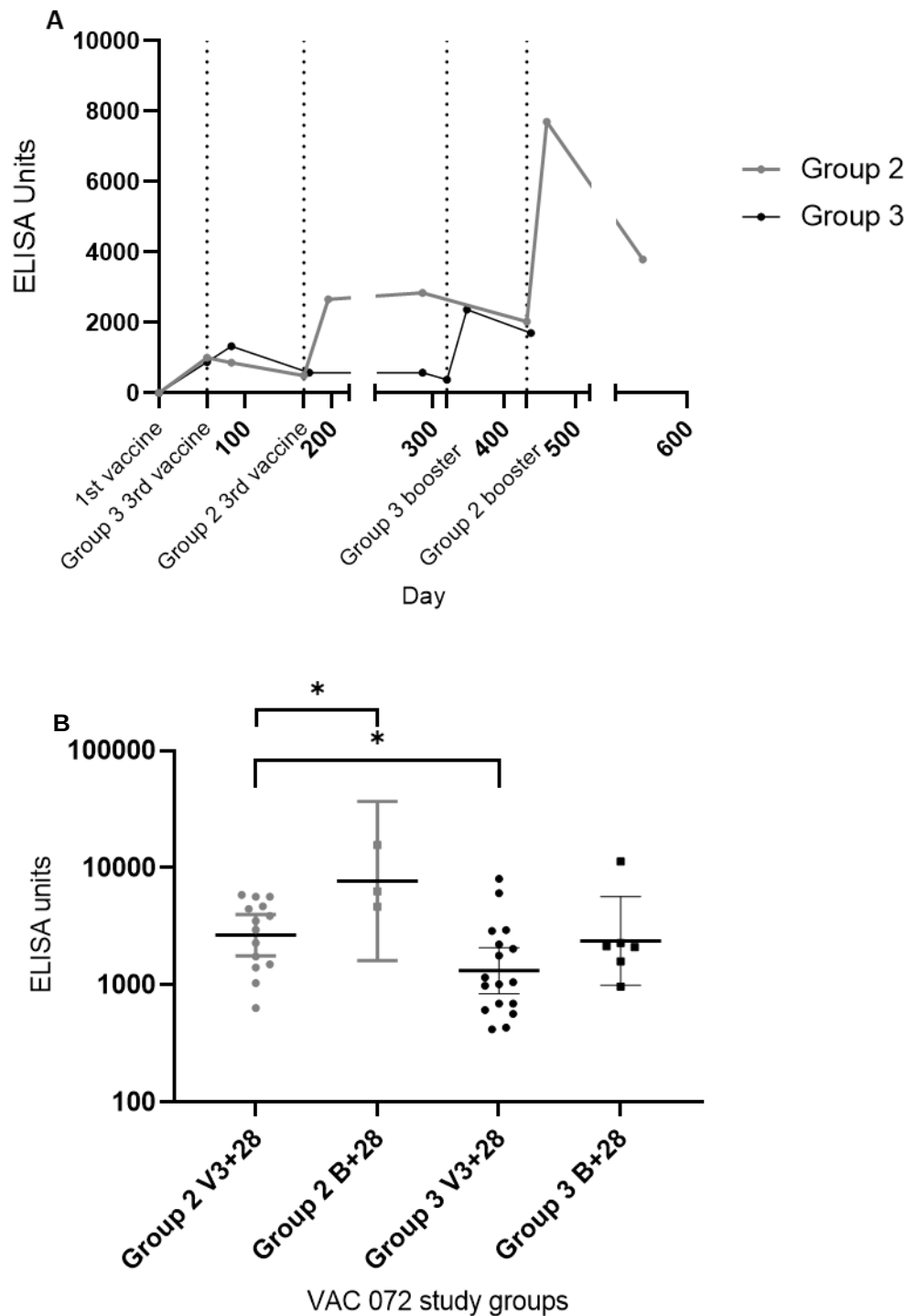


Figure 3K: Total NANP IgG as measured by ELISA.

Panel A shows GMT responses of groups 2 and 3 at baseline (day of 1st vaccine), day of 3rd vaccine and 28 days after 3rd vaccine, day of booster vaccine and 28 days after booster, and 4 months following booster vaccine. Participants underwent CHMI at 28 days following 3rd vaccination and 28 days following the booster vaccination. Panel B shows antibody responses at 28 days following primary series of vaccinations (V3+28) and 28 days following booster vaccinations (B+28). For the primary series of vaccinations, group 2 received two vaccinations four weeks apart and a third delayed dose.

Group 3 received three vaccinations four weeks apart. All doses of vaccine administered were 10µg R21/ 50µg Matrix-M. GMT and 95% CI are shown. Mann-Whitney test was used to calculate p values.

3.10.5.6 Comparison of humoral immune responses between R21c and R21

There was no significant difference in GMT of NANP-specific IgG between R21c produced at the Clinical Biomanufacturing Facility, University of Oxford, and R21, manufactured at SIIPL. When participants received three vaccinations of 10µg R21 with 50µg Matrix-M, at 28 days following the third dose, GMT in the R21c group was 1,561 EU (95% CI 1,122-2,174). In the R21 group, GMT was 1,389 EU (95% CI 1,031-1,871, $p = 0.41$, supplementary appendix table 16).

3.11 Discussion

I report here on the safety, immunogenicity and efficacy of the first administration of R21 manufactured by SIIPL, adjuvanted with Matrix-M, in a healthy UK adult population. All vaccine doses were safe and well tolerated, induced antibody responses to NANP, and many participants were protected against sporozoite challenge. Furthermore, a booster vaccination restored antibody responses resulting in durable efficacy.

It was encouraging that similar safety, immunogenicity, and efficacy results were achieved when comparing the R21c produced at the Clinical Biomanufacturing Facility, University of Oxford (57, 117) with the R21 vaccine produced by SIIPL.

When looking further at safety, the standard (10 µg) and high dose (50 µg) of R21 were safe, and there was no significant difference between them in terms of severe AEs ($p = 0.611$). Pain at the injection site was the most common local AE and was experienced by most participants. Fever was noted in only a few participants, with headache, fatigue and malaise the most common reported systemic AEs. However, these are common side effects of most licensed vaccines, and the AEs reported in this trial mostly resolved within 24-72 hours.

Efficacy of 75% was achieved with one vaccine schedule in this trial, some of the highest efficacy seen in malaria vaccine trials with sporozoite challenge. Previously in other clinical

trials, using the CHMI model, efficacy levels of 66.7% and 100% were reported using whole sporozoite vaccines intravenously (1.35×10^5 PfSPZ). The efficacy improved to 100% when increasing the number of vaccine doses from four to five (62). Further to this, efficacy with a higher dosing regimen (4.5×10^5 PfSPZ) was 86.7% at 3 weeks following third dose and 57.1% at 24 weeks following third dose (178). However, given the logistical challenges in terms of cold-chain maintenance, intravenous route of administration, multiple doses, potentially costly and large-scale manufacturing of aseptic, radiation-attenuated, purified, cryopreserved PfSPZ, it is unlikely these vaccinations will be deployable in malaria endemic settings.

Other malaria vaccine studies (47, 77) have shown that giving a delayed dose or a delayed, fractional dose of vaccine improved immunogenicity and efficacy in malaria naïve populations. This clinical trial (VAC 072) aimed to try and delineate if the improved immunogenicity, or efficacy, was as a result of simply delaying the third dose, or the combination of delaying and fractionating the third dose. Groups 2, 4 and 5 aimed to do this with group 3 as the comparator, where the standard dose (10µg) was given three times, four weeks apart. In the first malaria challenge we compared group 2 with group 3 and therefore, assessed the impact of delayed dosing alone. While VE was higher in group 2 (75%) than group 3 (62.5%), this difference did not reach statistical significance ($p = 0.687$). Interestingly, when both of these groups received a booster vaccination 8 months following their third vaccine and were challenged approximately one month later, 100% efficacy was noted in group 3 and 60% in group 2, the group which originally had the delayed third dose ($p = 0.333$). Our results show the delayed third dose does not significantly improve efficacy when participants were challenged after three vaccinations, or following a booster vaccination 8 months later, in the sample size assessed. However, numbers returning for a booster vaccination and repeat challenge were low.

When aiming to assess efficacy in the delayed and fractional dose groups (4 and 5), numbers were small as a result of the COVID-19 pandemic, therefore, no participants underwent CHMI in group 5. Nevertheless, VE of 50% was noted in group 4, which received two doses of 50µg R21 four weeks apart with a dose of 10µg R21 approximately 6 months later. Even though

this number was lower than the efficacy noted with group 3 in the first challenge, it is hard to compare as the number of participants challenged in group 4 was very small (n=4) and the challenge was done at a different time. Some participants from group 3 were challenged at the same time as group 4 but these were group 3 participants who had already been protected in one challenge and who had received a booster vaccination (fourth dose). High-level efficacy was observed with three doses of 10µg R21. Further studies are required to assess the efficacy when replacing the third 10µg dose from a standard vaccination regime with a fractional, third dose.

In the second malaria challenge, one control participant did not develop malaria. There were no aspects in the medical, travel or drug history that could explain this participant not developing malaria. Furthermore, the mean time to diagnosis in the control group was 8.8 days suggesting a successful infection challenge. The CHMI methodology used was comparable to other CHMI studies (23, 179). The sporozoite score following dissection of mosquito salivary glands was 15/20 suggesting sufficient infection. Therefore, this is likely a chance finding and failure to infect one control participant has previously been reported in CHMI trials in Oxford as well as other CHMI trials worldwide (57, 61, 62).

When looking at humoral responses across all the trial groups together, at 28 days following third vaccination, a significant increase ($p = 0.025$) in NANP-specific IgG was noted in the group that received the standard dose of R21 (10µg) for the first two doses and a delayed fractional third dose (group 5), compared with the vaccine groups that received three vaccines four weeks apart (groups 1 and 3). When comparing the standard vaccination regime (group 3) with the delayed, non-fractional third dose (group 2), there was no significant difference in efficacy despite significant difference in GMT of NANP-specific IgG at 28 days following third vaccination. Therefore, the magnitude of NANP-specific IgG may not be the predictor of efficacy, or larger numbers in the sample size may be required to estimate the efficacy more precisely. This may suggest functional assessment and the effect of further variables such as antibody avidity, isotype or subclass are required. This assessment is ongoing within our

research group but studies with RTS,S/AS01 formulations have conflicting evidence regarding antibody assessments. Regules et al. reported in malaria naïve participants that higher avidity was observed with a delayed, fractional vaccine regime, and a trend for higher avidity was associated with protection against malaria when considering anti-NANP responses (47). Similarly, Dobano et al. showed that in infants in low and high malaria transmission areas, avidity to the C-terminus following vaccination was significantly associated with vaccine efficacy, as well as the magnitude of anti-CSP IgG responses (177). Subsequent to the Regules study, Moon et al. noted that reduced antibody concentrations were associated with a reduction in VE, but anti-CSP avidity indices were within the same range across all their trial groups of malaria naïve adults receiving fractional, third doses; fractional second and third doses or a two-dose regime with a fractional second dose. In this study, higher avidity with NANP did not correlate with efficacy (180). Likewise, avidity of anti-CSP antibodies was not associated with protection from clinical malaria in Kenyan infants (181). Ajua et al. demonstrated in African infants that anti-CSP avidity increased between the second and third vaccine doses but there was no significant difference between a standard vaccination or a delayed, third vaccination regime. However, when applying statistical models to correct for trial site and vaccine schedule, participants with a high avidity index between second and third vaccination, were associated with a 54% risk reduction of clinical malaria (182). Our research group has also previously shown that while a fractional third dose of RTS,S/AS01 alone did not significantly improve VE, there was a significant improvement when assessing functional ability of antibodies using inhibition of sporozoite invasion assays (183).

The efficacy noted in this study in malaria naïve healthy adults against sporozoite challenge may not translate to malaria endemic settings against natural infection. Some reasons for this include genetic differences in populations, environmental factors, varying co-morbidities, previous malaria exposure, immunosuppression and differences in responses to vaccinations (36, 118, 133). Equally, the target population is infants and children under 5 years who accounted for more than two-thirds of malaria deaths in 2019 and 2020 (1, 2), therefore, the

vaccine needs to work in this population also. Encouragingly, with RTS,S/AS01, comparable efficacy was noted between trials using CHMI in malaria naïve populations and trials assessing field efficacy in malaria endemic regions. Pooled VE of approximately 60% was observed with RTS,S/AS01 against sporozoite challenge in malaria naïve adults (23, 47, 184, 185) and was 30% at 16 weeks in Kenyan adults, in a field efficacy trial (186). In African infants aged 5-17 months, over 12 months of follow-up, VE was 56% (102) but 31.3% in those aged 6-12 weeks (103).

There are limitations to this trial. The sample sizes for all the vaccine groups were small and participant retention and follow-up for the booster vaccination and re-challenge was low. This was thought to be mostly due to the COVID-19 pandemic. The trial was conducted in a malaria naïve population, when the target population for this vaccine would be in a malaria endemic setting. Also, CHMI was the method used to test efficacy which is not a true representation of malaria transmission in endemic countries.

In conclusion, this phase I/IIa clinical trial showed that the novel malaria vaccine, R21 adjuvanted with Matrix-M, was safe and well-tolerated in a healthy, malaria-naïve population. The immunogenicity profile showed good responses to NANP after three doses of vaccine as well as boosting of these responses to similar levels nine months later. Efficacy was demonstrated in all malaria vaccine groups that were challenged, with high-level efficacy (75% and 62.5%) noted in the groups that received three doses of 10µg R21 with 50µg Matrix-M. Furthermore, following a booster vaccination, 100% efficacy was noted in a small sample size at re-challenge, eight months following the first malaria challenge in group 3, demonstrating durable efficacy following a single booster vaccination.

Whilst this was an excellent result, a malaria vaccine is required to be safe, immunogenic and efficacious in the target age group of infants and children in malaria endemic settings. The data from this trial supported the progression to the age de-escalation trial of R21/Matrix-M, in a malaria endemic setting in adults, young children and infants, VAC 073, which is described in chapter 4.

- 4 A Phase 1b, open-label, age de-escalation, dose-escalation study to evaluate the safety and immunogenicity of different doses of a candidate malaria vaccine; adjuvanted R21(R21/Matrix-M) in adults, young children and infants in Kilifi, Kenya.**

4.1 Introduction

The WHO's Global Technical Strategy for Malaria (GTS) 2016-2030 targets a reduction in malaria cases and mortality by 90% before 2030, compared with 2015. A key element of the GTS strategy is the development and licensure of a malaria vaccine with protective efficacy of at least 75% by 2030 (8). No malaria vaccine candidate with this level of efficacy has progressed through phase III trials.

In the previous trial testing R21/Matrix-M in a UK adult population, a good safety profile and high-level efficacy was noted. Therefore, it was necessary to progress this vaccine candidate into the target population, firstly in terms of geographical location and secondly in terms of age. This allowed us not only to test if the vaccine is safe, but also, to assess induction of antibodies in our population of interest.

It is well documented that the immunogenicity of some vaccines is impaired in individuals living in LMICs, and often in infants compared with healthy adults (127, 187-192). A number of reasons have been hypothesised for this diminished immune response including host factors (age, sex, genetics, co-morbidities) and external factors (perinatal, nutritional, environmental, behavioural). These factors influence the quality and durability of vaccine immune responses and subsequent efficacy (118).

We selected Kilifi county, Kenya as the site for the phase Ib trial. High year-round transmission of *P. falciparum* is seen. The peaks of malaria transmission are after the long rains from April to June and the short rains from October to November every year.

In this chapter, I report on the safety and immunogenicity of R21/Matrix-M in a phase Ib trial in adults, young children and infants with different doses of vaccine in Kilifi, Kenya.

4.2 Methods

Detailed methods of assessment of safety data and assays used for immunology data collection can be found in chapter 2.

4.3 Study design

4.3.1 Overview

This was a phase Ib, open-label, non-randomised, age de-escalation and dose-escalation trial, evaluating the safety and immunogenicity of different doses of R21/Matrix-M in adults, young children and infants in Kilifi, Kenya.

4.3.2 Study site and population

Participants were recruited from Junju in the southern part of Kilifi county, Kenya. The demographic population under surveillance is 280,000 inhabitants. The local population is mostly from the Mijikenda (Chonyi) ethnic group with the main trade being in agriculture. Eligible participants were recruited according to three age categories: adults (18 – 45 years), young children (1 – 5 years) and infants (5 - <12 months).

4.3.3 Vaccines

All participants received three intramuscular vaccinations 4 weeks apart for the primary series of vaccinations. The doses of vaccine tested included 5µg R21/25µg Matrix-M, 5µg R21/50µg Matrix-M and 10µg R21/50µg Matrix-M. The same dose of vaccine was administered each time to the participant according to their group.

4.3.4 Study groups

4.3.4.1 Group 1 – Adults

Twenty healthy adults aged 18-45 were recruited to this group to receive 10µg R21/50µg Matrix-M. To ensure appropriate safety review, enrolment and vaccinations were staggered. Five adults (group 1a) were vaccinated initially and following adequate safety review at 72 hours, the remaining fifteen adults (group 1b) were vaccinated.

4.3.4.2 Group 2 – Young children

Twenty healthy young children aged 1-5 years were recruited to this group. This was the first time R21/Matrix-M was administered to children of any age. To ensure appropriate safety review and monitoring, three participants (group 2a) were initially administered half the adult

dose of vaccine, 5µg R21/25µg Matrix-M. Following adequate safety review and absence of safety concerns a minimum of ten days after their first vaccine, seventeen participants (group 2b) were vaccinated with the full dose of vaccine, 10µg R21/50µg Matrix-M.

4.3.4.3 Group 3 – Infants

Fifty-one healthy infants aged 5 - <12 months were recruited to this group. Similar to group 2, this was the first time R21/Matrix-M was administered to infants. Again, a staggered approach was adopted for dose escalation: three infants received half the adult dose of vaccine, 5µg R21/25µg Matrix-M and in the absence of any safety concerns, another three infants (group 3b) received the full dose of vaccine, 10µg R21/50µg Matrix-M. Following satisfactory safety data, forty-five infants (15 per group in groups 3c, 3d and 3e) were vaccinated with 5µg R21/25µg Matrix-M, 10µg R21/50µg Matrix-M and 5µg R21/50µg Matrix-M respectively. (Table 4a)

4.3.5 Study design amendment

An amendment to the trial design was submitted in November 2019 to give a booster vaccination, of the same dose initially received, to all participant groups. The booster dose was anticipated to be administered 9-14 months following the third dose in the primary series of vaccinations. Due to the COVID-19 pandemic, these booster vaccinations were delayed and administered 12-23 months following the third vaccination across all age groups.

Table 4a: VAC 073 trial design.

Group 1 were adults, group 2 were young children aged 1-5 and group 3 were infants aged 5 - <12 months at enrolment

Week	0	4	8	Boost
Group 1 n=20	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M
Group 2a n=3	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M
Group 2b n=17	10µg R21 50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M	10µg R21/50µg Matrix-M
Group 3a n=3	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M
Group 3b n=3	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M
Group 3c n=15	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M
Group 3d n=15	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M	10µg R21 50µg Matrix-M
Group 3e n=15	5µg R21 50µg Matrix-M	5µg R21 50µg Matrix-M	5µg R21 50µg Matrix-M	5µg R21 50µg Matrix-M

4.3.6 Data and Safety Monitoring Board (DSMB)

As this was the first time R21/Matrix-M was administered to adults in Kenya and for the first time to young children and infants anywhere, it was necessary to stagger the vaccinations to allow sufficient time to assess safety and to have suitable safety reviews conducted by a DSMB. Age de-escalation only occurred in each age group after a minimum of three weeks following the first vaccination in the previous group and assessment of safety by the DSMB. Within the young children and infant groups, there was a minimum of 10 days (over which safety data was collected and analysed) prior to dose escalation. Written approval for progression to the next dose or age group was given by the DSMB.

4.4 Study design rationale

There is still significant morbidity and mortality as a result of *P. falciparum* malaria and progress in control has stalled in recent years (1, 2). Another effective malaria vaccine, that is safe and has the same, or better efficacy than RTS,S/AS01 would help contribute to the toolkit to reduce malaria cases and deaths, and allow sufficient vaccine supply to reach the target population. The population most urgently in need of this intervention are children under five, as they account for more than two-thirds of the burden of malaria deaths (1, 2).

It is common practice to test safety and immunogenicity in phase I clinical trials in healthy adults. It is important that this is done in a place that allows for adequate assessment of safety and close monitoring. For this reason, safety, immunogenicity and efficacy of R21/Matrix-M was first assessed in the UK. The next step is to ensure that the vaccine is safe and induces immune responses in the target population in a malaria endemic area. This is the reason for conducting this trial in Kilifi, Kenya. Investigational medicinal products (IMPs) should first be tested in healthy adults prior to testing in children and infants. With advice from the DSMB and ethics committee, the trial design allowed for a process of age de-escalation and appropriate safety reviews.

For the adults (group 1), the same dose of antigen and adjuvant was used as in malaria naïve adults in the UK. This was the dose that was found to be safe, induce antibody responses to NANP and demonstrate efficacy when tested with CHMI. As this was the first administration of R21/Matrix-M vaccine to children and infants, the vaccine antigen and adjuvant doses were halved for initial dosing. This was similar to the dosing difference between adults and children/infants in the RTS,S/AS01 clinical trials. However, from phase I trials with R21, it has been demonstrated that the Matrix-M adjuvant increases the immune response significantly (117). Therefore, we wanted to test if this was the case when giving the half dose antigen with the higher dose of adjuvant, and also if the higher dose was safe in a paediatric population. Equally, we wanted to assess the difference in safety and immunogenicity when giving children/infants the full dose antigen and adjuvant (adult dose).

It is helpful to know these data as if the lower doses of antigen and adjuvant were safe and induced similar immune responses, the lower doses would be taken forward into the future trials and for potential licensure. In the longer term, this would have potential positive implications on cost and number of doses per vial. These are important factors when thinking about widespread deployment across sub-Saharan Africa.

Following good safety and immunogenicity with the primary series of vaccinations (3 doses, four weeks apart), an amendment was written to add a booster vaccination 12 months following the primary series of vaccinations. Experience from RTS,S/AS01 clinical trials has taught us that a booster vaccination administered at 20 months following the first dose improved immunogenicity and efficacy (41). We hoped this would also be the case with R21/Matrix-M.

4.5 Ethics and regulatory approval

The trial was approved by the national ethics committee in Kenya, the KEMRI Scientific and Ethics Review Unit (SERU ref: KEMRI/RES/7/3/1) and the national regulatory authority, the Pharmacy and Poisons Board (PPB ref: ECCT/18/12/01/2019(032)). Ethical approval was also granted in the UK by the Oxford Tropical Research Ethics Committee (OxTREC ref: 06-18). The trial was registered with ClinicalTrials.gov (ref: NCT03580824).

The trial was monitored for compliance with Good Clinical Practice (GCP) guidelines by independent monitors and an independent DSMB and Local Safety Monitor (LSM) provided safety oversight.

4.6 Objectives

4.6.1 Primary Objective

To evaluate the safety and tolerability of R21 with adjuvant Matrix-M in healthy adults followed by children and finally infants.

4.6.2 Secondary Objectives

4.6.2.1 Assessment of immunogenicity following primary series of vaccinations

To assess the humoral and cellular immunogenicity of R21 in humans with adjuvant Matrix-M in healthy adults, children and infants.

4.6.2.2 Assessment of immunogenicity following booster vaccination

To evaluate the immunological response and immune persistence induced by a booster dose of R21/Matrix-M in adults, children and infants.

4.7 Assessment of endpoints

4.7.1 Primary outcome measures

For safety and reactogenicity, the following were assessed:

- Occurrence of solicited local and systemic adverse events for 7 days following the vaccination.
- Occurrence of unsolicited adverse events for 28 days following vaccination.
- Clinically significant changes from baseline for laboratory measures assessing safety of the vaccine.
- Occurrence of serious adverse events for the study duration.

4.7.2 Secondary outcome measures

Quantification and comparison between age groups of NANP-specific IgG responses of the R21/Matrix-M vaccine doses and the longevity of these responses.

4.7.3 Tertiary outcome measures

Further immunological assays may be performed. These are still in progress and include:

- Quantification of antibodies to the vaccine components C terminus and HBsAb.
- Flow cytometry assays with intracellular cytokine staining to enumerate and functionally characterise immune cell populations such as effector and memory T cells (e.g. CD4+ and CD8+), T follicular helper cells, regulatory T cells, B cells, plasma cells and dendritic cells.

- ELISPOT for enumeration of antibody-secreting cells (e.g. B and plasma cells).

4.8 Study participants

All participants or parents/legal guardians of participants provided written, informed consent prior to participating in the trial. All informed consent forms were translated into Kiswahili and Giryama. Following this, participants were assessed and required to satisfy all inclusion criteria and not meet any exclusion criteria (supplementary appendix, section 8.2).

4.9 Study interventions

4.9.1 Vaccine manufacture

4.9.1.1 R21

R21 was manufactured under Good Manufacturing Practice (GMP) conditions by the Serum Institute of India Pvt. Ltd (SIPL). It was supplied in sterile 2ml glass vials and allowed for extraction of a single dose from each vial. It was stored frozen at a nominal temperature of -80°C for the primary series of vaccinations but was refrigerated at +2 - +8°C for the booster vaccinations, after further documentation of thermostability was available. Accountability, storage, shipment and handling of R21 were in accordance with relevant local SOPs and forms.

4.9.1.2 Matrix-M

Matrix-M was manufactured under GMP conditions by Novavax AB, Sweden. It was supplied in sterile 2ml glass vials at a concentration of 0.375mg/ml, allowing for more than one dose to be extracted from each vial. It was stored refrigerated at a temperature between +2 - +8°C and required protection from light. Accountability, storage, shipment and handling of Matrix-M were in accordance with relevant local SOPs and forms.

4.9.2 Vaccine preparation

Vaccine preparation was undertaken under aseptic conditions by the pharmacist according to the Jenner Institute SOPs 'VAC 073 Vaccine Dilutions' (supplementary appendix, section 8.9-8.11). On the day of vaccination, R21 was allowed to thaw to room temperature. Following

this, it was mixed with Matrix-M. R21 was administered within one hour of removal from the freezer.

4.9.3 Vaccine administration

Vaccines were administered into the non-dominant arm in adults and into the right deltoid in children and infants. During administration of the study vaccines, advanced life support drugs and resuscitation equipment were available immediately for the management of anaphylaxis. Administration of vaccines was performed according to local SOPs.

4.9.4 Assessment of safety

Participants were observed for sixty minutes following each vaccination. Daily visits were conducted at home by a field worker on days 1, 3, 4, 5 and 6 after each vaccination to record any solicited or unsolicited AEs and by a clinician or nurse on days 2 and 7 following each vaccination. Follow-up visits at the clinic took place at 14 days following the second vaccination, 28 days and 6 months following third vaccination, 28 days and one year following the booster vaccination.

A review of solicited, unsolicited and serious AEs occurred at each relevant follow-up visit. For adults, local AEs included pain, redness, swelling, warmth and itch. Systemic AEs included fever, arthralgia, myalgia, feverishness, headache, fatigue and malaise. For young children and infants, local AEs assessed were pain and swelling and systemic adverse events were fever, irritability, drowsiness and loss of appetite. Safety bloods included a full blood count, renal function and ALT, performed on days of each vaccination and 2 and 7 days following each vaccination in the primary series. Safety bloods were also taken 28 days post-booster vaccination.

As a result of the COVID-19 pandemic, the safety assessments were adjusted to facilitate local public health restrictions. Participant safety was paramount at all times. Therefore, at the booster vaccination visits, participants, parents and/or guardians, were taught how to measure body temperature and local AEs, and how to document these in the diary card. They were

provided with a thermometer, tape measure, diary card and pen so that if necessary, they could perform these assessments with the assistance of a field worker remotely (on the telephone). If there was any ambiguity or concern about severity of AEs and/or a grade 3 AE was recorded, they were asked to attend the clinic for assessment.

4.9.5 Assessment of immunogenicity

NANP-specific IgG were measured by enzyme-linked immunosorbent assay (ELISA). NANP is the central repeat of *P. falciparum* CS protein. Samples were obtained for immunogenicity on the day of first vaccination, 7 and 28 days following first vaccination, 14 and 28 days following second vaccination, 28 days, 6 months and 1 year following third vaccination, 28 days and one year following the booster vaccination.

As a result of the COVID-19 pandemic, at the time of writing, these immunogenicity assessments have only been performed and analysed on the samples taken at 28 days, 6 months and one year following the third vaccination. Additionally, for the infants, samples have been analysed at 28 days following the booster vaccination also to date.

4.10 Results

4.10.1 Participant flow

From April - August 2019, 43 adults, 20 young children and 125 infants were screened. Participant flow and study design are summarised in Figure 4A. The primary series of vaccinations was administered between May and December 2019. The booster doses were delayed for the adult and young children group as a result of the COVID-19 pandemic to June/July 2021 but infants received their booster vaccine doses during November – December 2020.

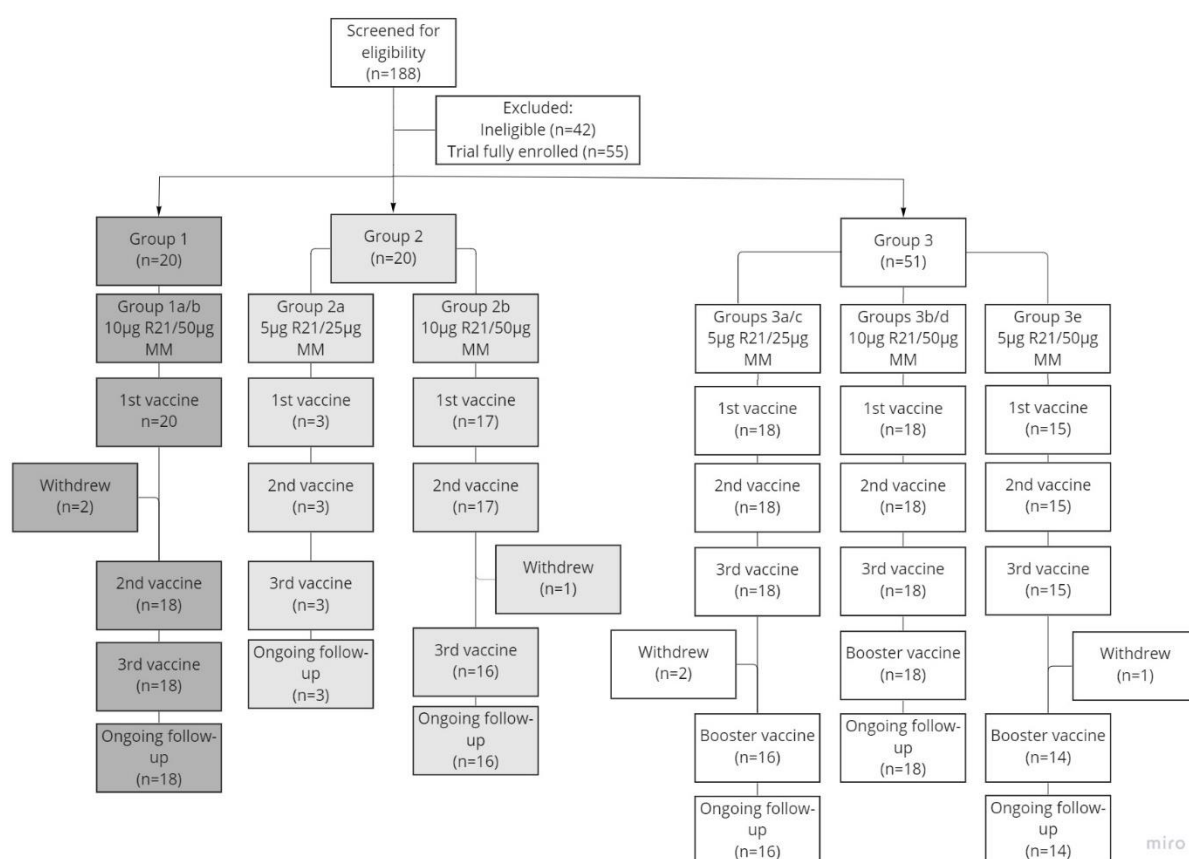


Figure 4A: VAC 073 flow chart of study design and participant recruitment.

Group 1 were adults, group 2 were young children aged 1-5 years and group 3 were infants aged 5 - <12 months. Primary series of vaccinations included 3 vaccinations, 4 weeks apart. Booster vaccination was administered at least 12 months following third vaccination.

4.10.2 Participant demographics

Baseline participant demographics and characteristics are summarised in Table 4b and Table 4c.

Table 4b: Baseline demographics of participants in group 1.

Numbers are number of participants (%) unless stated otherwise.

	Adults n = 20
Age in years	
Mean (SD)	28.2 (5.01)
Gender	
Male	15 (75)
Female	5 (20)
BMI	
Mean (SD)	21.98 (2.14)

Table 4c: Baseline demographics of participants in groups 2 and 3.

Numbers are number of participants (%) unless stated otherwise. Young children were aged 1 – 5 years and infants aged 5 – <12 months at enrolment.

	Young children n = 20	Infants n = 51
Age in months		
Mean (SD)	36.4 (12.71)	7.55 (1.89)
Gender		
Male	9 (45)	24 (47)
Female	11 (65)	27 (53)
Z-score		
+1	20 (100)	51 (100)

4.10.3 R21/Matrix-M safety

4.10.3.1 Serious Adverse Events (SAEs)

There have been two SAEs in this trial to date, occurring in the infant age group.

The first SAE occurred in a 14-month old participant, three months following the third dose of vaccination of 10µg R21/50µg Matrix-M. The child was admitted to hospital with a three-day history of fever, cough, difficulty in breathing and diarrhoea. Diagnoses of lower respiratory infection and gastroenteritis were made with treatment initiated. They made a full recovery with no sequelae. This SAE was assessed as not related to vaccination.

The second SAE occurred in a 13-month old participant, two months following the third dose of vaccination of 5µg R21/50µg Matrix-M. They were admitted to hospital with a seven-day history of fever, cough, difficulty in breathing and a febrile convulsion. Diagnoses of lower respiratory tract infection and iron deficiency anaemia were made and treatment was initiated. They made a full recovery with no sequelae. This SAE was assessed as not related to vaccination.

There have been no suspected unexpected serious adverse reactions (SUSARs) to date.

4.10.3.2 Solicited adverse events

4.10.3.2.1 Adults

Local and systemic AEs reported in the first seven days following each vaccination in the primary series are shown in Figure 4B and Figure 4C. All AEs reported were mild apart from

one participant who reported one moderate local AE of pain. The most common local AE across the primary series of vaccinations was pain at the injection site (Figure 4B). Systemic AEs experienced included feverishness, headache and fatigue. No participants had fever (Figure 4C).

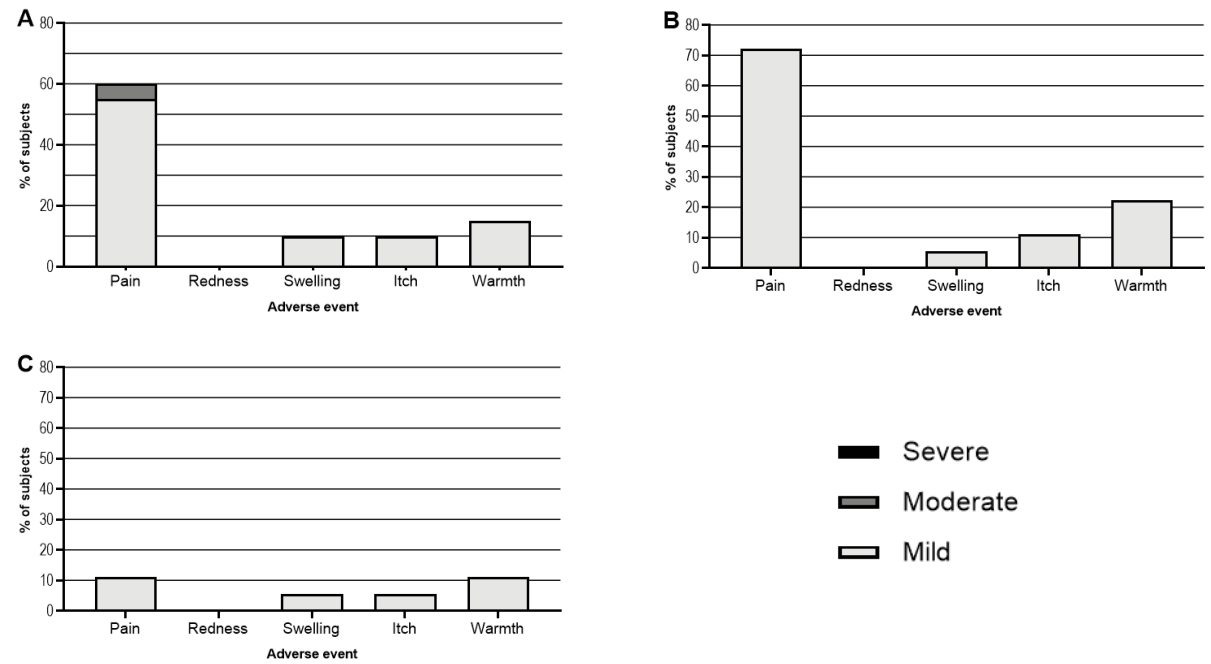


Figure 4B: Local solicited adverse events reported in the first seven days in group 1. These were adults who received three vaccinations of 10µg R21/ 50µg Matrix-M four weeks apart. Panel A is following the first dose (n=20), panel B following second dose (n=18) and panel C following third dose (n=18). After each dose, each adverse event per participant is counted once at the highest reported grade.

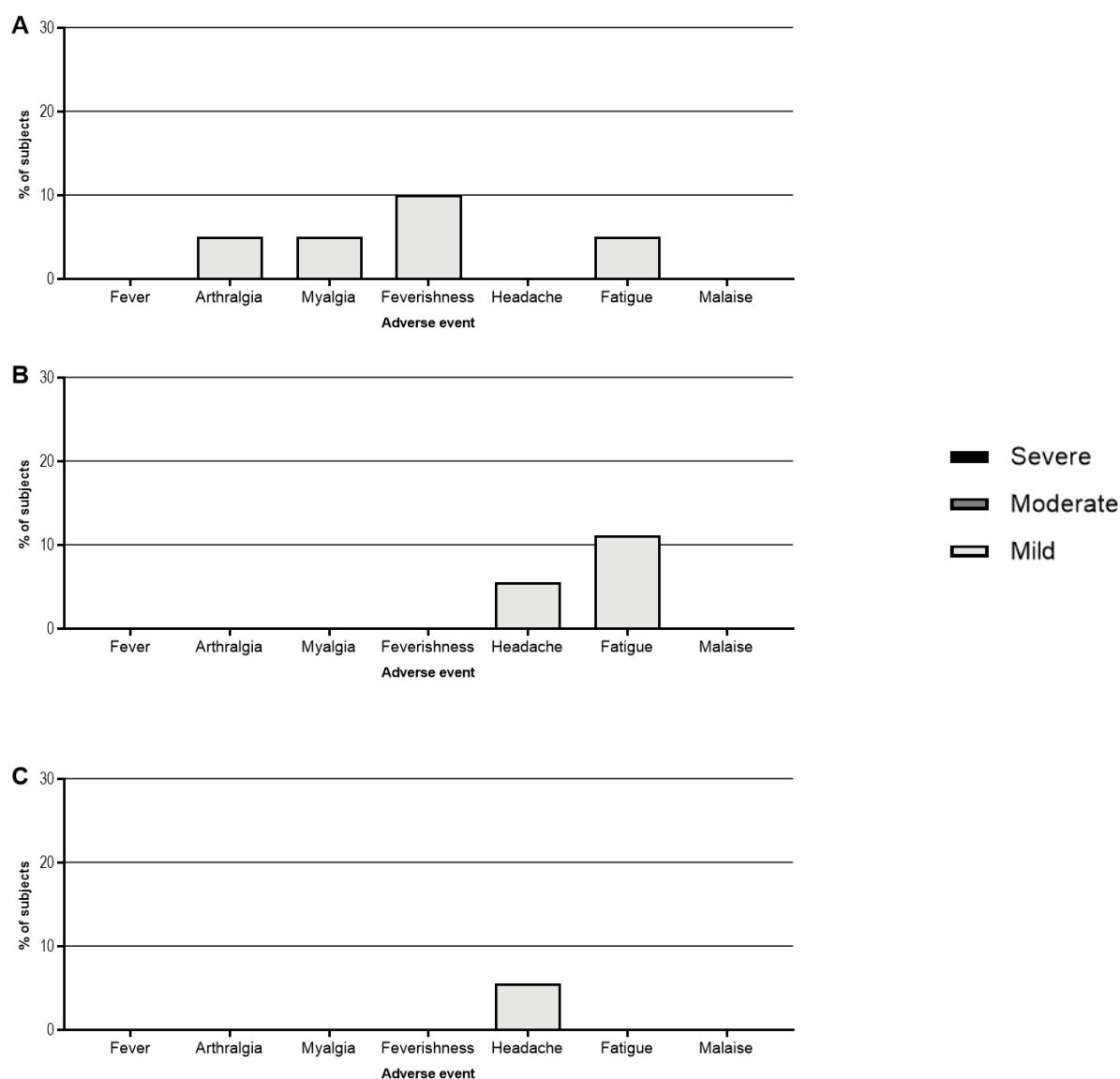


Figure 4C: Systemic solicited adverse events reported in the first seven days in group 1.

These were adults who received three vaccinations of 10µg R21/ 50µg Matrix-M four weeks apart. Panel A is following the first dose (n=20), panel B following second dose (n=18) and panel C following third dose (n=18). After each dose, each adverse event per participant is counted once at the highest reported grade. Fever was defined as a temperature of 37.5°C and above. The grading of adverse events can be found in the appendix.

4.10.3.2.2 Young children

Local and systemic AEs reported in the first seven days following each vaccination in the primary series are shown in Figure 4D and Figure 4E. Across the primary series of vaccinations in group 2a, one participant experienced one AE of pain at the injection site. This participant received the half dose antigen and adjuvant vaccine. In group 2b, where the full

dose of vaccine was administered, the most common local AE was pain and systemic AE was fever across the primary series of vaccinations. Fever was noted in seven participants after the second vaccination and in eight participants after the third vaccination. One of these participants had a fever of $>39^{\circ}\text{C}$, graded as severe. This severe fever was on day 7 following third vaccination and assessed as unrelated as the participant was diagnosed with malaria. All other participants experienced fever on the day of or the day following vaccination which resolved within 24 hours. None of those with a fever post vaccination had a febrile convulsion.

4.10.3.2.3 Infants

Local and systemic AEs reported in the first seven days following each vaccination are shown in Figure 4D and Figure 4E. Fever was the most common systemic AE in groups 3a and 3e: one participant had a fever after first vaccination, four participants after the second vaccination, four participants after the third vaccination and six participants after the booster vaccination. One participant experienced a severe fever but this was 7 days following the booster vaccination and associated with malaria.

In group 3e, where participants received half dose antigen and full dose adjuvant, two participants experienced the local adverse event of pain after the second dose. Fever was the most common systemic AE: one participant had a fever after first vaccination, nine participants after the second vaccination, nine participants after the third vaccination and seven participants after the booster vaccination. Two of these participants had severe fevers which occurred the day after the second dose and the day after the third dose.

In groups 3b and 3d, which received the full dose of vaccine, one participant experienced both local AEs of pain and swelling after the second dose. Fever was the most common systemic AE: one participant had a fever after first vaccination, three participants after the second vaccination, five participants after the third vaccination and six participants after the booster vaccination. There were no severe fevers recorded.

In this age category, there were also no febrile convulsions reported.

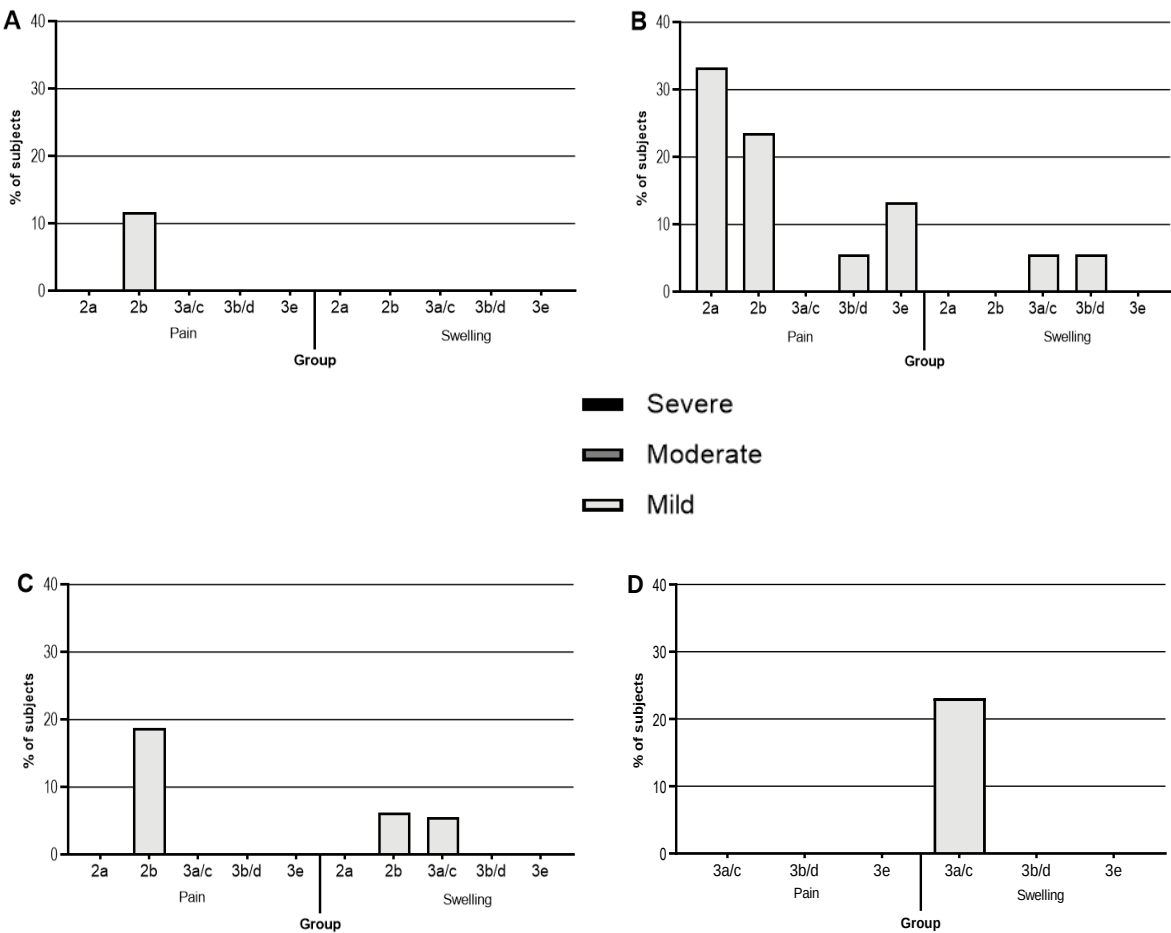


Figure 4D: Local solicited adverse events reported in the first seven days in groups 2 and 3.

All participants received three vaccinations four weeks apart. Group 2a received 5µg R21/ 25µg Matrix-M, group 2b received 10µg R21/ 50µg Matrix-M, groups 3a/c received 5µg R21/ 25µg Matrix-M, groups 3b/d received 10µg R21/ 50µg Matrix-M and group 3e received 5µg R21/ 50µg Matrix-M. Panel A is following the first dose, panel B following second dose and panel C following third dose. Data is only available for group 3 for the booster vaccination (panel D). After each dose, each adverse event per participant is counted once at the highest reported grade.

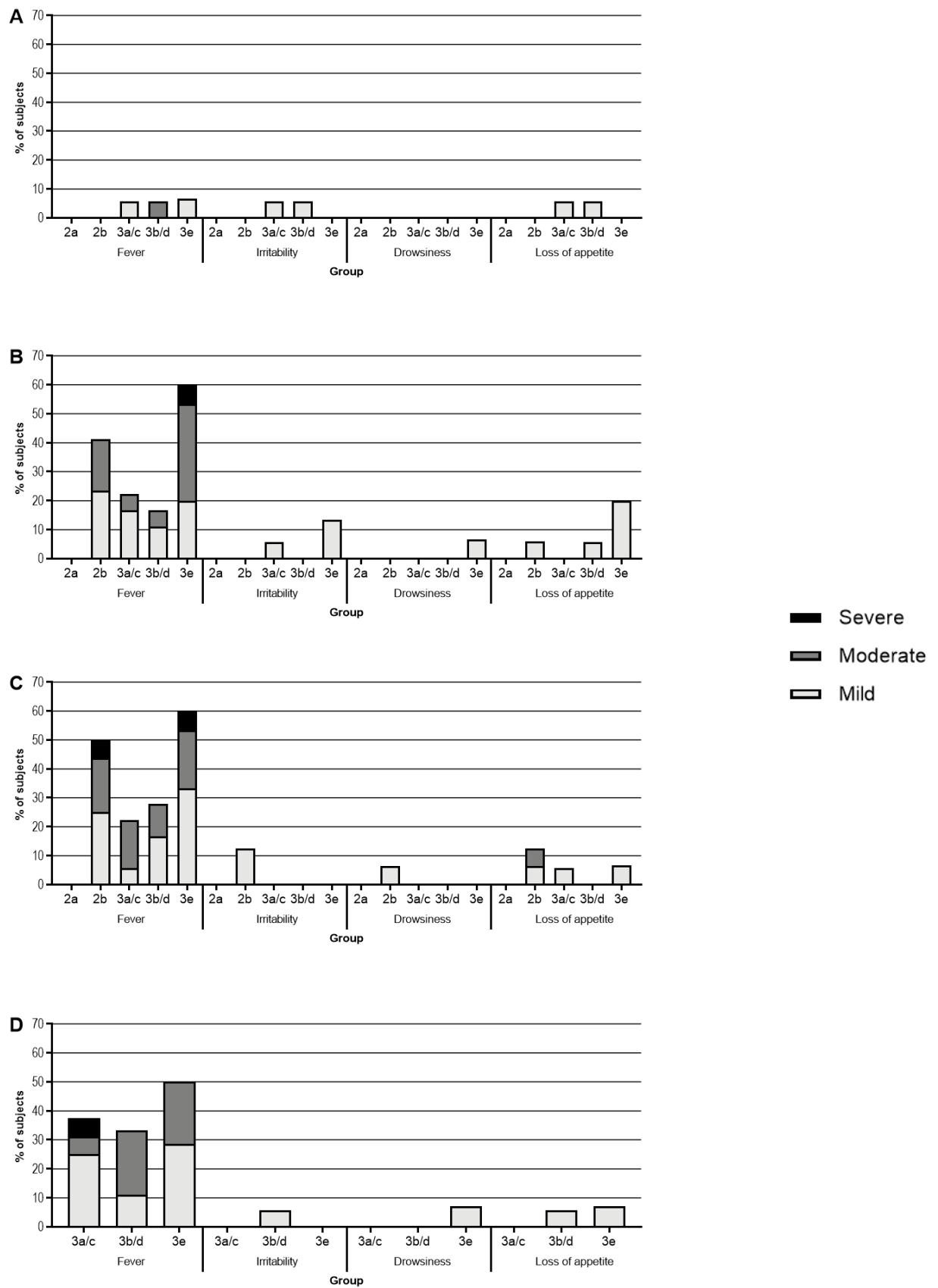


Figure 4E: Systemic solicited adverse events reported in the first seven days in groups 2 and 3.

(Figure 4E continued) All participants received three vaccinations four weeks apart. Group 2a received 5µg R21/ 25µg Matrix-M, group 2b received 10µg R21/ 50µg Matrix-M, groups 3a/c received 5µg R21/ 25µg Matrix-M, groups 3b/d received 10µg R21/ 50µg Matrix-M and group 3e received 5µg R21/ 50µg Matrix-M. Panel A is following the first dose, panel B following second dose and panel C following third dose. Data is only available for group 3 for the booster vaccination (panel D). After each dose, each adverse event per participant is counted once at the highest reported grade. Fever was defined as a temperature of 37.5°C and above. The grading of adverse events can be found in the appendix.

4.10.3.3 Unsolicited adverse events

There were no specific trends across the age groups noted with unsolicited AEs. Four participants had unsolicited AEs assessed as possibly related to vaccination: one adult, one young child and two infants. Details of these can be found in Table 4d.

Table 4d: Unsolicited adverse events reported in the 28 days following each vaccine dose.

AEs displayed are according to if definitely, probably or possibly related to vaccination. The maximum severity for each AE is shown. AEs have been coded according to the Medical Dictionary for Regulatory Activities (MedDRA) at the Preferred Term (PT) level.

MedDRA term	Age category	Grade	Day of symptom onset	Day of symptom resolution	Vaccination number	Causality
Pharyngitis	Adults	Moderate	12	19	1	Possibly related
Bronchiolitis	Young children	Mild	0	6	2	Possibly related
Rash	Infants	Mild	2	5	1	Possibly related
Infected skin ulcer	Infants	Mild	25	33	4	Possibly related

4.10.3.4 Laboratory adverse events

4.10.3.4.1 Adults

One participant had a mildly elevated ALT seven days following their first vaccination which was assessed as possibly related to vaccination. This participant was withdrawn from the trial due to excessive alcohol intake.

4.10.3.4.2 Young children

Three participants had mild hyponatremia and one participant had a mildly elevated creatinine assessed as possibly related to vaccination, all following second vaccination. All these AEs resolved without sequelae.

4.10.3.4.3 Infants

One participant had mild thrombocytopenia in the full dose vaccine group after their second vaccination, assessed as possibly related. This resolved prior to third vaccination.

Details of the laboratory adverse events are summarised in Table 4e.

Table 4e: Laboratory adverse events noted following each vaccine dose.

AEs displayed are according to if definitely, probably or possibly related to vaccination. The maximum severity for each AE is shown.

Laboratory AE	Age category	Grade	Day of onset	Vaccination number	Causality
Elevated ALT	Adults	Mild	7	1	Possibly related
Hyponatremia	Young children	Mild	2 2 7	2 2 2	Possibly related
Elevated creatinine	Young children	Mild	2	2	Possibly related
Thrombocytopenia	Infants	Mild	7	2	Possibly related

4.10.4 R21/Matrix-M immunogenicity

4.10.4.1 Humoral response before and after primary series of vaccinations

NANP-specific IgG responses peaked following three vaccinations in all participants (Figure 4F and supplementary appendix table 17). Of the eighteen participants in the adult group analysed, nine had baseline responses to NANP with the highest baseline response being 205 EU (Figure 4F and Figure 4G). No baseline responses were noted in the young children and only one infant had an antibody response at baseline.

Despite low baseline antibody responses in adults, at day 84 (28 days following third vaccination), titres were boosted at least six-fold with a geometric mean titre (GMT) of 1,392 EU (95% CI 984-1,969). Four adults with no detectable antibody response at baseline had some of the higher responses (Figure 4G).

At this time point, in the young children receiving the same dose of vaccine, the GMT response was 7,739 EU (95% CI 4,819-12,428) and in infants, the GMT was 11,271 EU (95% CI 6,995-

18,160, Figure 4F). The highest responses ever seen to NANP with R21/Matrix-M in our laboratory were noted in the infants, peak antibody response was 86,352 EU (5µg R21/50µg Matrix-M dose). Following the primary series of vaccinations, antibody titres dropped over the following 12 months (Figure 4F, supplementary appendix table 18).

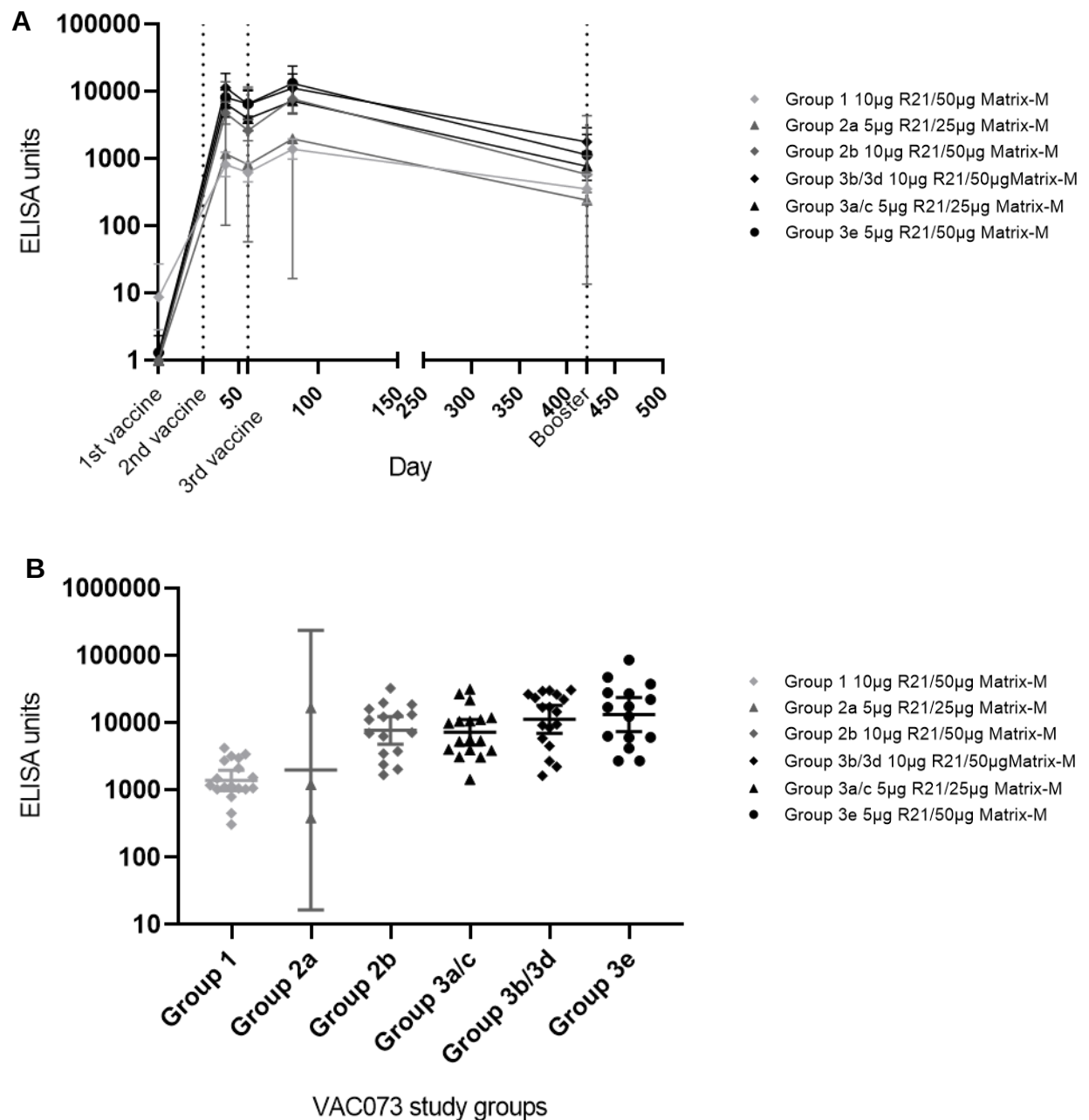


Figure 4F: Geometric mean antibody titres (95% CI) at baseline and total NANP IgG as measured by ELISA.

Panel A shows GMT and 95% CI at baseline; 14 days after second vaccination, 28 days and one year after third vaccination. Panel B shows antibody responses at day 84, 28 days following primary series of vaccinations in all groups. All participants received three vaccinations four weeks apart. Dunn's multiple comparisons test was used to calculate p values.

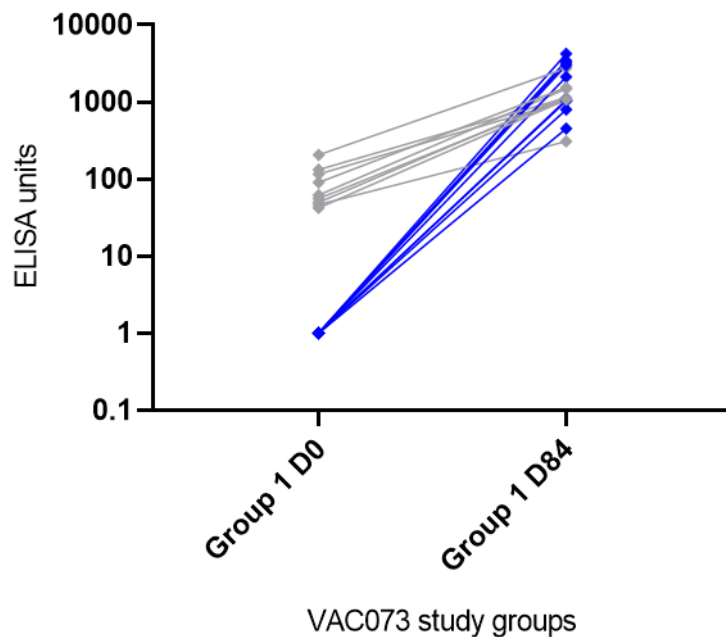


Figure 4G: Total NANP IgG as measured by ELISA.

Antibody responses in the adult group, group 1, at baseline (D0) and 28 days following primary series of vaccinations (D84). All participants received three vaccinations four weeks apart. Those marked in blue had no baseline responses.

4.10.4.2 Humoral response after first booster vaccination

At the time of writing, antibody titres after the first booster vaccination have only been analysed in the infant group. Across all three dose groups, NANP-specific IgG titres following the booster vaccination were similar to those seen at 28 days following the primary series of vaccinations (Figure 4H, supplementary appendix table 19 and supplementary appendix table 20). There was no significant difference in antibody titres at 28 days following the primary series compared with antibody titres at 28 days following the booster vaccination in any of the infant groups (Figure 4H, supplementary appendix table 19 and supplementary appendix table 20).

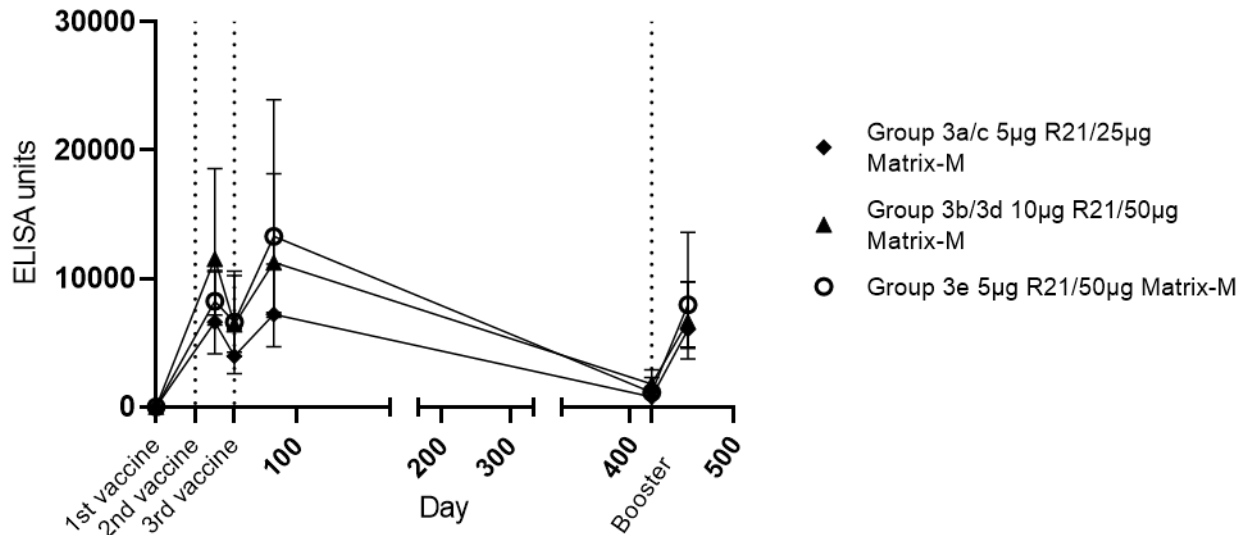


Figure 4H: Geometric mean antibody titres (95% CI) for infants.

Antibody responses measured at baseline; 14 days after second vaccination; 28 days and one year after third vaccination; and 28 days after the booster vaccination. The booster vaccination was administered approximately one year after the third vaccination. Dunn's multiple comparisons test was used to calculate p values.

4.10.4.3 Impact of age on antibody responses

All adults, young children in group 2b and infants in groups 3b and 3d received 10µg R21/50µg Matrix-M. When comparing NANP antibody titres across the age groups at 28 days following the primary series of vaccinations, there was a significant increase in the young children ($p = 0.0011$) and in the infants ($p < 0.0001$) when compared with the adults. There was no significant difference in antibody titres between the young children and infants (Figure 4I). There was also no significant difference in antibody responses between the young children and infants who received 5µg R21/25µg Matrix-M (Figure 4I, $p > 0.9999$).

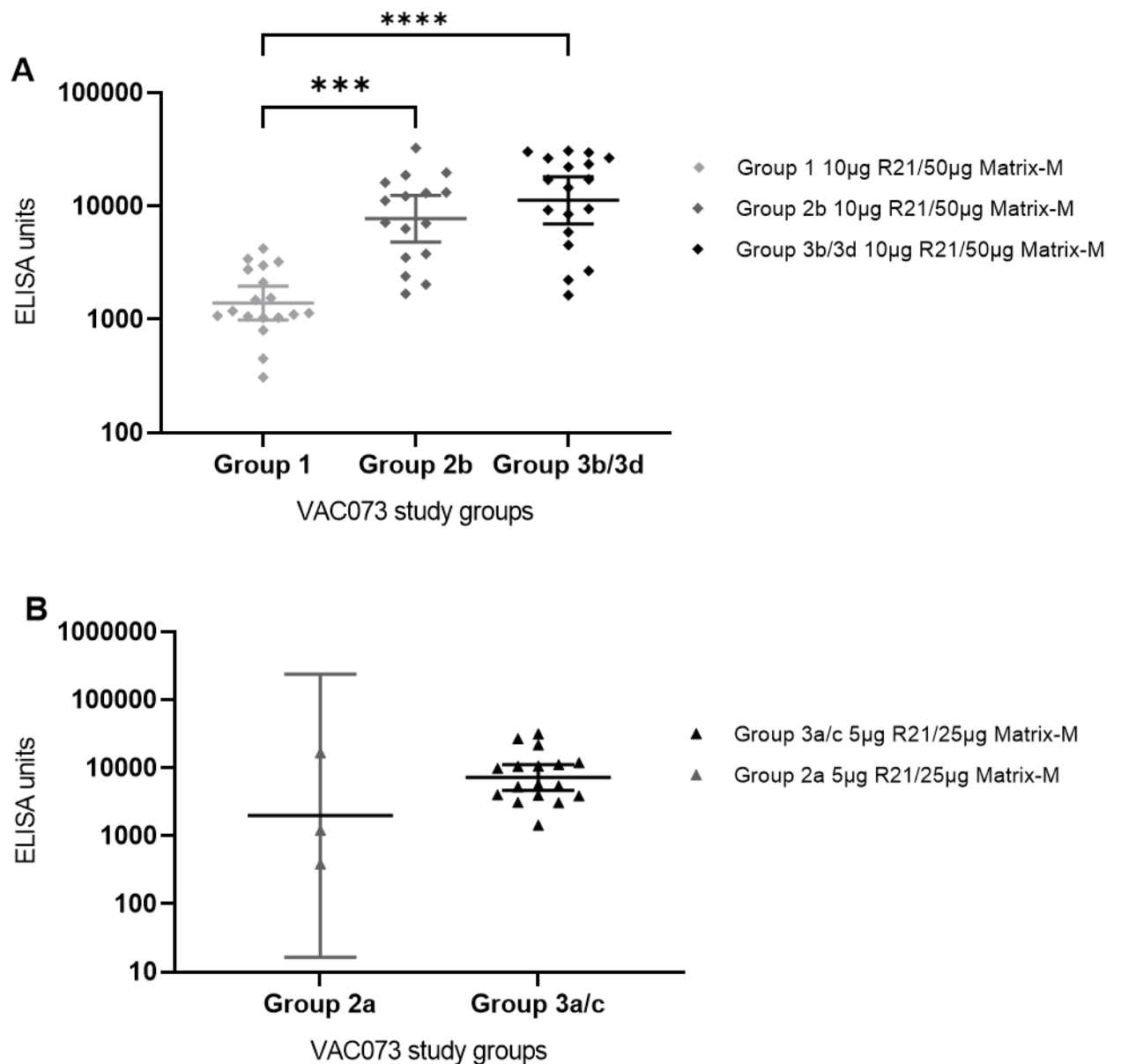


Figure 4I: Total NANP IgG as measured by ELISA at day 84, 28 days following primary series of vaccinations.

Panel A shows antibody responses for adults (group 1), young children (group 2b) and infants (group 3b/d) who received 10µg R21/ 50µg Matrix-M. Panel B shows antibody responses in the young children (group 2a) and infants (groups 3a/c) who received 5µg R21/ 25µg Matrix-M. GMT and 95% CI are shown. Dunn's multiple comparisons test was used to calculate p values.

4.10.4.4 Impact of vaccine dose on antibody responses

In group 3 when looking specifically at the infant age group, the three different dose combinations of vaccine and adjuvant can be compared: 5µg R21/25µg Matrix-M (groups 3a and 3c), 10µg R21/50µg Matrix-M (groups 3b and 3d) and 5µg R21/50µg Matrix-M (group 3e).

There was no significant difference in NANP-specific IgG titres between any of these doses at 28 days following the primary series of vaccinations ($p > 0.9999$ for all comparisons, supplementary appendix table 17 and supplementary appendix table 20). Three young children (group 2a) also received the half dose of vaccine compared with the remaining young children (group 2b) who received full dose vaccine. No significant difference in antibody responses was noted ($p > 0.9999$, Figure 4J and Supplementary appendix table 17).

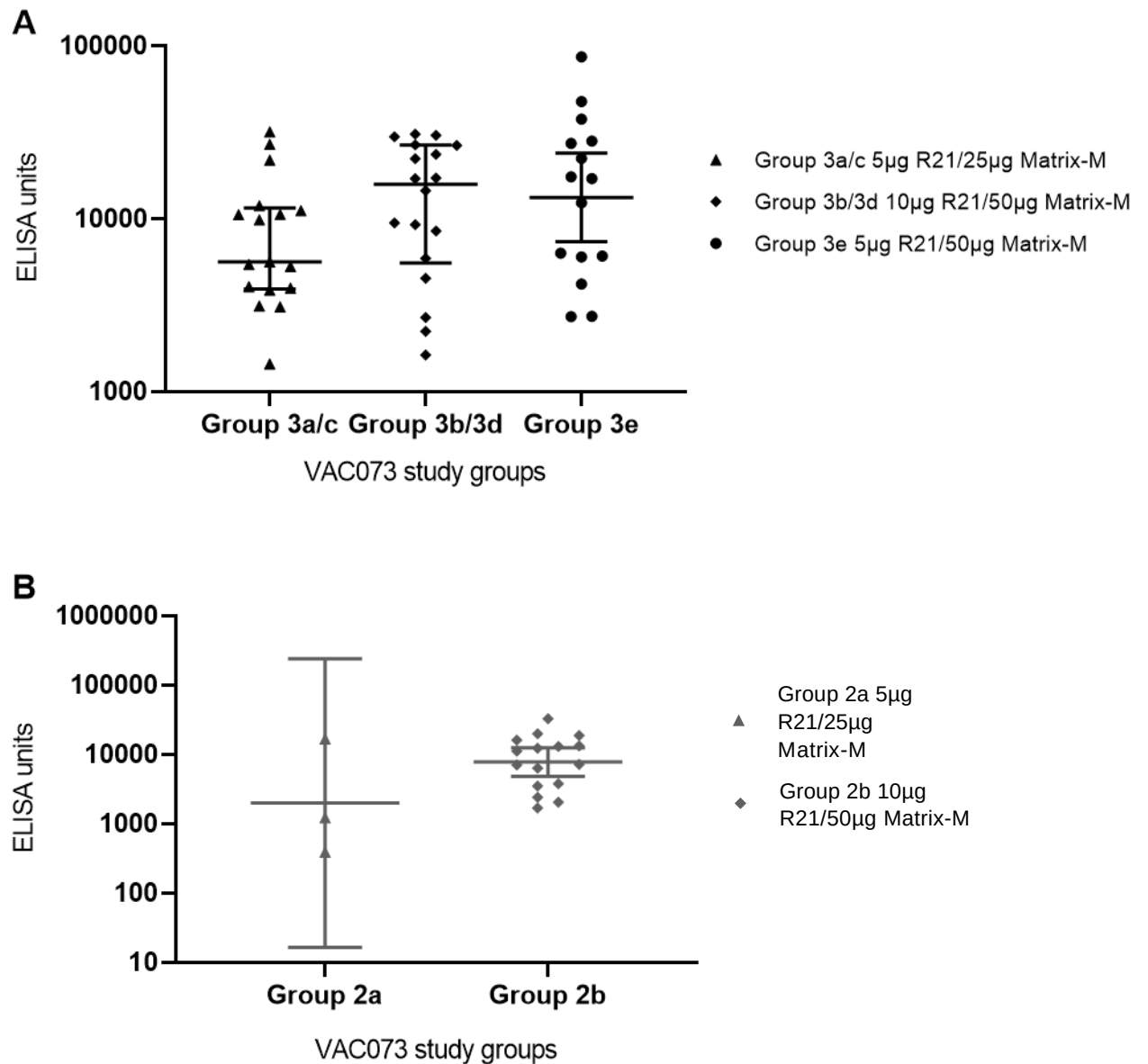


Figure 4J: Total NANP IgG as measured by ELISA at day 84, 28 days following primary series of vaccinations.

Panel A shows antibody responses for infants who received R21/Matrix-M at different doses. Panel B shows antibody responses in the young children who received R21/Matrix-M at different doses. GMT and 95% CI are shown. Dunn's multiple comparisons test were used to calculate p values.

4.11 Comparison of data between UK adults and Kenyan adults

4.11.1 Impact of different populations on safety of vaccine

In chapter 3, I have previously discussed the clinical trial, VAC 072, where, in two study vaccine groups, healthy, malaria naïve adults in the UK received the same vaccine dose and schedule as the adults in this trial. When comparing the number of adult participants who had severe

AEs in the UK (VAC 072) and Kenya (VAC 073), there was no significant difference ($p = 0.50$).

Pain was the most common local AE in both the UK and Kenya, but overall, more systemic AEs were reported in the UK compared with Kenya (6.1 AEs per person in the UK, compared with 0.45 AEs per person in Kenya).

4.11.2 Impact of different populations on antibody responses

NANP-specific IgG measured in both UK and Kenyan adults 28 days after third vaccination showed no significant difference in antibody titres (Figure 4K, $p = 0.73$). The highest response induced in UK adults was 8,076 EU, GMT 1,389 EU (95% CI 1,031-1,871). In Kenyan adults, the highest response was 4,239 EU, GMT 1,392 EU (95% CI 984-1,969).

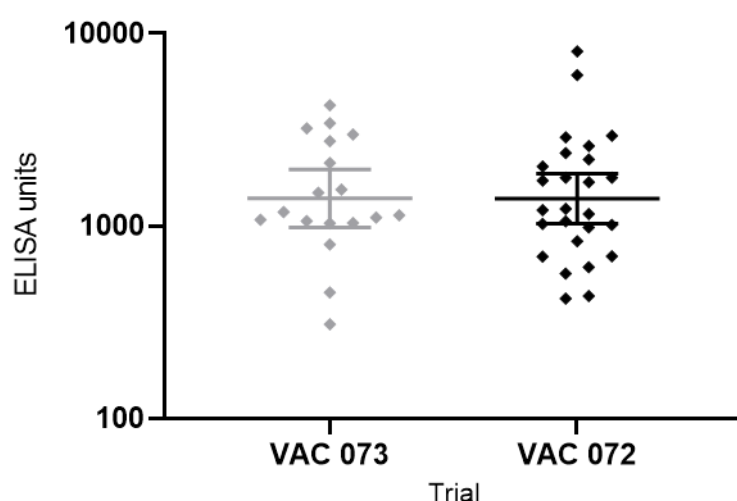


Figure 4K: Total NANP IgG as measured by ELISA at day 84, 28 days following primary series of vaccinations in Kenyan adults (VAC 073) and UK adults (VAC 072).

All participants received 10µg R21/50µg Matrix-M. GMT and 95% CI are shown. Mann-Whitney test was used to calculate p value.

4.12 Discussion

I report here on the safety and immunogenicity of the first administration of R21/Matrix-M in an African population of adults, young children and infants. All vaccine doses were safe and well tolerated and induced high antibody responses to NANP.

As this was the first administration of R21 and Matrix-M in a paediatric population, half doses of both antigen and adjuvant were administered together as well as half dose of antigen with a full dose of adjuvant. R21/Matrix-M was well tolerated at all doses and there were no SUSARs or SAEs related to vaccination. With RTS,S/AS01, the current leading malaria vaccine candidate, the paediatric dose is half of the adult dose. Pain and fever were the most common AEs noted with RTS,S/AS01 (102) and this was similar to the young children and infants in our trial. With the RTS,S/AS01 vaccine, the incidence of febrile convulsions in the 5-17-month age group was higher than in the control group, and were more likely to occur following the third dose (41, 193, 194). Importantly, no febrile convulsions occurred in the infant age group in our trial assessing R21/Matrix-M, although our sample size would have been unlikely to detect one event of febrile convulsion if the rate of febrile convulsions (1 per 1000 doses administered) was similar to RTS,S/AS01 (194).

There were fewer systemic solicited AEs reported in the Kenya adult group than in the UK adult group. This is consistent with data that reporting of adverse drug reactions is very low, even for diseases that are more prevalent in Africa (195).

Due to chronic malaria exposure in a malaria endemic area, it is not surprising that there were baseline responses to NANP in the adult population that were further boosted by the vaccine. Given the magnitude of response, it is hoped this would lead to efficacy against disease. There was no specific correlation in the magnitude of response between those participants who had baseline responses to NANP and those who did not.

There was no significant difference in NANP responses between Kenyan adults and UK adults. The UK adult, malaria naïve, population was sterilely protected from malaria following a malaria challenge. This is encouraging when considering potential efficacy of the R21/Matrix-M vaccine in a malaria endemic region. Moreover, when young children and infant immune responses were compared with adults in Kenya, who were administered the same dose (10µg R21/50µg Matrix-M) of vaccine, the young children and infant NANP responses were significantly higher.

Some of the highest NANP antibody responses ever seen in our laboratory were induced in these Kenyan young children and infants with the full dose of R21/Matrix-M (10µg R21/50µg Matrix-M). The reasons for this are unclear. Many factors are thought to be implicated in infant immune responses including interference from maternal antibodies (125); birth weight (196); limited B cell production potentially causing short-lived immune responses (197); and prematurity, resulting in diminished pathogen recognition by macrophages and dendritic cells, diminished B cell interaction with T cells, and diminished T cell activity, mainly Th1 activity (198).

It is imperative that durability of antibody responses is measured over a number of months and years, as well as assessing the impact of the booster vaccination when the infant is older, given the significant burden of malaria in children up to five years of age. Furthermore, qualitative evaluation of the immune responses, measuring avidity and affinity of antibodies, is required to understand this high immunogenicity and possible future efficacy. Regules et al. found that in malaria naïve adults, antibody avidity with RTS,S/AS01 was higher in those protected against malaria challenge when measuring anti-NANP but no other CS antigen targets (anti-CS full-length or C-terminus) (47). In infants from the phase III trial of RTS,S/AS01 in one low and two high malaria transmission sites, Dobano et al. demonstrated that avidity to C-terminus after vaccination was significantly associated with vaccine efficacy (177).

The use of a novel adjuvant, Matrix-M, has not only resulted in a favourable safety profile but also is an adjuvant that can be manufactured at low-cost and large-scale. The Serum Institute of India have committed to producing >200 million doses of R21/Matrix-M annually if licensed. This is necessary if a malaria vaccine is to reduce cases in countries where malaria is a major public health concern.

There are some limitations to this trial including overall sample size number, and the wide age range among young children. This is important as the immune system is developing rapidly over the first few years of life (199). The specific age-range of one target population (5-17 months), as well as sample size, has been addressed in a larger phase IIb trial evaluating

R21/Matrix-M discussed in chapter 5. Also, due to the COVID-19 pandemic, it was difficult to boost the participants and analyse these samples in the planned timeline. When these additional results become available they will improve our understanding of the immune responses and this will inform future trial designs.

In conclusion, this phase Ib clinical trial showed that the novel malaria vaccine, R21 adjuvanted with Matrix-M, was safe in an African population: Kenyan adults, young children and infants. This was the first administration of R21/Matrix-M in this population, as well as the first administration to different age groups, including the target age group of infants. The immunogenicity profile showed very high responses to NANP in the young children and infants. While this is encouraging, durability of this immune response is yet to be assessed in Kenya, including duration of responses after the booster vaccination.

The goal of a malaria vaccine is to protect against malaria, therefore, it is important to test efficacy of R21/Matrix-M. Additionally, it would be helpful to see if efficacy correlates with responses to NANP. If so, NANP levels can be used as a proxy for clinical efficacy as well as monitoring of vaccine durability.

The data from this trial supported the progression to the field-efficacy trial of R21/Matrix-M in infants, VAC 076, which is described in chapter 5.

- 5 A Phase I/IIb randomised controlled trial of the safety, immunogenicity and efficacy of a candidate malaria vaccine, R21 adjuvanted with Matrix-M (R21/MM), in 5 – 17-month-old children in Nanoro, Burkina Faso (VAC 076)**

5.1 Introduction

The most effective malaria vaccine candidate to date, RTS,S/AS01, demonstrated 55.8% efficacy over 12 months in African infants aged 5-17 months, following three vaccinations (102), and 36.3% efficacy after four vaccinations over a median of 48 months follow-up (41). The phase III trial evaluating this took place at eleven sites across seven African countries. Due to possible safety signals of meningitis and increased female mortality (43, 44), this vaccine is currently being assessed in implementation studies. Results from the pilot implementation studies have resulted in the WHO recommendation for four doses of RTS,S/AS01 among children from 5 months of age living in areas of moderate to high *P. falciparum* malaria transmission. In addition, countries with highly seasonal malaria or perennial transmission with seasonal peaks can consider providing the vaccine seasonally with a five-dose strategy (110). Subsequently, RTS,S/AS01 has also been granted prequalification by WHO in 2022 (113).

I have shown in chapter 4 that R21/Matrix-M is safe and immunogenic in an African population, with highest antibody responses noted in infants, the target age group. I have also shown in chapter 3 that this vaccine is protective against malaria, however, this was in a malaria naïve adult population. Therefore, it was important to further assess if vaccine efficacy is similar in our target population of African infants.

We selected Nanoro, Burkina Faso as the site to run this phase IIb trial evaluating safety and field-efficacy of R21/Matrix-M. In Nanoro, malaria transmission occurs throughout the year, with the peak overlapping with the rainy season. The rainy season is June - November and the peak malaria season is September – October with minor changes in seasonal patterns seen each year. Significant malaria transmission still occurs in December due to the high numbers of mosquitoes circulating.

P. falciparum is responsible for more than 90% of all clinical malaria cases (200). Recent data has shown an overall incidence of clinical malaria during the first year of life of 1.03 per child-year. However, within this first year of life, this varies according to age. One study reported

that two-thirds of first malaria episodes occurred at 6 - 12 months of age compared with one-third occurring from birth – 6 months (200).

This site in Nanoro was also a testing site for RTS,S/AS01 in their phase III trial, facilitating potential comparison of vaccine efficacy of R21/Matrix-M and RTS,S/AS01 in the same population.

In this chapter, I assess the efficacy, safety and immunogenicity of R21/Matrix-M in a phase IIb trial in African infants in Nanoro, Burkina Faso.

5.2 Methods

Detailed methods of assessment of safety data, assays used for immunology data collection, efficacy data collection and case definitions can be found in chapter 2.

5.3 Study design

5.3.1 Overview

This was a phase I/IIb double-blinded, randomised controlled trial assessing the safety, immunogenicity and field-efficacy of R21/Matrix-M in 5 – 17-month-old children in Nanoro, Burkina Faso. Administration of the primary series of vaccinations occurred prior to and early in the malaria season.

5.3.2 Study site and population

Participants were recruited from Nanoro. Nanoro is a rural area located about 90 km from Ouagadougou, the capital city of Burkina Faso. The Nanoro Health and Demographic Surveillance System (HDSS) covers 24 villages. In this HDSS catchment area, healthcare is provided by 11 peripheral health posts and one referral hospital. The population under surveillance is just over 63,000 inhabitants. The local population is mostly from the Mossi ethnic group with the main trade being in agriculture.

Seasonal Malaria Chemoprevention (SMC) in children from 3 to 59 months of age has been implemented since 2017. However, there is no implementation of Indoor Residual Spraying (IRS) in households in the area (201).

5.3.3 Vaccines

All participants received three intramuscular vaccinations 4 weeks apart for the primary series of vaccinations. The doses of vaccine tested included 5µg R21/25µg Matrix-M and 5µg R21/50µg Matrix-M. The same dose of vaccine was administered each time to the participant according to their group. The control vaccine was Rabivax-S, a licensed vaccine to protect against rabies.

5.3.4 Randomisation

Participants were randomly assigned 1:1:1 to groups 1 - 3. An independent statistician generated an allocation list using block randomisation with variable block sizes. Using this list, a person independent of the trial prepared and sealed envelopes which the pharmacist then used to assign participants.

5.3.5 Blinding

This trial was double-blinded. Participants, their families, and the trial study team were all blinded to group allocation. Only the pharmacists preparing the vaccine were unmasked to group allocation.

Both R21/Matrix-M and Rabivax-S were prepared by the pharmacist using the same type of syringe, and the contents of the syringe were covered with an opaque label (supplementary appendix, section 8.12 and 8.13).

5.3.6 Study groups

There were 150 participants in each group and all groups received three vaccinations 4 weeks apart. Recruitment was to one of three groups where group 1 received 5µg R21/25µg Matrix-M; group 2 received 5µg R21/50µg Matrix-M; and group 3 were the control group and received

Rabivax-S (Table 5a). The study was powered to provide an estimate of the efficacy in either group 1 or 2, assuming that vaccine efficacy over six months was >50%.

5.3.7 Study design amendments

An amendment to the trial design was submitted in December 2019 to add a booster vaccination of the same dose initially received by all participants. The booster dose was administered approximately 12 months following the third vaccination, in June 2020, prior to the malaria season (Table 5a).

Following promising results and additional funding, a further amendment to the trial design was submitted in May 2021 to add further yearly booster vaccinations prior to the malaria season for another two years. Malaria vaccine group participants, groups 1 and 2, were randomised 2:1 each year to receive 5µg /50µg Matrix-M or Rabivax-S, the control vaccine (Table 5b).

Table 5a: VAC 076 trial design with first booster vaccination.

All participants were aged 5-17 months at enrolment. The control vaccine was Rabivax-S. Randomisation to groups 1 – 3 was 1:1:1. The primary series of vaccinations (first 3 vaccinations) were administered prior to the peak malaria season. The booster vaccine was administered approximately 12 months later in June 2020.

Week	0	4	8	First booster vaccination
Group 1 (n=150)	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M	5µg R21 25µg Matrix-M
Group 2 (n=150)	5µg R21 50µg Matrix-M	5µg R21 50µg Matrix-M	5µg R21 50µg Matrix-M	5µg R21 50µg Matrix-M
Group 3 (n=150)	Control	Control	Control	Control

Table 5b: VAC 076 trial design following amendment for further booster vaccinations.

The control vaccine was Rabivax-S. Participants in groups 1 and 2 were randomised 2:1 to receive R21/Matrix-M: Control vaccine. Participants in groups 1a and 2a were further randomised 1:1 to receive R21/Matrix-M: Control vaccine for the third booster. The second booster vaccine was administered in June/July 2021, prior to the malaria season. Third booster vaccinations were administered in June 2022.

Booster vaccination	Second booster		Third booster
Group 1a	5µg R21 50µg Matrix-M	Group 1aa	5µg R21 50µg Matrix-M
		Group 1ab	Control
Group 1b	Control		Control
Group 2a	5µg R21 50µg Matrix-M	Group 2aa	5µg R21 50µg Matrix-M
		Group 2ab	Control
Group 2b	Control		Control
Group 3	Control		Control

5.3.8 Data and Safety Monitoring Board (DSMB)

A DSMB reviewed the safety data to day 14 of trial participation after the first 30 participants had received their first vaccinations. Due to randomisation, this allowed for safety data review across all three groups. There were no concerns following this and the trial progressed. Data were also reviewed after the primary series of vaccinations had been completed in all participants. The DSMB were notified of all SAEs and were able to provide feedback or have their queries addressed if necessary.

5.4 Study design rationale

We planned to test field-efficacy of R21/Matrix-M for the first time in the target population of African infants. At the same time, we would test safety and immunogenicity to expand on these data collected from the phase Ib. Two adjuvant doses, 25 µg and 50µg of Matrix-M, were tested as Matrix-M had only previously been given to a small number of paediatric participants: those in the phase I trial assessing R21/Matrix-M (chapter 4). If this vaccine and

adjuvant are found to be safe and efficacious, another malaria vaccine could progress to licensure and contribute to malaria elimination. In partnership with the Serum Institute of India Pvt. Ltd (SIPL), R21/Matrix-M has the advantage of being available at low-cost and can be manufactured and deployed at large scale.

It was observed with RTS,S/AS01 that there was evidence of waning immunity in the 5-17-month olds. Over a median of 48 months, vaccine efficacy was 28.3% in those participants who received only three doses of RTS,S/AS01 compared with 36.3% efficacy in those who received four doses. The fourth dose, given as a booster, did induce an increase in anti-circumsporozoite geometric mean titres, although not to levels achieved after the primary series of vaccinations (41). As an acceptable safety profile was noted following the primary series of vaccinations with R21/Matrix-M, an amendment for a first booster vaccination was submitted. As discussed later in the chapter, immunogenicity and efficacy results were also promising. It is demonstrated by the Expanded Programme of Immunisation (EPI) that most childhood vaccinations require boosting in the second year of life or later, and such regimes have been shown to be successful at producing good immune responses with pneumococcal conjugate vaccines (202), meningococcal (203) and haemophilus influenza vaccines (204).

I present later in the chapter that our data clearly show that NANP-specific IgG do wane over the course of a year but are strongly boosted and restored to similar levels by vaccination a year later. As malaria is a significant burden in children up to the age of 5 years (2), it is important that vaccines induce a sufficient immune responses in order for young children to be protected for as long as possible. The durability of response to R21/Matrix-M is currently unknown. It is also imperative to evaluate the potential rebound phenomenon (107) to ensure children who do not receive further booster vaccinations are not at increased risk of malaria compared with those who received no vaccines at all. For this reason, the second and third amendment to the trial design were submitted for the malaria vaccine groups to be further randomised each year to test the efficacy of four doses compared with five and six doses of R21/Matrix-M, when boosting annually prior to the malaria season. The dose chosen for

boosting was the same for all groups. The higher adjuvant dose of 50µg was used as this dose produced significantly increased antibody responses compared with 25µg Matrix-M, with no evident safety concern. This meant that the dose used to boost those randomised in group 1 for second and third R21/Matrix-M boosters was different to the low-dose adjuvant used in the primary series and first booster for group 1.

5.5 Ethics and regulatory approval

The trial was approved by the national ethics committee in Burkina Faso, Comite d’Ethique pour la Recherche en Sante, Burkina Faso (CERS ref: 2019-01-012) and the national regulatory authority, Agence National de Regulation Pharmaceutique, Burkina Faso (ANRP ref: 5005420193EC0000). Ethical approval was also granted in the UK by the Oxford Tropical Research Ethics Committee (OxTREC ref: 19-19). The trial was registered with ClinicalTrials.gov (ref: NCT03896724).

The trial was monitored for compliance with Good Clinical Practice (GCP) guidelines by an independent, external company, Pharmedica, and an independent Data and Safety Monitoring Board (DSMB) and Local Safety Monitor (LSM) provided safety oversight.

5.6 Objectives

5.6.1 Primary Objective

To assess the protective efficacy against clinical malaria of R21 adjuvanted with Matrix-M in 5-17-month-old children living in a malaria endemic area, for 6 months after the third vaccination.

5.6.2 Secondary Objectives

5.6.2.1 Duration of protective efficacy against clinical malaria

To assess the protective efficacy against clinical malaria of R21 adjuvanted with Matrix-M in 5-17 months old children living in a malaria endemic area, for 12 months after administration of the third dose of vaccine.

To assess the protective efficacy against clinical malaria of R21 adjuvanted with Matrix-M in 5-17 months old children living in a malaria endemic area, for 6 and 12 months after each booster vaccination.

5.6.2.2 Efficacy against asymptomatic *P. falciparum* infection

To assess the protective efficacy against asymptomatic *P. falciparum* infection of R21 adjuvanted with Matrix-M in 5-17 months old children living in a malaria endemic area, at 6 and at 12 months after administration of the third dose of vaccine and at 12 months following administration of each booster vaccine.

5.6.2.3 Safety Objective

To assess the safety and reactogenicity of R21 adjuvanted with Matrix-M in 5-17 months old children living in a malaria endemic area, in the month following each vaccination and at 6 and 12 months after administration of the third dose of vaccine and following administration of each booster vaccine.

5.6.2.4 Immunogenicity Objective

To assess the humoral immunogenicity of R21 adjuvanted with Matrix-M in 5-17 months old children living in a malaria endemic area.

5.6.3 Exploratory Objectives

5.6.3.1 Efficacy against incident cases of severe malaria

To assess the protective efficacy against severe malaria of R21 adjuvanted with Matrix-M in 5-17 months old infants living in a malaria endemic area, at 6 and 12 months after administration of the third dose and the booster dose of vaccine.

5.6.3.2 To evaluate cellular immunogenicity and other exploratory immunological endpoints.

5.7 Assessment of endpoints

5.7.1 Primary outcome measures

To assess efficacy against clinical malaria, the following case definitions were used:

Primary case definition of clinical malaria episode

Presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ AND *P. falciparum* parasites density > 5000 asexual forms/ μL

Secondary case definitions of clinical malaria episode

Presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ and/ or history of fever within the last 24 hours AND *P. falciparum* parasites density > 0 asexual forms/ μL

5.7.2 Secondary outcome measures

To assess efficacy against asymptomatic malaria, the following case definition was used:

Primary case definition of asymptomatic *P. falciparum* infection

Presence of axillary temperature < 37.5°C and absence of history of fever within the last 24 hours; AND *P. falciparum* parasites density > 0 asexual forms/ μL

To assess efficacy against severe malaria, the following case definition was used:

Primary case definition of severe malaria

Presence of *P. falciparum* parasites density > 5000 asexual forms/ μL ; AND one of the criteria of disease severity as described in further detail in chapter 2.

For safety and reactogenicity, the following were assessed:

- Occurrence of solicited local reactogenicity signs and symptoms for 7 days following the vaccination
- Occurrence of solicited systemic reactogenicity signs and symptoms for 7 days following the vaccination
- Occurrence of unsolicited adverse events for 28 days following the vaccination
- Change from baseline for safety laboratory measures
- Occurrence of serious adverse events for the whole study duration.

For humoral immunogenicity, the following were and are still being assessed:

- Comparison of immunogenicity (antibody responses to CSP) in the R21/Matrix-M vaccination group with those in the rabies vaccine group and the durability of responses
- Enzyme-linked immunosorbent assay (ELISA) to quantify antibodies to the vaccine components (regions of the CS antigen including the NANP repeat region and other elements of the protein, specifically the C-terminus, as well as anti-HBs).

5.7.3 Exploratory outcome measures

The following has been performed:

- DNA extraction and sequencing to determine differences in gut microbiome between those who respond to vaccination and those who do not respond.

It is hoped that the following analyses will take place on samples from this trial:

- Flow cytometry assays with intracellular cytokine staining to enumerate and functionally characterise immune cell populations such as effector and memory T cells (e.g. CD4+ and CD8+), T follicular helper cells, regulatory T cells, B cells, plasma cells and dendritic cells.
- ELISPOT for enumeration of antibody-secreting cells (e.g. B and T cells).
- Assays to measure antibody function such as inhibition of sporozoite invasion of hepatocytes.
- Assays to assess immunological aging, dysregulation and senescence, such as telomere length or expression of relevant markers and transcription factors.
- Assays to assess presence or absence of other factors affecting vaccine immunogenicity, such as antibodies against viral pathogens including cytomegalovirus.
- Genetic testing of the DNA of malaria parasites identified during the study to determine if the vaccine preferentially protects against specific genetic types of *P. falciparum*.

5.8 Study participants

All parents/guardians of participants provided written, informed consent, prior to participating in the trial. All informed consent forms were translated into French. If parents/guardians were illiterate, a literate, impartial witness was present for the consent process and the

parent/guardian thumb printed the consent form. Following this, participants were assessed and required to satisfy all inclusion criteria and not meet any exclusion criteria (supplementary appendix section 8.3).

5.9 Study interventions

5.9.1 Vaccine manufacture

5.9.1.1 R21

R21 was manufactured under Good Manufacturing Practice (GMP) conditions by the Serum Institute of India Pvt. Ltd. It was supplied in sterile 2ml glass vials and allowed for extraction of a single dose from each vial. It was stored frozen at a nominal temperature of -80°C but was refrigerated at +2 - +8°C for the booster vaccinations, after further documentation of thermostability was available. Accountability, storage, shipment and handling of R21 were in accordance with relevant local SOPs and forms.

5.9.1.2 Matrix-M

Matrix-M was manufactured under GMP conditions by Novavax AB, Sweden. It was supplied in sterile 2ml glass vials at a concentration of 0.375mg/ml, allowing for more than one dose to be extracted from each vial. It was refrigerated, at a temperature between +2 - +8°C, and required protection from light. Accountability, storage, shipment and handling of Matrix-M were in accordance with relevant local SOPs and forms.

5.9.2 Vaccine preparation

Vaccine preparation was undertaken under aseptic conditions by the pharmacist according to the Jenner Institute SOPs, 'VAC 076 Vaccine Dilutions' (supplementary appendix, section 8.12 and 8.13). On the day of vaccination, for the frozen preparation, R21 was allowed to thaw to room temperature. Following this, it was mixed with Matrix-M. R21 was administered within one hour of its removal from the freezer. With the refrigerated preparation of R21, following its removal from the refrigerator, it was mixed with Matrix-M and administered within 30 minutes.

5.9.3 Vaccine administration

Vaccines were administered into a thigh of the participant. During administration of the study vaccines, advanced life support drugs and resuscitation equipment were available immediately for the management of anaphylaxis. Administration of vaccines was performed according to local SOPs.

5.9.4 Assessment of efficacy

Parents of participants were advised by the study team to contact or attend the community health facility if their child had any illness, history of fever in the preceding 24 hours or a temperature of $\geq 37.5^{\circ}\text{C}$ at any point during the trial. Participants would be reviewed, and if clinically indicated, assessed for malaria. Following the primary series of vaccinations up to 6 months, field workers visited the participants at home every 30 days. If there was history of fever in the preceding 24 hours or a temperature of $\geq 37.5^{\circ}\text{C}$, blood sampling was performed for blood film microscopy to detect *Plasmodium* species. Episodes of malaria within seven days of a previous episode were not counted as more than one event.

For asymptomatic malaria, blood sampling for microscopy was performed at 6 and 12 months following the primary series of vaccinations and the booster vaccination.

Each blood film was analysed by two independent microscopists who were blinded to the participant group. A third microscopist was available to adjudicate in case of discrepancy.

5.9.5 Assessment of safety

Participants were observed for sixty minutes following each vaccination. Daily visits were conducted at home by a field worker for 6 days after each vaccination to record any solicited or unsolicited AEs. Follow-up visits at the clinic took place at 7 days following the first, second and third vaccinations, 28 days and 6 months following third vaccination, and 7, 28 days, 6 months and one year following the booster vaccination. Additional safety visits are planned following the second and third booster vaccination.

A review of solicited, unsolicited and SAEs occurred at each relevant follow-up visit. Local AEs assessed were pain, redness/discoloration and swelling at the injection site, and systemic adverse events were fever, irritability, drowsiness and loss of appetite. Safety bloods included a full blood count, creatinine, liver enzymes and glucose which were performed at baseline, 28 days following first vaccination and 28 days, 6 months and one year following third and booster vaccinations.

5.9.6 Assessment of immunogenicity

NANP-specific IgG were measured by enzyme-linked immunosorbent assay (ELISA). NANP is the central repeat of *P. falciparum* CS protein. Samples were obtained for immunogenicity at baseline, 28 days following third vaccination, 6 months and 1 year following third vaccination, and 28 days, 6 months and one year following the booster vaccinations.

5.10 Results

5.10.1 Participant flow

From May – June 2019, 498 children were screened. Participant flow and study design are summarised in Figure 5A. The primary series of vaccinations was administered between May and August 2019.

Primary analyses were based on a modified intention-to-treat population. This included all participants who received three vaccinations, even if the wrong vaccination was administered at any timepoint. As all vaccines were administered correctly, this was equivalent to a per-protocol analysis. Eight participants were withdrawn before the third vaccination and three participants at 7 days following the third vaccination.

Four hundred and nine participants returned to receive the booster vaccination although one was withdrawn on the day of vaccination and therefore, 408 participants are included in the analysis at 12 months following the booster vaccination.

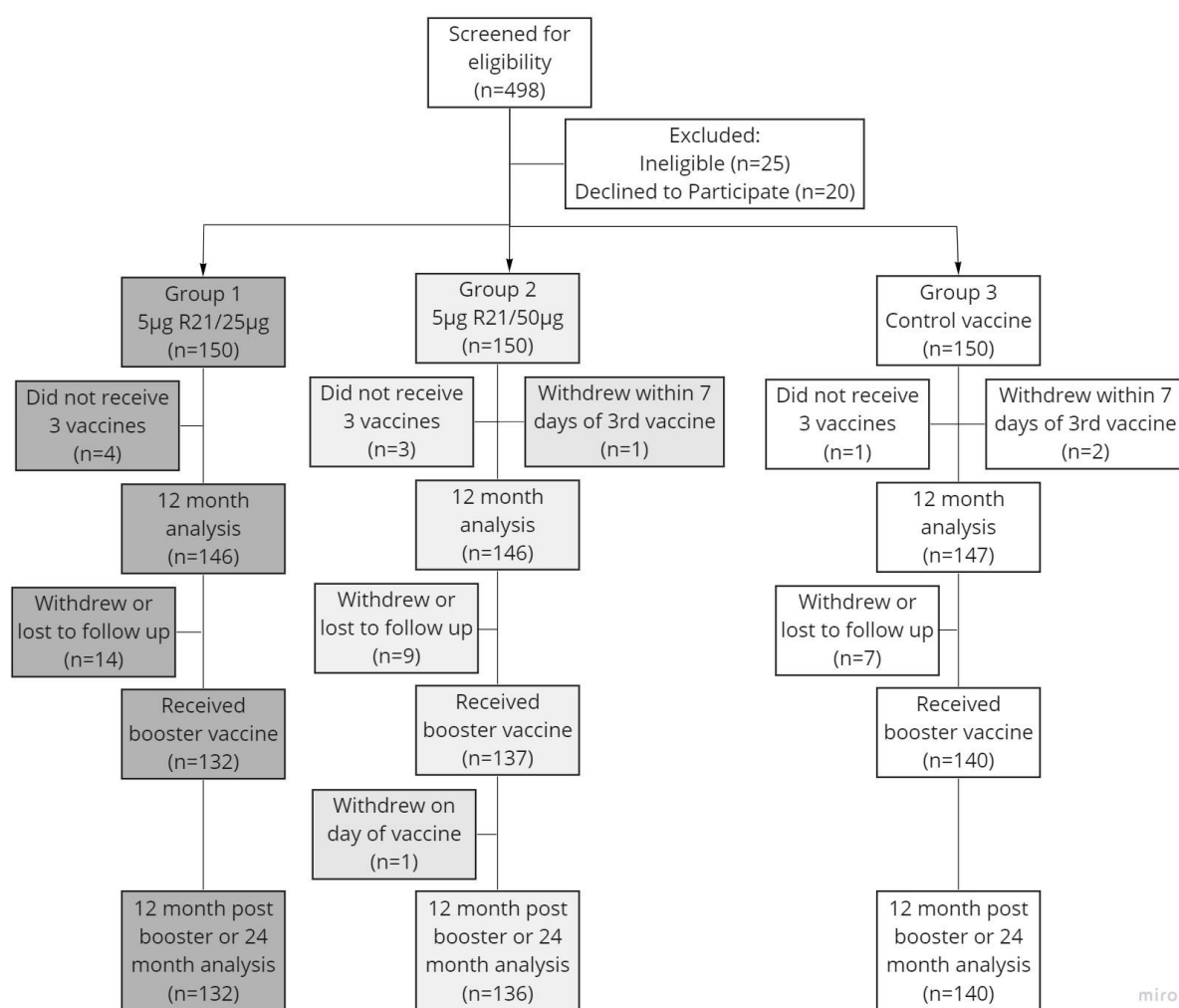


Figure 5A: VAC 076 flow chart of study design and participant recruitment.

Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received Rabivax-S. Primary series of vaccinations included 3 vaccinations, 4 weeks apart. Participants received a booster vaccination of the same vaccine 12 months following the primary series of vaccinations.

Baseline demographics and characteristics of participants are summarised in Table 5c and were similar across the groups. These included age, gender, ITN use, IRS in household, SMC use and Z-score. The combined mean age of participants at enrolment was 11.6 months (SD 3.8). There were 220 male participants and 222 female participants. Prior to the malaria season, adequate ITN use was present in 383/442 (87%) participants. The data on ITN use was collected at the third vaccination. During the malaria season 300/442 (68%) had at least one round (3 consecutive doses daily in one month) of SMC (Table 5c) (171). There was no significant difference between baseline characteristics of those lost to follow-up after the

booster vaccination and those still remaining in the trial (supplementary appendix table 21) (205). SMC should be given at the start of the malaria transmission season in areas of highly seasonal malaria transmission, with a maximum of four doses given during the same season (19). Our study in Nanoro showed that uptake of this regime was very poor in comparison with studies assessing SMC coverage across multiple regions in Burkina Faso, where >90% uptake was noted (206).

Table 5c: Demographics and characteristics of participants.

This includes all participants who received the primary series of vaccinations (3 doses). The eight participants who did not receive the third vaccination have not been included. Numbers are number of participants (%). Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received Rabivax-S. *ITN – insecticide- treated net % A round of seasonal malaria chemoprevention (SMC) is 3 doses of treatment received per month

	Group 1 n=146	Group 2 n=147	Group 3 n=149	Overall n=442
Age in months				
Mean (SD)	11.5 (3.7)	11.1 (3.8)	12.1 (3.8)	11.6 (3.8)
Age category in months				
5-9	50 (34.3)	57 (38.8)	40 (26.9)	147 (33.3)
10-12	19 (13.0)	21 (14.3)	19 (12.8)	59 (13.4)
>12	77 (52.7)	69 (46.9)	90 (60.4)	236 (53.4)
Sex				
Male	68 (46.6)	83 (56.5)	69 (46.3)	220 (49.8)
Female	78 (53.4)	64 (43.5)	80 (53.7)	222 (50.2)
Indoor residual spraying				
Yes	19 (13.0)	18 (12.2)	28 (18.8)	65 (14.7)
Missing		1 (0.7)		1 (0.2)
Adequate bed net use				
Yes	125 (85.6)	130 (88.4)	128 (85.9)	383 (86.7)
Missing		1 (0.7)		1 (0.2)
Bed net use Absent				
ITN* no holes	15 (10.3)	10 (6.8)	17 (11.4)	42 (9.5)
ITN* with holes	124 (84.9)	128 (87.1)	126 (84.6)	378 (85.5)
Missing	7 (4.8)	8 (5.4)	6 (4.0)	21 (4.8)
		1 (0.7)		1 (0.2)
At least one round% of SMC				
Yes	107 (73.3)	91 (61.9)	102 (68.5)	300 (67.9)
Number of rounds% of SMC				
0	39 (26.7)	56 (38.1)	47 (31.5)	142 (32.1)
1	75 (51.4)	57 (38.8)	78 (52.4)	210 (47.5)
2	26 (17.8)	28 (19.1)	22 (14.8)	76 (17.2)
3	4 (2.7)	5 (3.4)	2 (1.3)	11 (2.5)
4	1 (0.7)	1 (0.7)	0	2 (0.5)
5	1 (0.7)	0	0	1 (0.2)
Z score				
≥-3SD and <-2SD	32 (21.9)	29 (19.7)	33 (22.2)	94 (21.3)
≥-2SD and <-1SD	63 (43.2)	53 (36.1)	55 (36.9)	171 (38.7)
≥-1SD and <median	37 (25.3)	47 (32.0)	40 (26.9)	124 (28.1)
≥ median and < 1SD	10 (6.9)	17 (11.6)	17 (11.4)	44 (10.0)
≥ 1SD and < 2SD	4 (2.7)	1 (0.7)	4 (2.7)	9 (2.0)

5.10.2 Efficacy

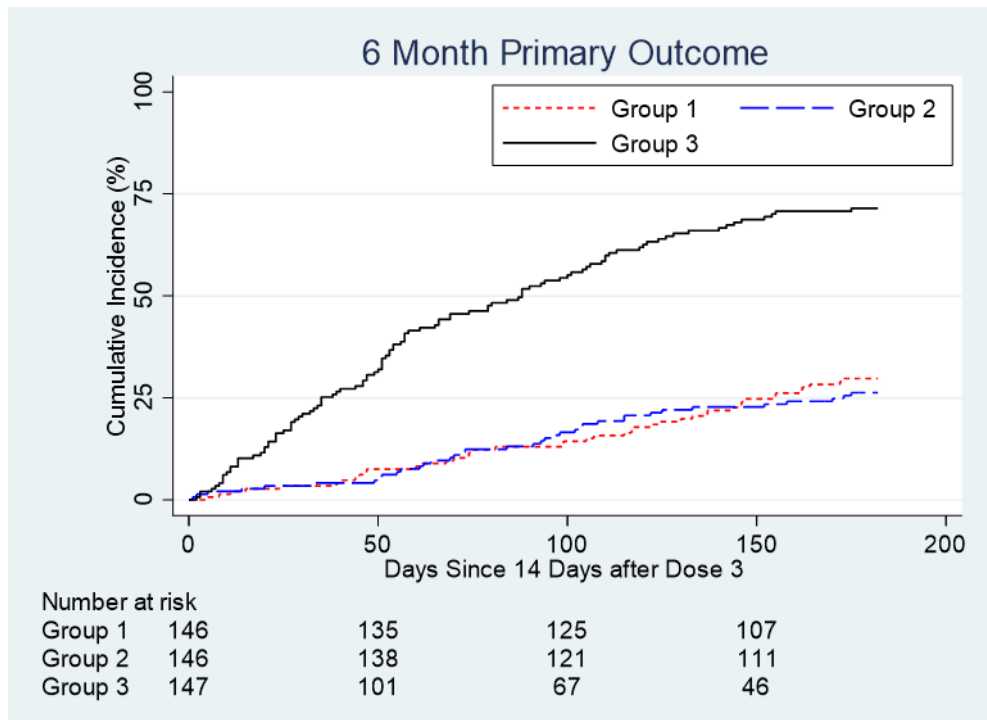
5.10.2.1 Clinical malaria

5.10.2.1.1 Primary case definition

According to the definition of fever $\geq 37.5^{\circ}\text{C}$ and a parasitaemia $>5000/\mu\text{l}$, 186 participants had a first episode of clinical malaria by 6 months following the primary series of vaccinations. These cases occurred in 43/146 (29%) participants in group 1, 38/146 (26%) participants in group 2 and 105/147 (71%) participants in group 3. A Cox regression model comparing group 1 with group 3 and group 2 with group 3 resulted in vaccine efficacy (VE) of 74% (95% CI 63-82; $p < 0.0001$) and 77% (95% CI 67-84; $p < 0.0001$) respectively (Figure 5B, supplementary appendix table 22) (171).

At 12 months (range 327-398 days), 195 participants had at least one episode of clinical malaria. These cases occurred in 50/146 (34%) participants in group 1, 39/146 (27%) participants in group 2 and 106/147 (72%) participants in group 3. VE was 71% (95% CI 59-79; $p < 0.0001$) in group 1 and 77% (95% CI 67-84; $p < 0.0001$) in group 2 (Figure 5B, supplementary appendix table 22). When adjusting for potential confounders such as age at randomisation, gender, adequate bed net use, and if participants received at least one round (three doses in one month) of SMC, efficacy was similar at 12 months: 71% (95% CI 59-79; $p < 0.0001$) in group 1 and 76% (95% CI 65-84; $p < 0.0001$) in group 2 (supplementary appendix table 22) (171).

A



B

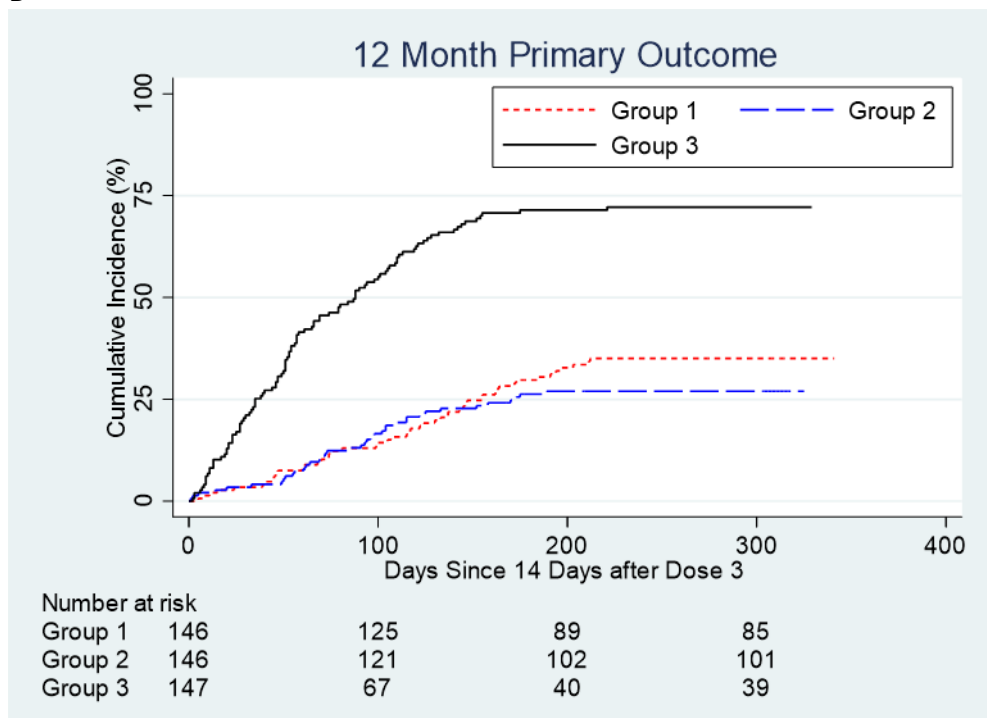


Figure 5B: Kaplan – Meier estimates of the time to first episode of clinical malaria, primary case definition.

Primary analysis was based on a modified intention-to-treat population. Panel A shows the data from 14 days to 6 months after third vaccination. Panel B shows the data from 14 days to 12 months after

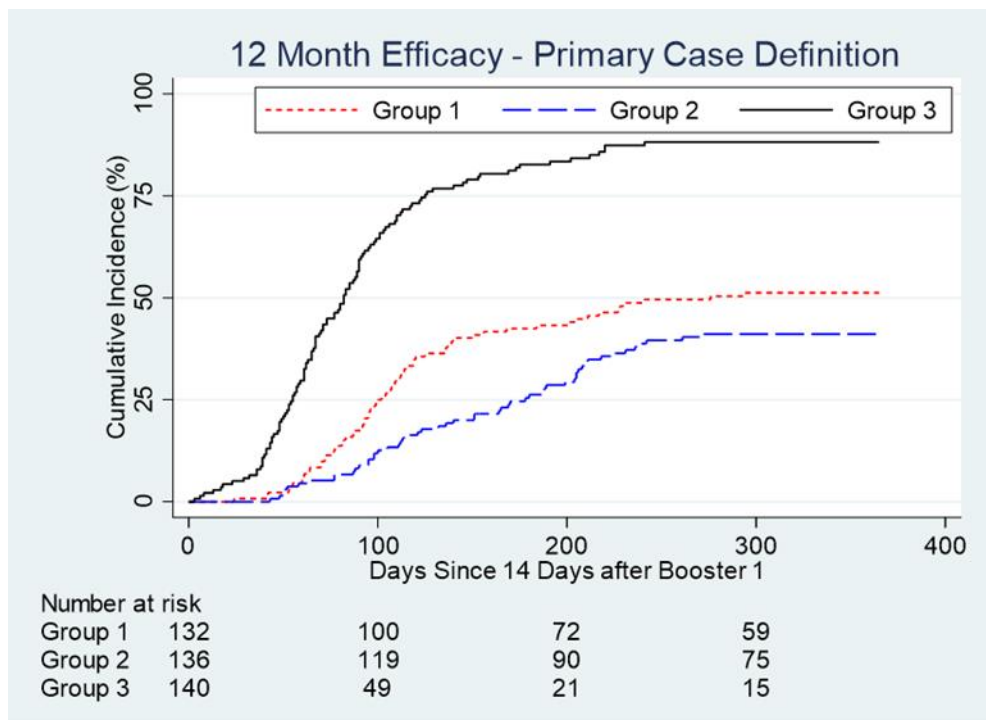
third vaccination. Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received rabies vaccinations.

At 6 months following the first booster vaccination, 196 participants had at least one episode of clinical malaria. These cases occurred in 54/132 (41%) participants in group 1, 31/137 (23%) participants in group 2 and 111/140 (79%) participants in group 3. VE was 69% (95% CI 57-78; $p < 0.0001$) in group 1 and 85% (95% CI 77-90; $p < 0.0001$) in group 2 (supplementary appendix table 23) (205).

At 12 months following the first booster vaccination (range 327-398), 242 participants had at least one episode of clinical malaria. These cases occurred in 67/132 (51%) participants in group 1, 54/137 (39%) participants in group 2 and 121/140 (86%) participants in group 3. VE was 71% (95% CI 60-78; $p < 0.0001$) in group 1 and 80% (95% CI 72-85; $p < 0.0001$) in group 2. When adjusting for the same potential confounders described above, efficacy was similar. VE in group 1 was 70% (95% CI 59-78; $p < 0.0001$) in group 1 and 81% (95% CI 74-87; $p < 0.0001$) in group 2 (Figure 5C, supplementary appendix table 23) (205).

At 24 months following the primary series of vaccinations (range 660-731), 280 participants had at least one episode of clinical malaria. These cases occurred in 82/132 (62%) participants in group 1, 70/137 (51%) participants in group 2 and 128/140 (91%) participants in group 3. VE was 66% (95% CI 55-74; $p < 0.0001$) in group 1 and 75% (95% CI 66-81; $p < 0.0001$) in group 2. When adjusting for the potential confounders, VE in group 1 was 66% (95% CI 55-74; $p < 0.0001$) in group 1 and 76% (95% CI 68-83; $p < 0.0001$) in group 2 (Figure 5C, supplementary appendix table 22) (205).

A



B

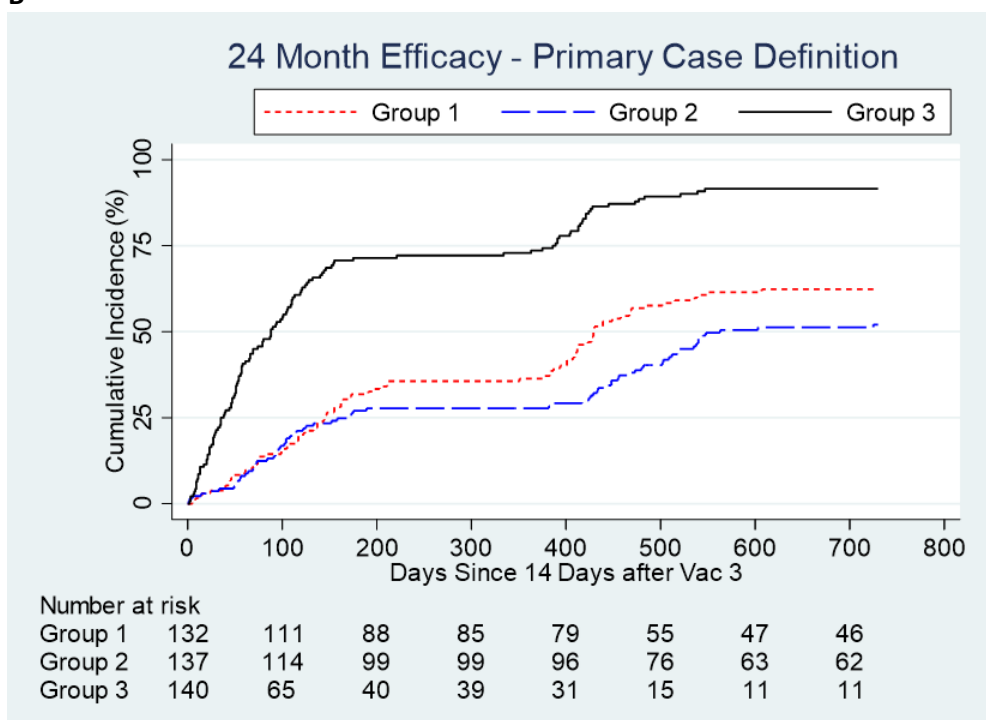


Figure 5C: Kaplan – Meier estimates of the time to first episode of clinical malaria, primary case definition.

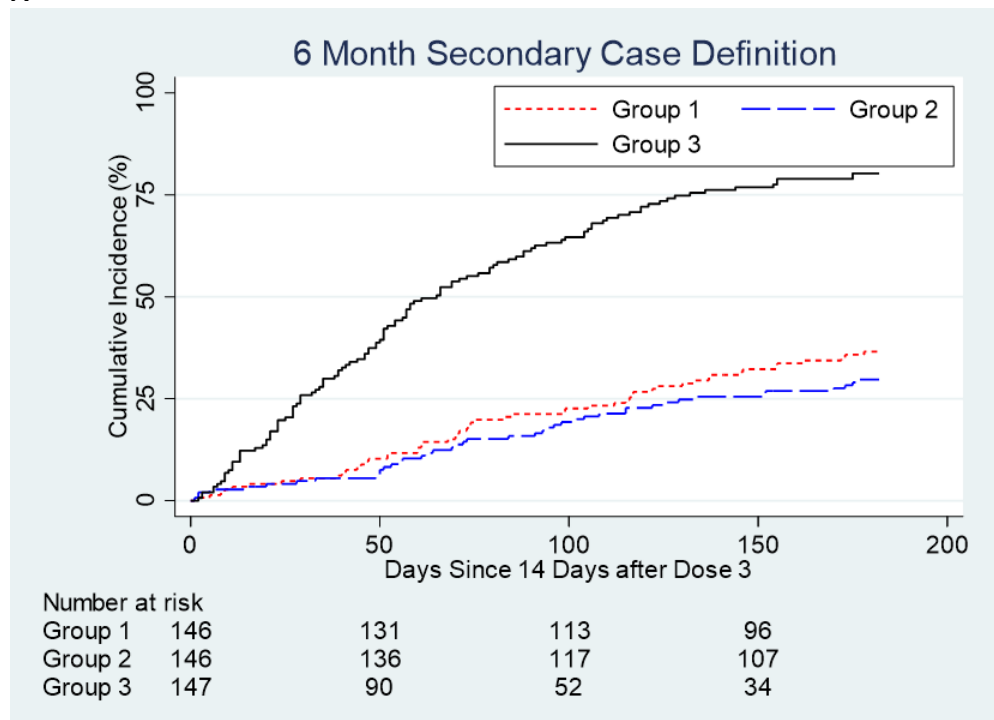
Primary analysis was based on a modified intention-to-treat population. Panel A shows the data from 14 days to 12 months after booster vaccination. Panel B shows the data from 14 days to 24 months

after third vaccination. Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received rabies vaccinations.

5.10.2.1.2 Secondary case definition

According to the definition of fever $\geq 37.5^{\circ}\text{C}$ and a parasitaemia $>0/\mu\text{l}$, 214 participants had a first episode of clinical malaria at 6 months following the primary series of vaccinations. A Cox regression model comparing group 1 with group 3 and group 2 with group 3 resulted in VE of 73% (95% CI 63-81; $p < 0.0001$) and 79% (95% CI 70-85; $p < 0.0001$) respectively (Figure 5D). At 12 months, 224 participants met the secondary case definition and VE was 70% (95% CI 60-78; $p < 0.0001$) in group 1 and 80% (95% CI 72-86; $p < 0.0001$) in group 2 (Figure 5D, supplementary appendix table 24) (171).

A



B

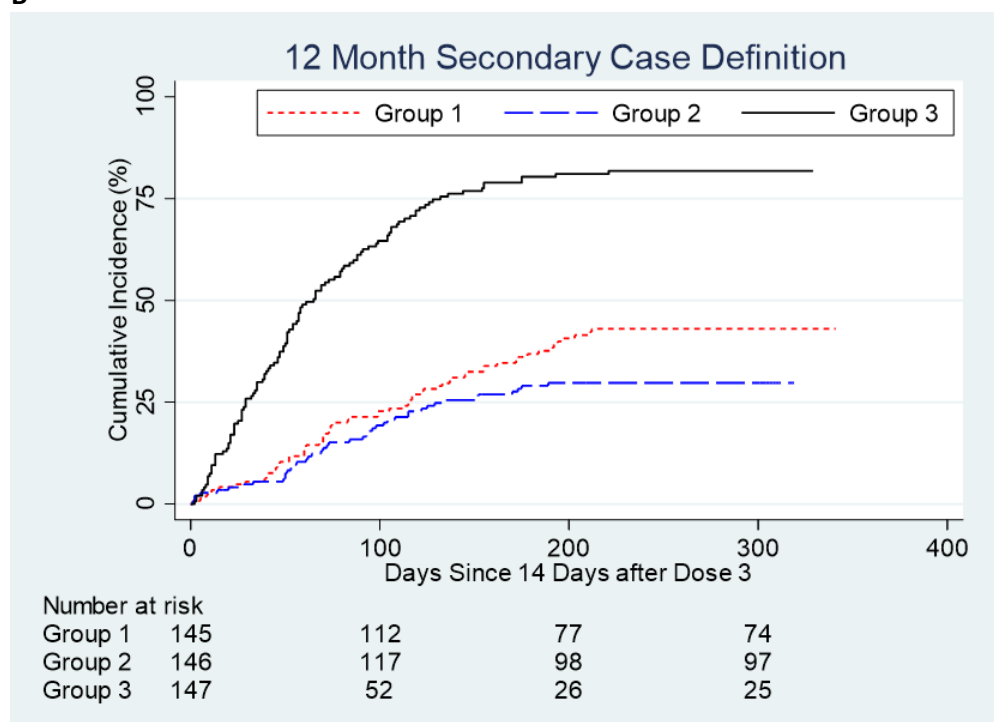
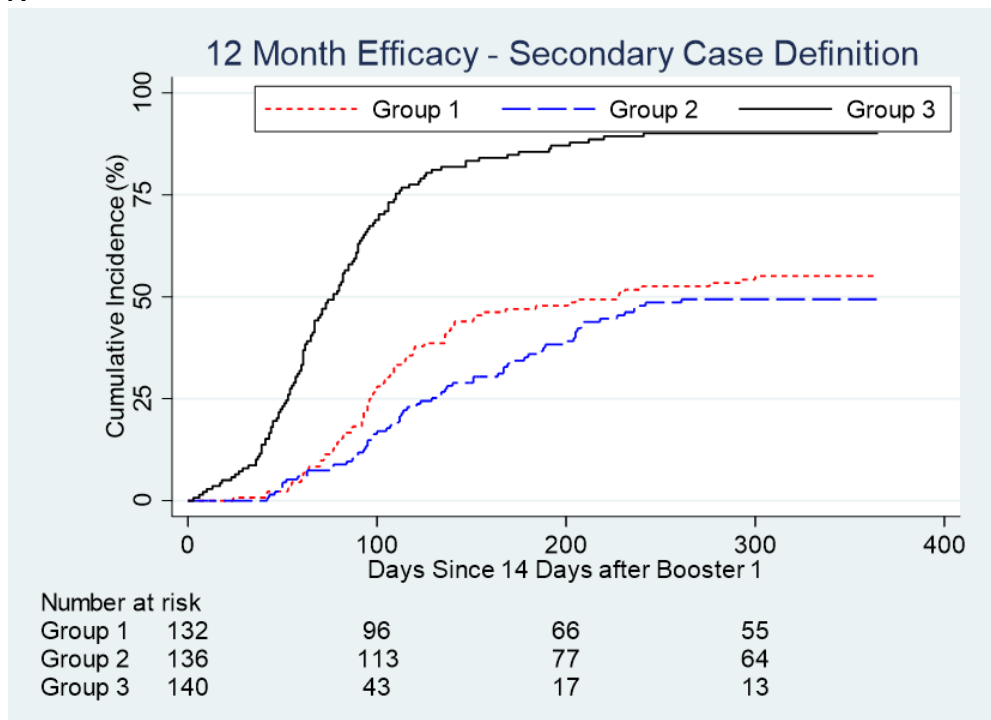


Figure 5D: Kaplan – Meier estimates of the time to first episode of clinical malaria, secondary case definition.

Panel A shows the data from 14 days to 6 months after third vaccination. Panel B shows the data from 14 days to 12 months after third vaccination. Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received rabies vaccinations.

At 12 months following the first booster vaccination, 261 participants met the secondary case definition and VE was 71% (95% CI 60-78; $p < 0.0001$) in group 1 and 77% (95% CI 69-83; $p < 0.0001$) in group 2 supplementary appendix table 23. At 24 months following the primary series of vaccinations, 301 participants met this case definition. VE was 67% (95% CI 56-75; $p < 0.0001$) in group 1 and 75% (95% CI 67-81; $p < 0.0001$) in group 2. (Figure 5E, supplementary appendix table 24) (205).

A



B

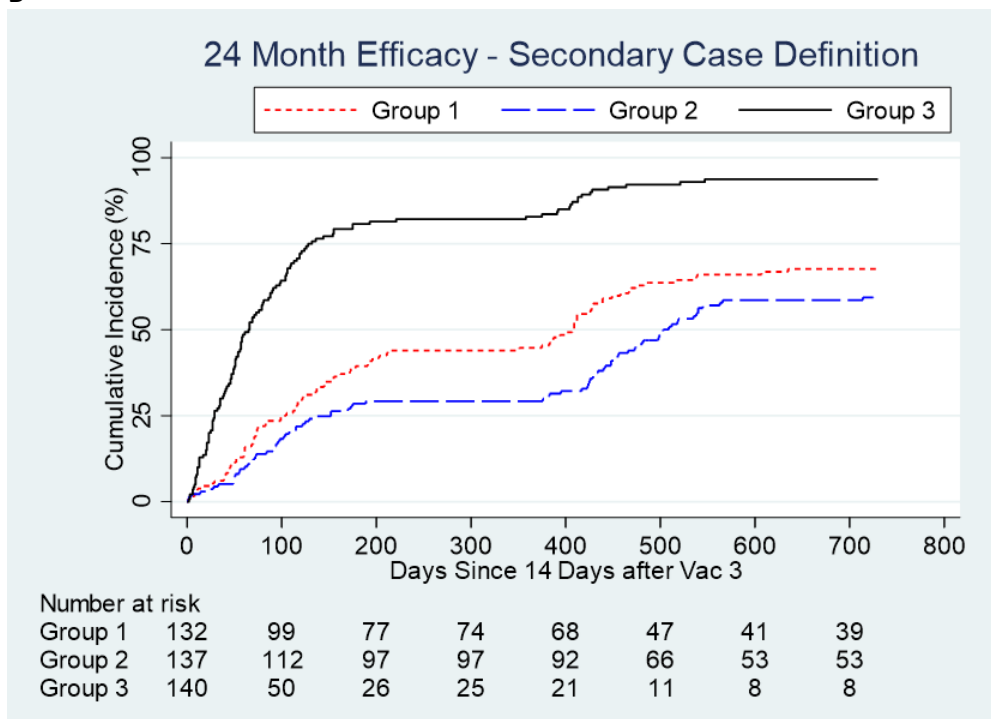


Figure 5E: Kaplan – Meier estimates of the time to first episode of clinical malaria, secondary case definition.

Panel A shows the data from 14 days to 12 months after booster vaccination. Panel B shows the data from 14 days to 24 months after third vaccination. Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received rabies vaccinations.

5.10.2.2 Multiple episodes of malaria

The highest number of malaria episodes noted per participant at 12 months follow-up following the primary series of vaccinations was eight and this was for a participant in the control group.

In group 1, no participant experienced more than three malaria episodes. In group 2, five malaria episodes was the highest with only one participant experiencing four and five episodes (Table 5d).

Table 5d: Number of episodes of clinical malaria per treatment group.

Numbers are number of participants (%). Those who withdrew before 12-month follow-up were not included as they did not have the potential for as many episodes as participants followed up for 12 months. Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received Rabivax-S.

Number of episodes	Group 1 (n=134) n (%)	Group 2 (n=139) n (%)	Group 3 (n=142) n (%)
0	85 (63.4)	101 (72.7)	39 (27.5)
1	30 (22.4)	25 (18.0)	33 (22.5)
2	13 (9.7)	8 (5.8)	34 (23.9)
3	6 (4.5)	3 (2.2)	20 (14.1)
4	0	1 (0.7)	6 (4.2)
5	0	1 (0.7)	6 (4.2)
6	0	0	2 (1.4)
7	0	0	1 (0.7)
8	0	0	1 (0.7)
Total number of episodes	74	59	242

When calculating VE against multiple episodes of malaria at 12 months following the primary series of vaccinations, this was similar to efficacy seen with first episode of clinical malaria (Table 5e). In group 1, VE was 70% (95% CI 60-78; $p < 0.001$) and in group 2, VE was 76% (95% CI 65-83; $p < 0.0001$) (171).

During this period, over the 6-12-month period following the primary series of vaccinations (the dry season with less malaria), VE against multiple episodes of malaria was 45% (95% CI -2-70; $p < 0.06$) in group 1, and 68% (95% CI 36-84; $p < 0.0001$) in group 2.

Table 5e: Calculation of case rate per 1000 person-years and vaccine efficacy against all malaria episodes.

Case incidence rates for all episodes were calculated using negative binomial regression over 12 months following the primary series of vaccinations for groups 1 to 3(207). Wald Test p value comparison to group 3 was used (control group).

Group	Person-years at risk	Events	Rate per 1000 person-years (95% CI)	Protective Efficacy % (95% CI)	Wald Test P-value
Group 1	120.5	75	622.6 (496.5-780.8)	69.6 (58.7-77.6)	<0.0001
Group 2	121.4	60	494.4 (383.9-636.8)	75.7 (66.3-82.4)	<0.0001
Group 3	124.0	250	2015.9 (1780.8-2281.9)		

At 6 months following the booster vaccination (during the malaria season), VE against multiple episodes of malaria was 65% (95% CI 56-72; $p < 0.001$) in group 1, and 78% (95% CI 72-83; $p < 0.0001$) in group 2 (supplementary appendix table 25).

Over 6-12 months following the booster vaccination (the dry season), VE against multiple episodes of malaria was 56% (95% CI 35-70; $p < 0.001$) in group 1, and 67% (95% CI 49-78; $p < 0.0001$) in group 2 (supplementary appendix table 26).

Overall, at 12 months following the booster vaccination, VE against multiple episodes of malaria was 61% (95% CI 50-69; $p < 0.001$) in group 1 and 78% (95% CI 71-83; $p < 0.0001$) in group 2. At 24 months following the primary series of vaccinations, VE against multiple episodes of malaria was 63% (95% CI 55-71; $p < 0.0001$) in group 1 and 77% (95% CI 69-83; $p < 0.0001$) in group 2 (Table 5f) (205).

Table 5f: Calculation of case rate per 1000 person-years and vaccine efficacy against all malaria episodes.

Case incidence rates for all episodes were calculated using negative binomial regression over 12 months following booster vaccination for groups 1 to 3 (207). Wald Test p value comparison to group 3 was used (control group).

Group	Person-years at risk	Events	Rate per 1000 person-years (95% CI)	Protective Efficacy % (95% CI)	Wald Test P-value
Group 1	123.2	144	1168.8 (992.7-1376.2)	60.5 (50.3-68.5)	<0.0001
Group 2	124.8	83	665.1 (536.3-824.7)	77.5 (70.6-82.8)	<0.0001
Group 3	129.2	381	2949.6 (2667.8-3261.2)		

When combining the analyses of both the dry seasons for the first year following the primary series of vaccinations and the second year following the booster vaccination (i.e. January-June 2020 and 2021), VE against multiple episodes of malaria was 53% (95% CI 31-67; $p < 0.0001$) in group 1, and 67% (95% CI 51-78; $p < 0.0001$) in group 2 (supplementary appendix table 26).

5.10.2.3 Asymptomatic infection

Primary analysis was based on a modified-intention-to-treat population. At 6 and 12 months cross-sectional blood films were performed and showed that at 6 months, at least double the number of participants in the control group had asymptomatic malaria. These were 28/147 (19.1%) in group 3 compared with 12/140 (8.6%) in group 1 and 13/145 (9%) in group 2 (Table 5g). When comparing group 1 and group 2 with the control group, significant difference was noted, $p < 0.0001$ and $p = 0.017$ respectively. At 12 months, following the dry season, there were far fewer cases (171). The same was also noted at 12 months following the booster vaccination.

Table 5g: Asymptomatic *P. falciparum* infection.

Asymptomatic infection was defined as the presence of axillary temperature <37.5°C, absence of history of fever within the last 24 hours, and *P. falciparum* parasite density >0 parasites/ µl. Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received Rabivax-S. These were analysed using a log binomial regression model, including randomised group as a covariate. Relative risks (RR) and 95% confidence intervals (CI) were reported comparing groups 1 and 3 and groups 2 and 3. *adjusted for confounding factors of sex, age category (5-9 months, 10-12 months and >12 months), bed net use (adequate or not at day 57) and receiving at least one round (3 doses in one month) of SMC.

Group	Time point	Number with at least one episode of asymptomatic malaria (%)	Unadjusted			Adjusted*		
			RR	95% CI	P-value	RR	95% CI	P-value
Group 1	6 months	12/140 (8.6)	0.44	0.23-0.84	0.012	0.44	0.24-0.84	<0.0001
	12 months	3/132 (2.3)	0.54	0.14-2.11	0.373	0.56	0.14-2.19	0.412
	12 months post booster	2/122 (1.6)	0.30	0.06-1.40	0.125	0.33	0.07-1.58	0.166
Group 2	6 months	13/145 (9.0)	0.46	0.25-0.86	0.013	0.48	0.26-0.89	0.017
	12 months	2/141 (1.4)	0.34	0.07-1.64	0.177	0.38	0.08-1.88	0.229
	12 months post booster	2/125 (1.6)	0.29	0.06-1.39	0.123	0.25	0.05-1.24	0.090
Group 3	6 months	28/147 (19.1)						
	12 months	6/142 (4.2)						
	12 months post booster	7/129 (5.4)						

5.10.2.4 Severe malaria

Two episodes of severe malaria were noted up to 12 months following the booster vaccination and are described further under SAEs in section 1.10.3.1.

5.10.2.5 Impact of adjuvant dose on efficacy

There was no significant difference in number of malaria cases meeting the primary case definition between group 1 and 2 at 6, 12 or 24 months following the primary series of vaccinations ($p > 0.9999$ and $p = 0.061$). However, at 12 months following the booster vaccination, there was a significant difference in number of malaria cases between group 1 and 2 ($p = 0.038$), with fewer malaria cases recorded in group 2 (supplementary appendix table 23).

5.10.2.6 Impact of participant factors on number of malaria episodes

In the malaria vaccine groups (group 1 and group 2), age did not affect the number of malaria episodes at 12 months following the primary series of vaccinations ($p = 0.234$ and $p = 0.182$ respectively). When assessing if gender affected the number of malaria episodes at 12 months, male participants had a significantly higher number of malaria episodes ($p = 0.047$) in group 2. In group 1, female participants had a higher number of malaria episodes but this did not reach statistical significance ($p = 0.111$, supplementary appendix table 27).

When assessing the control group, group 3, neither age ($p = 0.603$) nor gender ($p = 0.754$) affected the number of malaria episodes a participant experienced at 12 months following the primary series of vaccinations.

5.10.3 Safety

5.10.3.1 Serious Adverse Events (SAEs)

There have been nine SAEs in this trial to date, all assessed as not related to vaccination. Further details of these can be found in Table 5h. One SAE was a participant who had severe anaemia (Hb 3.4g/dL) secondary to severe *P. falciparum* malaria. They presented seventeen days following their first vaccination with R21/Matrix-M and following diagnosis were transferred to the referral hospital but sadly died shortly after (205).

Table 5h: Serious Adverse Events to date.

All terms are coded according to MedDRA preferred terms (PT). Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received Rabivax-S. All SAEs were reviewed by the DSMB. Age is age at onset of SAE. M/F corresponds to male/female. Causality was assigned according to criteria set out in chapter 2. IMP – Investigational Medicinal Product.

Group	Number of vaccinations	Age (months)	Gender	Serious Adverse Event (SAE)	Outcome	Number of days following last vaccination to start of SAE	Causality to IMP
1	1	14.3	F	Malaria	Fatal	17	Not related
1	3	20.8	M	Anaemia secondary to pneumonia and enteritis	Resolved	161	Not related
1	3	22.4	M	Acute lymphocytic leukaemia	Fatal	210	Not related
1	4	24.9	F	Meningitis	Resolved	19	Not related
2	2	6.6	F	Bronchiolitis	Resolved	12	Not related
2	3	13.6	M	Diarrhoea haemorrhagic and dehydration	Resolved	205	Not related
2	4	25.7	F	Malaria and pneumonia	Resolved	104	Not related
3	3	12.8	M	Malnutrition and Anaemia	Resolved	184	Not related
3	4		F	Intussusception	Resolved	220	Not related

5.10.3.2 Solicited AEs

Local and systemic AEs reported in the first seven days following each vaccination are shown in Figure 5F and Figure 5G.

5.10.3.2.1 Local AEs

Pain, redness and swelling were experienced across all groups after the primary series of vaccinations but overall the number of participants experiencing each AE were low. The highest proportion of participants experiencing a local AE after any given vaccine dose was 15.6%. Swelling was the most common AE across all groups and experienced in more participants after their second dose of the vaccine. There were no severe local adverse events

reported. Following the first booster vaccination, only a very small proportion experienced local AEs and these were all reported in group 1 participants (Figure 5F).

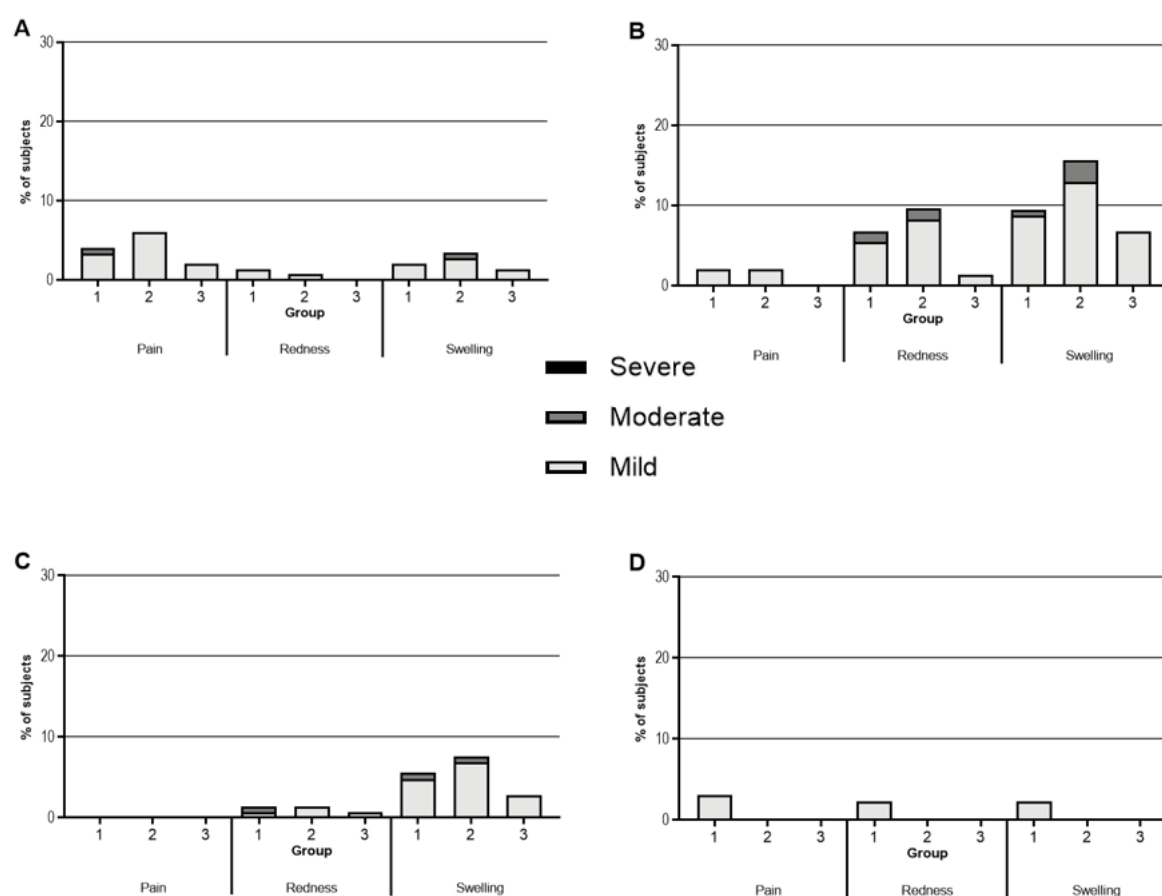


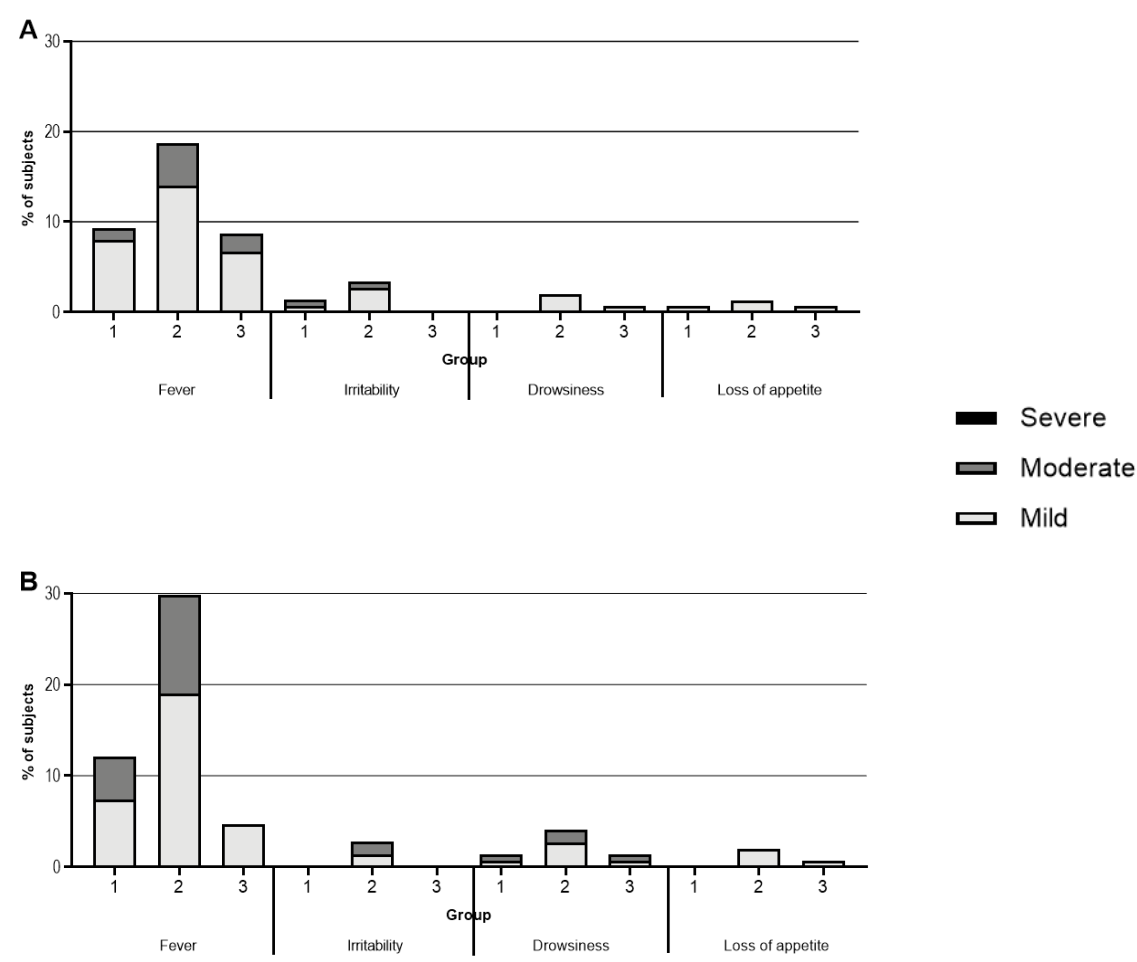
Figure 5F: Local solicited adverse events reported in the first seven days.

Group 1 received 5 μ g R21/25 μ g Matrix-M, Group 2 received 5 μ g R21/50 μ g Matrix-M and Group 3, the control group, received Rabivax-S. Panel A is following the first dose (n=450), panel B following second dose (n=445), panel C following third dose (n=442) and panel D following the booster dose (n=410). After each dose, each adverse event per participant is counted once at the highest reported grade.

5.10.3.3 Systemic AEs

Across the primary series of vaccinations and following the booster dose, fever was the most common systemic AE. Following each dose of vaccine, there were significantly more fevers in group 2, the higher adjuvant dose malaria vaccine group, than in group 1 or 3 (supplementary appendix table 28). The highest proportion of participants (30%) who had a fever after any given dose was in group 2 after the second dose. Overall, the majority of fevers

were mild, $\geq 37.5^{\circ}\text{C} - 38.0^{\circ}\text{C}$. Two participants had a severe fever of $>39.0^{\circ}\text{C}$ and these were both following the third dose of vaccine in malaria vaccine groups (Figure 5G) (171).



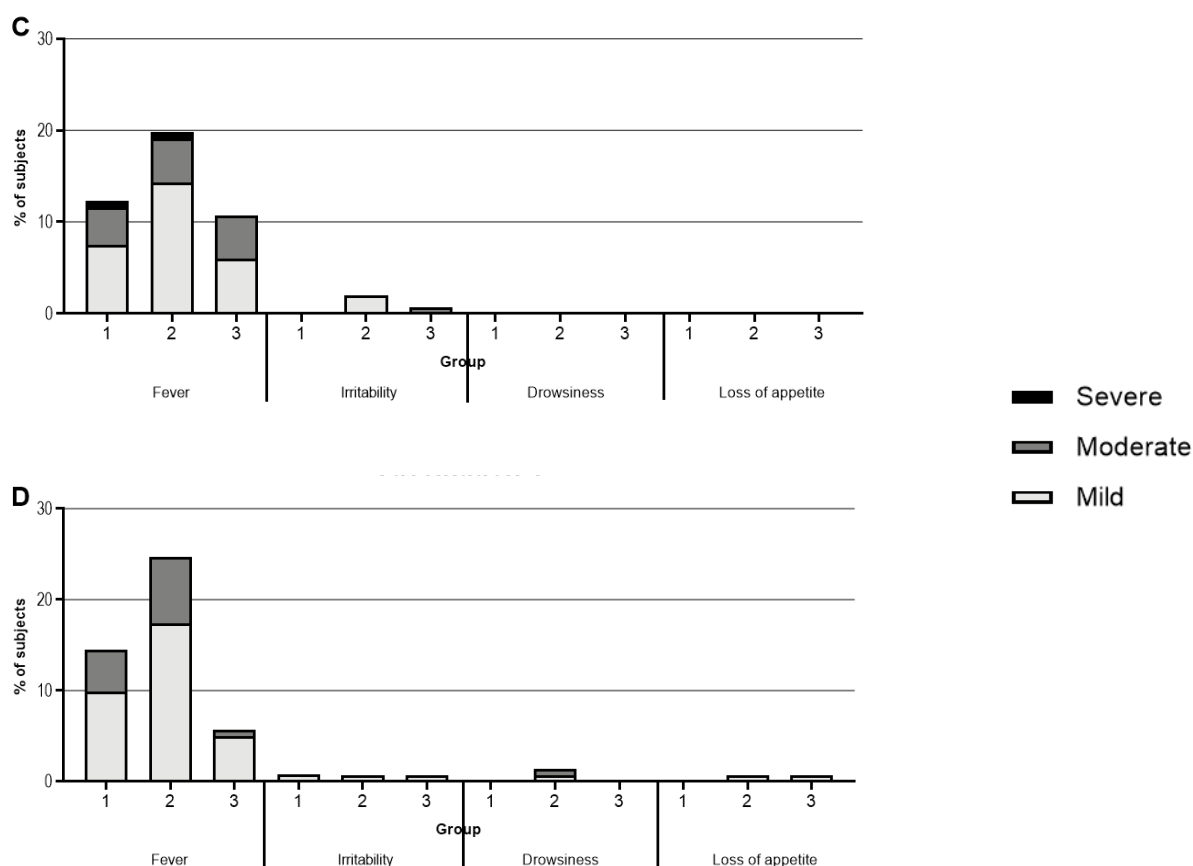


Figure 5G: Systemic solicited adverse events reported in the first seven days.

Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received Rabivax-S. Panel A is following the first dose (n=450), panel B following second dose (n=445), panel C following third dose (n=442) and panel D following the booster dose (n=410). After each dose, each adverse event per participant is counted once at the highest reported grade. Fever was defined as a temperature of 37.5°C and above. The grading of adverse events can be found in the chapter 2.

5.10.3.4 Unsolicited AEs

There were no trends noted across the unsolicited AEs following the primary series of vaccinations or the booster vaccination. None of the AEs were assessed as related to vaccinations. There were 811 terms assigned following the primary series of vaccinations and 203 following the booster vaccination. The number of events were similar per group. Details of all assigned events can be found in supplementary appendix table 29 - supplementary appendix table 31 (171).

5.10.3.5 Laboratory AEs

Following the primary series of vaccinations, anaemia was noted across all three vaccine groups and leucocytosis in one participant in group 1. None of these abnormalities were assessed as related to vaccination. Severe anaemia was also noted in relation to SAEs. Details of these laboratory events can be found in supplementary appendix table 32 (171).

5.10.4 R21/Matrix-M immunogenicity

5.10.4.1 Humoral response before and after primary series of vaccinations

NANP-specific IgG was measured at baseline and following vaccinations. No participants had detectable NANP IgG levels at baseline. All participants in malaria vaccine groups mounted a response, with highest responses noted at 28 days following third vaccination. These antibody titres dropped significantly over 6 months and further at one year in both low and high adjuvant dose groups (Figure 5H, supplementary appendix table 33) (171).

5.10.4.2 Humoral response after first booster vaccination

All participants who were still part of the trial received a booster vaccination approximately 12 months after the third vaccination from the primary series. In both low and high adjuvant dose groups, NANP-specific IgG titres were boosted to similar levels seen at 28 days following the primary series of vaccinations (Figure 5H, supplementary appendix table 33). There was no significant difference in titres at day 84 or 28 days following the booster vaccination in each vaccine group (205).

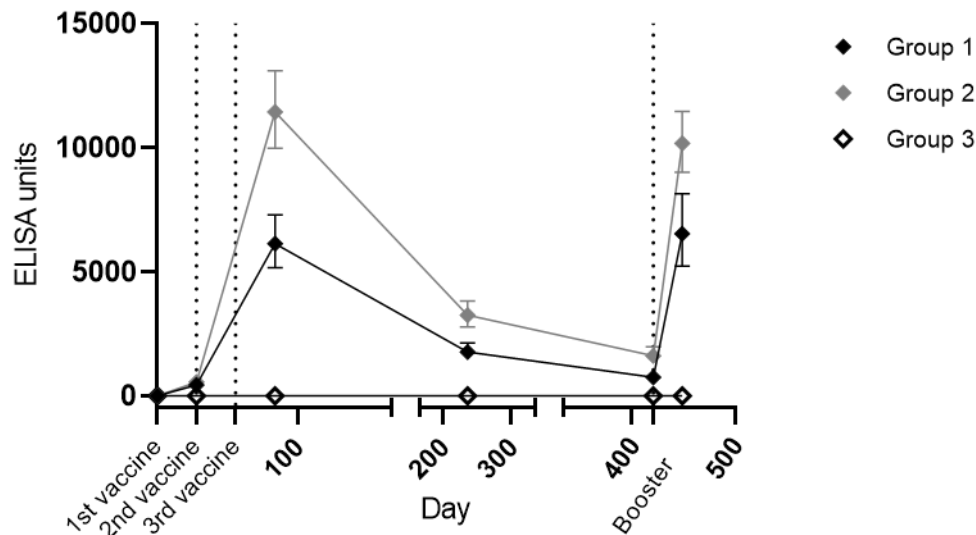


Figure 5H: Geometric mean titres (GMT) of total NANP-specific IgG as measured by ELISA.

Group 1 received 5µg R21/25µg Matrix-M, Group 2 received 5µg R21/50µg Matrix-M and Group 3, the control group, received RabiVax-S. The first three doses were administered 4 weeks apart and the booster dose approximately 12 months later.

5.10.4.3 Impact of adjuvant dose on antibody responses

Antibody titres at 28 days following the primary series of vaccinations were significantly higher in the high adjuvant dose group compared with the low adjuvant dose group ($p < 0.0001$). The GMT for group 1 was 6,133 EU (95% CI 5,161 – 7,289) and for group 2, 11,438 EU (95% CI 9,985 – 13,102). Significantly higher antibody titres were also seen 28 days following the booster vaccination, $p = 0.0006$. The GMT 28 days following booster vaccinations for group 1 was 6,529 EU (95% CI 5,230 – 8,151) and for group 2, 10,170 EU (95% CI 9,016 – 11,472, supplementary appendix table 33).

5.10.4.4 Impact of participant factors on antibody responses

5.10.4.4.1 Age

Age did not significantly affect antibody response at 28 days following the primary series of vaccinations in either group 1 ($p = 0.099$) or group 2 ($p = 0.892$).

5.10.4.4.2 Gender

Gender did not significantly affect antibody response at 28 days following the primary series of vaccinations in group 1 ($p = 0.320$) or group 2 ($p = 0.191$).

5.10.4.5 Impact of antibody responses on clinical malaria episodes

5.10.4.5.1 Group 1 (low adjuvant dose)

Using a linear regression model, the difference in mean antibody titres between participants with or without at least one malaria episode was calculated (supplementary table 12). At 12 months following the primary series of vaccinations and 12 months following the booster vaccination, there was no significant difference in antibody levels when comparing those participants who had a malaria episode with those who did not ($p = 0.514$ and $p = 0.099$ respectively, supplementary appendix table 34).

5.10.4.5.2 Group 2 (high adjuvant dose)

When comparing differences in mean antibody titres in this group, there was a significant increase in those participants who did not experience a malaria episode compared with those who did at 12 months following the primary series of vaccinations ($p = 0.0004$), and 12 months following the booster vaccination ($p = 0.032$, supplementary appendix table 34).

5.10.4.6 Correlating antibody responses with efficacy

In group 2, antibody levels correlated negatively with number of malaria episodes at 12 months following the primary series of vaccinations (Spearman's $\rho = -0.32$ (-0.45 - -0.19 , $p = 0.0001$) and 12 months following the booster vaccination (Spearman's $\rho = -0.20$ (-0.34 - -0.06 , $p = 0.02$). Using reverse cumulative distribution curves, a threshold of NANP antibodies was identified as a correlate of protection. This was 6618 (95% CI, 5565 – 8397) ELISA units for the first 12 months following the primary series of vaccinations and 6130 (95% CI, 5347 – 7179) ELISA units for the 12 months following the booster vaccination (supplementary appendix figure 1).

5.11 Comparison of data between Kenyan infants and Burkinabe infants

5.11.1 Impact of different populations on safety of vaccine

In chapter 4, I discussed the clinical trial, VAC 073, where infants in three study vaccine groups received the same doses and schedule of R21/Matrix-M as in this trial. The infants were 5-12 months in Kenya and 5-17 months in Burkina Faso. For the primary series of vaccinations, in the vaccine groups with the low adjuvant dose group (5µg R21/25µg Matrix-M), one participant in Burkina Faso had a severe AE while none were observed in Kenya. In Burkina Faso, there were 0.37 AEs reported per participant and in Kenya, there were 0.72 AEs per participant. For the high adjuvant dose group (5µg R21/50µg Matrix-M), one participant in Burkina Faso had a severe AE, and two participants in Kenya had a severe AE. There were 0.83 AEs reported per participant in Burkina Faso and 1.73 AEs per participant in Kenya. All severe AEs recorded were fevers.

5.11.2 Impact of different populations on antibody responses

The highest response induced in Kenyan infants receiving R21/25µg Matrix-M was 31,858 EU, GMT 7,219 EU (95% CI 4,684-11,126). In Burkinabe infants, the highest response was 44,061 EU, GMT 6,133 EU (95% CI 5,161-7,289). In Kenyan infants receiving R21/50µg Matrix-M, the highest response was 86,352 EU, GMT 13,280 EU (95% CI 7,366-23,942). In Burkinabe infants, the highest response was 159,350 EU, GMT 11,438 EU (95% CI 9,985-13,102). When making the comparison between the two populations of Kenyan and Burkinabe infants receiving the same vaccine dose, there was no significant difference at either adjuvant dose ($p = 0.77$ with 5µg R21/25µg Matrix-M and $p = 0.55$ with 5µg R21/50µg Matrix-M, Figure 5I).

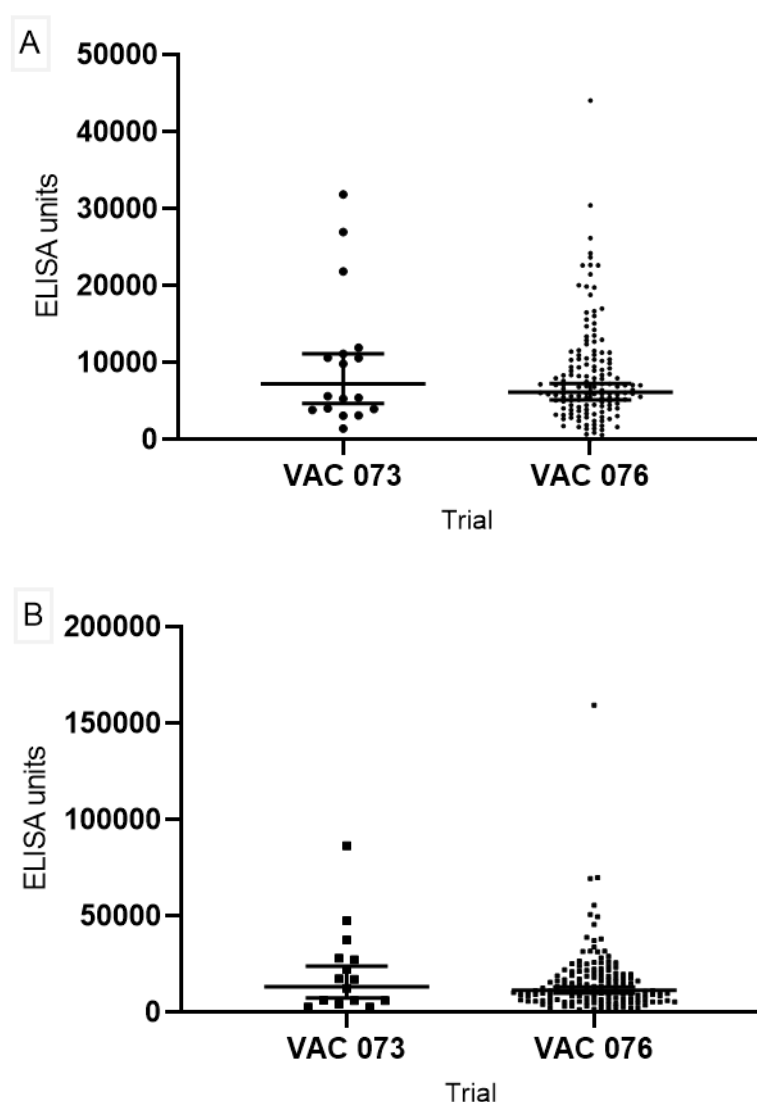


Figure 5I: Total NANP IgG as measured by ELISA at day 84, 28 days following primary series of vaccinations in Kenyan infants (VAC 073) and Burkinabe infants (VAC 076).

All participants in panel A received 5 μ g R21/ 25 μ g Matrix-M and 5 μ g R21/ 50 μ g Matrix-M in panel B. GMT and 95% CI are shown. Mann-Whitney test was used to calculate p value.

5.12 Discussion

I report here on the safety, immunogenicity and efficacy of R21/Matrix-M in a target population of African infants. This is the first malaria vaccine to reach the WHO-specified efficacy goal of $\geq 75\%$, with 77% efficacy seen over 12 months in one of the malaria vaccine groups and 80% efficacy at 12 months following a booster vaccination. Moreover, vaccine efficacy was similar against multiple episodes of malaria, which is particularly important as most children living in endemic areas will experience many episodes of clinical malaria (208-210). Our data showed

that infants experienced up to eight episodes of malaria over the course of the first year. The study was not powered to show efficacy against severe malaria, but it is hoped this endpoint will be assessed further in the phase III.

In the clinical trial setting, clinical malaria is assessed according to primary and secondary case definitions. When using the secondary case definition, even higher VE is noted in the higher adjuvant dose group after the primary series of vaccinations (80%) and 12 months following the booster vaccination (77%), compared with VE according to the primary case definition. While the high parasitaemia in the primary case definition helps to confirm that the clinical picture is due to malaria, in this context, any child presenting with a fever and a positive blood film is likely to be treated for malaria. Therefore, VE against the secondary case definition is probably more applicable in practice. However, this VE must be interpreted with caution, as malaria may not be the key reason for the presentation. In these secondary endpoint cases, treatment or prevention (in the form of R21/Matrix-M vaccine) for malaria may not be the solution to curing the child or protecting them from malaria.

This vaccine was studied in an area of highly seasonal malaria transmission, with the primary series of vaccinations administered prior to or at the start of the malaria season. For this reason, the majority of clinical malaria episodes and asymptomatic malaria episodes were recorded in the first six months of the data collected for the year. It is unclear if this seasonal administration of vaccines increased VE significantly. However, when analysing multiple episodes of malaria over the second 6 months of the malaria season following the booster vaccination (i.e. restricted to the dry season from January to June), high VE was still noted at 67% (95% CI 49-78) in group 2 suggesting that efficacy can be maintained all year round. This is also despite the reduction in antibody titres in the second six months after vaccinations, potentially suggesting that there would be similar VE in areas of non-seasonal malaria transmission. The trial site in Nanoro, Burkina Faso, was one of the eleven sites where RTS,S/AS01 was tested in the large phase III trial. At that time, VE of 44% (95% CI 37-50) was reported compared with the VE seen with R21/Matrix-M in the higher adjuvant dose group

of 77% (95% CI 67-84). It should be noted that vaccinations with R21/Matrix-M were given with a seasonal approach but RTS,S/AS01 vaccinations were administered all year round. To test if seasonal administration does have a significant impact on vaccine efficacy, in the phase III trial evaluating R21/Matrix-M, trial sites are in countries experiencing different transmission patterns, and vaccinations are being administered at different times of year.

A recent non-inferiority trial in Mali and Burkina Faso comparing seasonal administration of RTS,S/AS01 alone, seasonal malaria chemoprevention (SMC) alone, or both in combination, found that the seasonal administration of vaccine was non-inferior to chemoprevention. Chandramohan et al. demonstrated that the RTS,S/AS01 vaccine in combination with SMC resulted in substantially lower incidence of malaria compared with vaccine or SMC alone (46). Policy recommendations in Burkina Faso suggest there should be four monthly courses of SMC (19) and a recent study concluded that a fifth monthly course would be of value (211). However, it was noted in our trial that the vast majority of children did not receive the recommended four courses in contrast to the above study and previously reported high coverage of SMC across Burkina Faso (206). As our trial aimed to test vaccine efficacy in a real-world setting, SMC delivery was not part of our trial design. We recorded SMC, which was routinely delivered by the local healthcare teams. Adjusting for SMC did not significantly alter R21/Matrix-M vaccine efficacy. In the ongoing phase III trial assessing R21/Matrix-M, SMC is being given as part of the trial in one of the seasonal trial sites (Bougouni, Mali) and as standard of care in the other seasonal trial site (Nanoro, Burkina Faso) with the aim to assess SMC coverage and the potential of coverage to affect vaccine efficacy. It will be interesting to assess if similar results are seen with R21/Matrix-M and SMC as were noted with RTS,S/AS01 and SMC (46).

This study, in a larger cohort of the target population than the phase I trial, showed that R21/Matrix-M was safe and well-tolerated. There were no SUSARs or SAEs assessed as related to vaccinations. We did not see an increased rate of meningitis or increased female mortality, as had been noted with RTS,S/AS01 in its large phase III trial (43, 44). While it is

difficult to assess subjective AEs in infants, we sought to observe them by adopting a standard scale used in vaccine trials and which was the same as that used in RTS,S/AS01 (212). Swelling and fever were the AEs most commonly experienced in this trial. However, after any given dose, no more than 16% of participants in any group had swelling and no more than 30% in any group had fever. Even though fever was significantly higher in the high adjuvant dose group, overall numbers were low, and the safety profile of R21/Matrix-M is in line with reported safety profiles for common childhood immunisations. The most common local and systemic AEs with RTS,S/AS01 in the phase III were pain at the injection site: 12% (95% CI 11.4-13.4) of participants experienced this after primary series of vaccinations, with 31% (95% CI 30-33) experiencing fever (212). These AEs were less frequent with R21/Matrix-M: for pain 2% and 3% of participants experienced this in the low and high adjuvant dose groups respectively, and for fever, 12% and 23%. When assessing the safety of R21/Matrix-M in two different African infant populations across the phase I and phase II trials, similar profiles in Kenya and Burkina Faso were noted and very few severe AEs were reported.

All participants in malaria vaccine groups developed antibodies to NANP, the central repeat of the *P. falciparum* CS protein, and at 28 days following third vaccination, these were significantly higher in the high adjuvant dose group compared with the low adjuvant dose group. Antibody titres dropped over the 12 months following the primary series of vaccinations in both groups, but after a booster vaccination, were similar to those following the third vaccination. Both malaria vaccine groups in this trial demonstrated high-level efficacy with no significant difference noted according to adjuvant dose after the primary series of vaccinations but a difference in VE was noted after the booster vaccination. This further suggests that NANP-specific IgG quality or function may be related to efficacy. When RTS,S/AS01 participants received a booster dose, antibodies peaked at half the level of that following the third vaccination and efficacy waned considerably over time (41). R21 lacks the excess HBsAg, resulting in a higher proportion of CSP in the vaccine (49) and therefore, potentially creating a greater immune response to CSP. In light of these immunogenicity results with R21/Matrix-M,

it is hoped that efficacy might be maintained if booster vaccinations are administered annually, and prior to the malaria season annually. This is currently under investigation in this trial.

Antibody titres following R21/Matrix-M vaccinations correlated with protection against malaria in the higher adjuvant dose group during both the first and second year of follow-up. Analyses of reverse cumulative distribution data suggest a 77% reduction in the risk of malaria if NANP levels were 6,600 ELISA units following the primary series of vaccinations (205). This threshold correlate will need to be assessed further in future trials for this age group but may provide a useful biomarker of protection.

At 28 days following third vaccination, antibody titres in the high adjuvant dose group were also noted to be about eight times higher in these African infants compared with malaria naïve adults from the UK, who were protected in CHMI trials. As discussed in chapter 3, R21/Matrix-M tested in the UK population using controlled human malaria infection (CHMI) showed efficacy of 62.5-75%. The comparable efficacy between the CHMI trial and field-efficacy trial demonstrates that the CHMI model can inform VE in the field. This is important when thinking about testing different doses and schedules of this vaccine, but also new malaria vaccine candidates entering clinical development.

It is unclear why the antibody responses are so varied between adults, young children and infants. Neither age nor gender had an impact on antibody responses in Burkina Faso within the target infant group. Higher antibody avidity correlated with protection in malaria naïve adults with a specific RTS,S/AS01 immunisation regime (47), therefore, further work on investigating antibodies in terms of avidity and affinity may help to answer these questions. Malaria is a significant burden across sub-Saharan Africa and, therefore, it is important that a malaria vaccine induces good antibody responses and is efficacious in all populations.

There are some limitations with this study, for example, the modest sample size to evaluate the safety of a relatively new vaccine adjuvant, the short period of follow-up, the limited age-range of 5-17-month-old infants at enrolment, and testing the vaccine using seasonal

administration in one area of highly seasonal malaria transmission. The follow-up of this trial has now been extended for another two years to enable efficacy to be measured over a longer period of time and assess the value of further booster vaccinations prior to the malaria season (on an annual basis). This not only needs to be assessed with respect to safety and efficacy, but also with respect to the feasibility of reaching the target children each year.

The encouraging data from this trial supported the progression to a larger safety and efficacy phase III trial assessing R21/Matrix-M in infants, VAC 078, which is fully recruited, with 12-month efficacy data anticipated later this year. This has enabled a larger sample size to be evaluated, not only for efficacy but also for safety, which is of importance, given the relatively new adjuvant formulation. The significant difference in antibody titres, as well as the possibility of NANP-specific IgG representing a correlate of protection, has informed the dose of vaccine and adjuvant that has been used in VAC 078, the phase III trial: 5µg R21 with the higher adjuvant dose, 50µg Matrix-M. The phase III trial is taking place across five African sites of differing malaria transmission and seasonality, assessing efficacy with both seasonal and non-seasonal vaccine administration.

6 Further investigation into participant antibody responses to R21/Matrix-M vaccination and number of clinical malaria episodes by analysing the gut microbiome

6.1 Introduction

In the previous chapter, we saw good efficacy of R21/Matrix-M in African infants and the possibility, with a higher dose of adjuvant, that a certain threshold of NANP-specific IgG may be required for protection. However, throughout this thesis, there was variability in responses to NANP within the individual populations assessed and according to age. Equally not all participants who had good responses to NANP were protected from malaria. Therefore, I wanted to investigate a possible reason for variation in responses and efficacy, specifically differences in the gut microbiome. I explored the relationship between vaccine responses and the gut microbiome among the Burkinabe infant population from the phase IIb trial.

In the first year of life, an infant's dietary and environmental exposure increases, enhancing the gut microbiome (213). Factors such as genetics, delivery mode, diet, sanitary habits, environment and antibiotic use are all thought to alter the microbiome environment (118). The gut microbiome has the highest intra- and inter- individual variability up to the age of 2- 3 years, when it starts to reach a composition likened to the stable adult microbiome (142, 213). Due to this, infants are more susceptible to environmental factors affecting the microbiome, causing a long-lasting impact on immunity (214). Many interactions have been identified between the microbiome and the innate, as well as adaptive, immune systems (215). In infant germ-free mice, reduced quantity and function of lymphoid cells were noted (216) and this has been echoed in human studies (217). Increased maturation of IgA-secreting cells was associated with presence of *Bacteroides fragilis* in the gut microbiome of one- and two-month old infants. Equally, maturation of IgM-secreting cells was noted in comparison with other bacterial genera studied or diet (218). These results suggest the potential of the gut microbiome to prime the immune system or modulate it. Studies in germ-free mice and infants have shown a skew towards T-helper cell (Th) 2 response (145, 219) although this imbalance can be corrected in germ-free mice with colonisation of *B. fragilis* (220). Other examples of microbiome composition affecting the immune response include the colonisation of germ-free mice with segmented filamentous bacteria to induce Th17 cells (221) or the administration of probiotics

in infants. One such study has shown the administration of *Lactobacillus acidophilus* in the first six months of life reduced IL-10 responses to tetanus toxoid (TT) vaccine (222).

In recent years, given the potential links between the microbiome and function of the immune system, there has been evidence implicating the gut microbiome with vaccine immune responses. Mechanisms are not well understood, and currently, the few studies that have been published have inconsistent findings. These studies have also been in small sample numbers, different age groups and various geographical locations. Infants administered oral rotavirus vaccine in India and Nicaragua showed no significant difference in gut microbiome composition between vaccine responders and non-responders (146, 147), however, in Pakistan, increased abundance of *Firmicutes* and *Proteobacteria* were found in vaccine responders with increased *Escherichia coli* and *Serratia* in non-responders (145). In Ghana, vaccine responders had increased *Bacilli* and non-responders had increased *Bacteroidetes* (144). In Bangladesh, infants were administered oral polio vaccine (OPV), Bacillus Calmette-Guérin (BCG), TT and hepatitis B vaccine (HBV) in two studies. One showed that relative abundance of *Actinobacteria* positively correlated with T-cell responses to OPV, BCG and TT and high levels of *Enterobacteriales*, *Pseudomonales* and *Clostridiales* suggested lower vaccine responses at 15 weeks of age (223). The second study showed that specifically *Bifidobacterium* was positively correlated with CD4 T-cell responses to BCG and TT as well as TT-specific IgG at two years (150).

In addition to the composition of the gut microbiome, it has been hypothesised that it may act as an adjuvant to systemic vaccine responses. This was demonstrated through a study in mice, showing that Toll Like Receptor 5 (TLR5) signalling of flagellin, from the gut microbiome, was required to produce an immune response to the seasonal influenza vaccination. Germ-free mice or mice deficient in TLR5 had significantly reduced IgG and IgM responses to an influenza vaccine (156).

The gut microbiome has been described in Burkinabe children aged 1 - 6 years (n = 14) (224) and 2 - 8 years (n = 24) (225). More recently, a larger study of infants aged 6 – 59 months

was conducted (n=155) (226). Unfortunately, these sample numbers are small, encompassing a wide age range when many changes are expected in the gut microbiome; and participants were recruited from different areas within Burkina Faso representing rural and urban settings. Differences in the gut microbiome were also found between the rural and urban children in Burkina Faso (225). In order to understand specific differences in microbiome composition that may lead to changes in immune responses and vaccine-specific responses, we need to understand the gut microbiome of our target population and potential individual factors affecting it.

We can see from published data that there have been potential links identified between the immune system and the gut microbiome, as well as links with vaccine responses. However, there is limited literature assessing the gut microbiome and vaccine responses with intramuscular vaccines and African infant populations. Therefore, from participants in the VAC 076 clinical trial, rectal swabs were collected from participants at baseline and 306 were sequenced. The objectives of the study and the analysis are discussed below.

6.2 Objectives

6.2.1 Composition of gut microbiome

Describe the composition of gut microbiome in Burkinabe infants aged 5-17 months at baseline.

6.2.2 Gut microbiome and participant factors

Is the composition of the baseline gut microbiome affected by any specific participant factors?

6.2.3 Gut microbiome and immune responses to vaccine

Does the composition of the baseline gut microbiome have any impact on antibody responses to NANP after the primary series of R21/Matrix-M vaccinations?

6.2.4 Gut microbiome and number of clinical malaria episodes

Does the baseline composition of the gut microbiome have any impact on the total number of malaria episodes at 12 months?

6.3 Methods

Rectal swabs were collected prior to first vaccinations in the VAC 076 clinical trial discussed in the previous chapter. All participants were resident in Nanoro, Burkina Faso, and aged 5-17 months.

Detailed methods of DNA isolation and sequencing, and bioinformatic processing can be found in chapter 2.

6.3.1 Sample inclusion

Sampling was performed on all 450 participants enrolled in the VAC 076 malaria vaccine trial. Unfortunately, due to issues with storage, temperature control and transport, sufficient DNA extraction only took place on 306 samples. Therefore, in total, 306 samples were available for sequencing, however, due to missing data, six samples were excluded. Altogether, 300 samples are included in this microbiome analysis when looking at alpha- and beta- diversity. When specifically analysing for responses to NANP, only group 1 and 2 participants could be used as they received the malaria vaccine, R21/Matrix-M (different dose of adjuvant according to group) in the trial. This was a subset of 216 samples. When specifically looking at malaria episodes at 12 months following the primary series of vaccinations, all three trial groups were included (group 3 was the control group), but those participants who had withdrawn, or for whom data were unavailable, were excluded. In total, this was a subset of 274 samples.

Data on baseline participant factors collected in the trial were analysed. This data included age at baseline, gender, delivery mode, nutritional z-score, number of older siblings in household, method of feeding, and age of weaning.

6.3.2 Enterotype analysis

The microbiome was then characterised according to the population characteristics and using principle coordinates analysis (PCoA) with Bray-Curtis distance (to examine sample dissimilarity by microbiome). Enterotypes were identified using hierarchical clustering of Bray-Curtis distances.

6.3.3 Alpha-diversity analysis

Alpha-diversity was assessed by looking at the evenness and richness of the microbiome enabling Shannon diversity index calculation (used to measure the diversity of species within the samples). Multiple linear regression (Shannon diversity ~ age at baseline + z-score + number of older siblings + enterotype) was performed on this diversity index to take into account multiple factors that may affect how diverse each sample was.

6.3.4 Beta-diversity analysis

Mixed effects linear models were used to assess the potential effects of participant factors, NANP-specific IgG levels 28 days following the primary series of vaccinations and the number of malaria episodes at 12 months on taxa abundance. Confounders between trial groups were assessed to help inform the analysis model. This was performed using chi-square or Kruskal-Wallis with Dunn's multiple comparisons tests, PCoA and permutational multivariate analysis of variance (PERMANOVA).

Effects on NANP-specific IgG levels and malaria episodes were assessed independently of each other using multiple linear modelling. The first model (model 1) applied contained the fixed effects of NANP-specific IgG levels 28 days following the primary series of vaccinations, age at baseline, and the number of older siblings in their household with the random effect of enterotype [model 1 equation: NANP-specific IgG levels Day 84 + age at baseline + number of older siblings; random effect: enterotype]. The second model (model 2) applied contained the fixed effects of clinical malaria episodes for 12 months following the primary series of vaccinations, age at baseline and the number of older siblings in their household with the random effect of enterotype [model 2 equation: total number of malaria episodes at 12 months + age at baseline + number of older siblings; random effect: enterotype]. After both models were applied, bacterial features (bacterial phyla, families and genera) that were significant in both models were assessed. P-values were adjusted for multiple testing using Benjamini-Hochberg method.

6.4 Results

6.4.1 Population characteristics

Baseline measures were collected on several participant factors including age, gender, delivery mode, nutritional z-score, number of older siblings in household, method of feeding and age of weaning. Due to the distribution of answers to questions related to these factors, there was not enough power to assess the impact of factors on the gut microbiome such as delivery mode and age of weaning.

When assessing for confounders between trial groups, there was no significant difference between trial groups according to gender, number of older siblings in household, method of feeding and z-score (supplementary appendix section 8.15, supplementary appendix figure 2 - supplementary appendix figure 6) at baseline. While in the VAC 076 whole trial data set there was no significant difference in age between the groups, in the subset of 300 samples used for this microbiome study, using Dunn's multiple comparisons test, difference in age was noted between group 2 and 3 ($p = 0.02$) (supplementary appendix figure 4). As expected from the clinical trial results, there was a significant difference between trial groups for NANP- specific IgG at 28 days following the primary series of vaccinations ($p < 0.0001$) and the number of malaria episodes at 12 months following the primary series of vaccinations ($p < 0.0001$, supplementary appendix figure 7 - supplementary appendix figure 8) when comparing malaria vaccine groups to the control group.

Baseline characteristics are summarised in Table 6a.

Table 6a: Baseline characteristics of participants samples analysed.

Groups 1 and 2 received R21/Matrix-M and group 3 were the control group which received the Rabies vaccine. *Z-score calculated using WHO weight-for-age Child Growth Standards.

	Group 1 n=111	Group 2 n=112	Group 3 n=77	Overall n=300
Age in months Mean (SD)	10.8 (3.7)	10.5 (3.6)	11.9 (3.8)	11.1 (3.7)
Sex Male Female Unknown	53 (47.7) 58 (52.3) 0 (0)	65 (58) 46 (41.1) 1 (0.9)	36 (46.8) 41 (53.2) 0 (0)	154 (51.3) 145 (48.3) 1 (0.3)
Number of older siblings in household 0 1 2 3 4 5 6 7 8 Unknown	12 (47.7) 15 (13.5) 14 (12.6) 27 (24.3) 17 (15.3) 6 (5.4) 3 (2.7) 2 (1.8) 1 (0.9) 14 (12.6)	12 (10.7) 17 (15.2) 21 (18.8) 18 (16.1) 17 (15.2) 14 (12.5) 3 (2.7) 2 (1.8) 0 (0) 8 (7.1)	16 (20.8) 6 (7.8) 10 (13.0) 11 (14.3) 12 (15.6) 10 (13.0) 4 (5.2) 0 (0) 0 (0) 8 (10.4)	40 (13.3) 38 (12.7) 45 (15.0) 56 (18.7) 46 (15.3) 30 (10) 10 (3.3) 4 (1.3) 1 (0.3) 30 (10)
Mode of delivery Vaginal Caesarean Unknown	93 (83.8) 4 (3.6) 14 (12.6)	97 (86.6) 7 (6.3) 8 (7.1)	67 (87) 2 (2.6) 8 (10.4)	257 (85.7) 13 (4.3) 30 (10)
Feeding Breast-fed exclusively Mixed Unknown	107 (96.4) 4 (3.6) 0 (0)	111 (99.1) 0 (0) 1 (0.9)	77 (100) 0 (0) 0 (0)	295(98.3) 4 (1.3) 1 (0.3)
Z score ≤ -3SD -3SD and ≤ -2SD -2SD and ≤ median > median Unknown	21 (18.9) 50 (45) 30 (27) 10 (9) 0 (0)	23 (20.5) 35 (31.3) 43 (38.4) 10 (8.9) 1 (0.9)	17 (22.1) 31 (40.3) 21 (27.3) 8 (10.4) 0 (0)	61 (20.3) 116 (38.7) 94 (31.3) 28 (9.3) 1 (0.3)

6.4.2 Characterisation of the gut microbiome in the Burkinabe infants in the VAC 076 clinical trial

6.4.2.1 Microbiome profile

At the phylum level, when looking at the mean relative abundance across all the trial groups, twenty-three different phyla were noted (supplementary appendix table 35). The four main phyla identified were Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. The ratios of each phylum were similar across all trial groups with Firmicutes and Bacteroidetes being the predominant ones (Figure 6A).

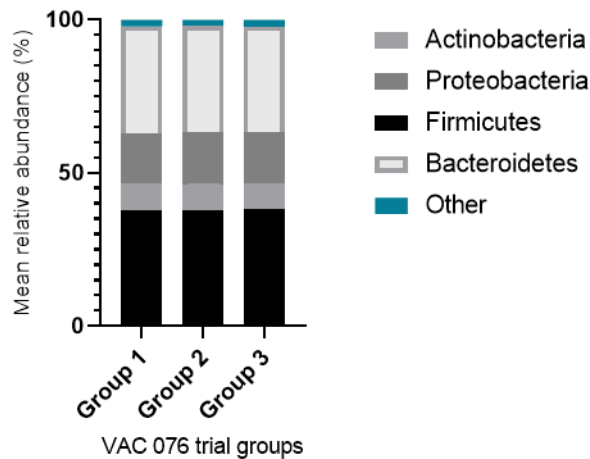


Figure 6A: Mean relative abundance (%) of the four main phyla all gut microbiome samples as baseline.

The 'other' category incorporated 19 different phyla.

6.4.2.2 Enterotypes

Diversity across the entire data set was assessed and which bacteria (if any) were changing across the samples. When using PCoA to look at how similar the samples were with Bray-Curtis distance metric, and looking at the beta-diversity according to each participant factor, there appeared to be two distinct enterotypes. The majority clustered into one enterotype (278 samples (93%), enterotype 1) and a small number (22 samples, (7%), enterotype 2) clustered into another (Figure 6B). The data available on participant factors could not explain the two different enterotypes. When looking at the phylum relative abundance, the samples in enterotype 2 were high in *Proteobacteria* and low in *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. In enterotype 1, the samples were high in *Firmicutes* and *Bacteroidetes* and low in *Actinobacteria* and *Proteobacteria* (Figure 6B). Due to these significant differences, these samples were set as a random effect in the microbiome model, discussed later.

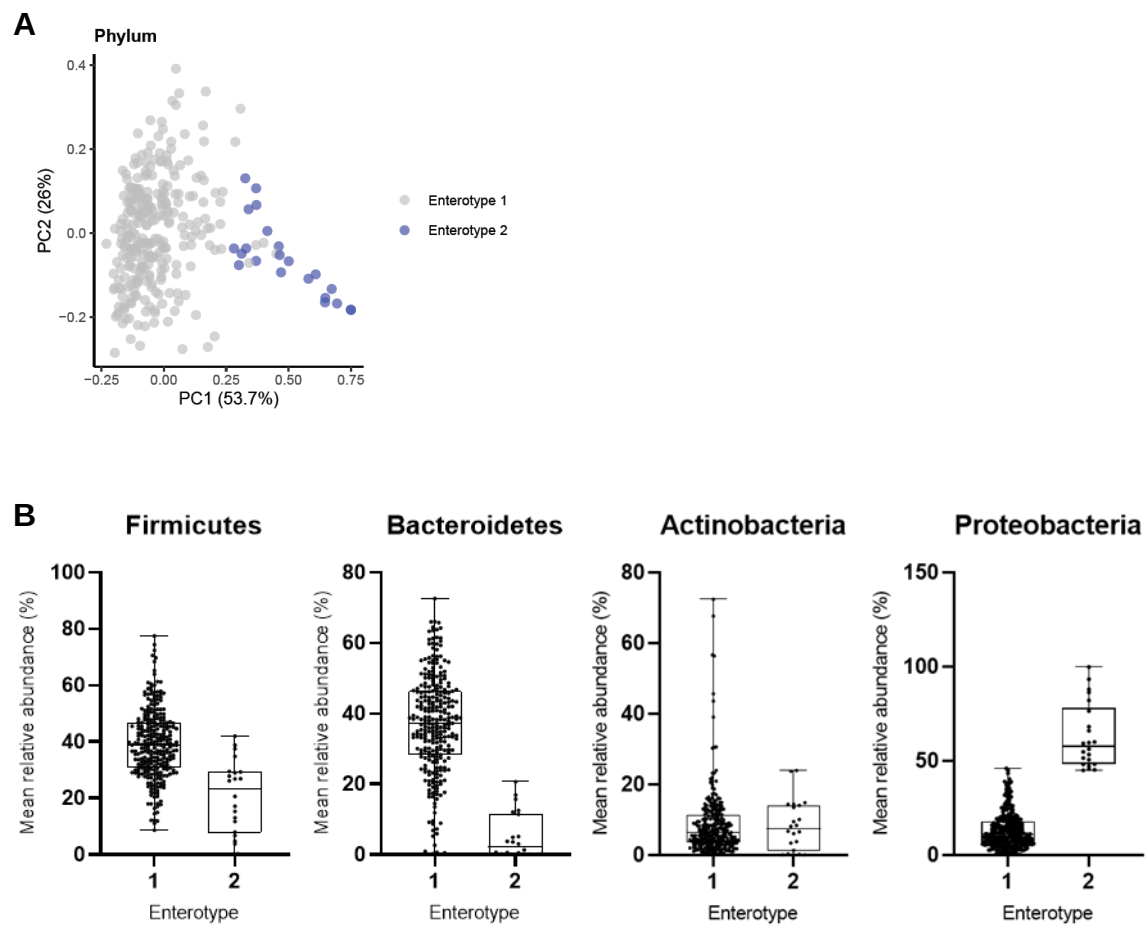


Figure 6B: Enterotypes: Principal Coordinates Analysis of all samples at the phylum level (panel A) and mean relative abundance (%) of the four main phyla according to enterotype (panel B). Enterotype 1 included 278 samples and enterotype 2 included 22 samples.

6.4.3 Alpha- diversity

Diversity of each individual sample was measured according to evenness, richness and the Shannon diversity index. Participant factors including age at baseline, method of feeding at baseline, z-score and number of older siblings in the household suggested a potential effect on alpha-diversity when using ANOVA to look at factor at a time (supplementary appendix table 36). Method of feeding at baseline was not included in the multiple linear regression model because all participants, except five (four in group 3, one in group 1), were exclusively breastfed.

When using these significant factors, as well as taking into account the difference noted with enterotype 2 in a multiple linear regression model, only the age at baseline and the enterotype factors were significant (supplementary appendix table 37). Z-score was not significant ($p = 0.39$) and number of older siblings was not significant ($p = 0.09$). Following this, we saw that alpha-diversity was significantly higher if the child was older at baseline and that enterotype 2 had significantly lower alpha-diversity than enterotype 1 (Figure 6C and Figure 6D).

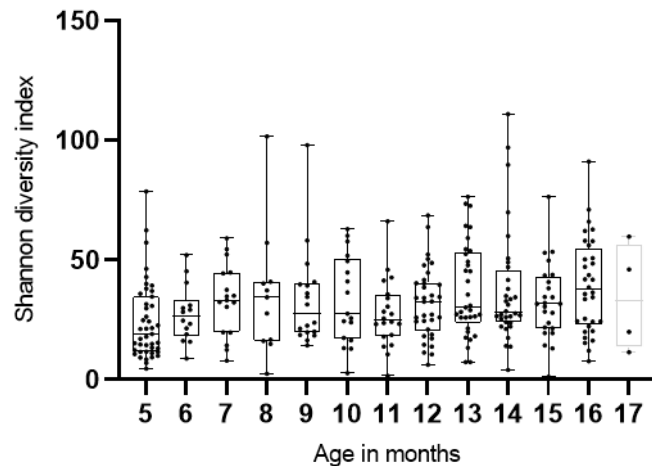


Figure 6C: Shannon diversity index according to age. Alpha diversity.

When exploring the alpha diversity further in enterotype 2 it was low in richness (Figure 6D).

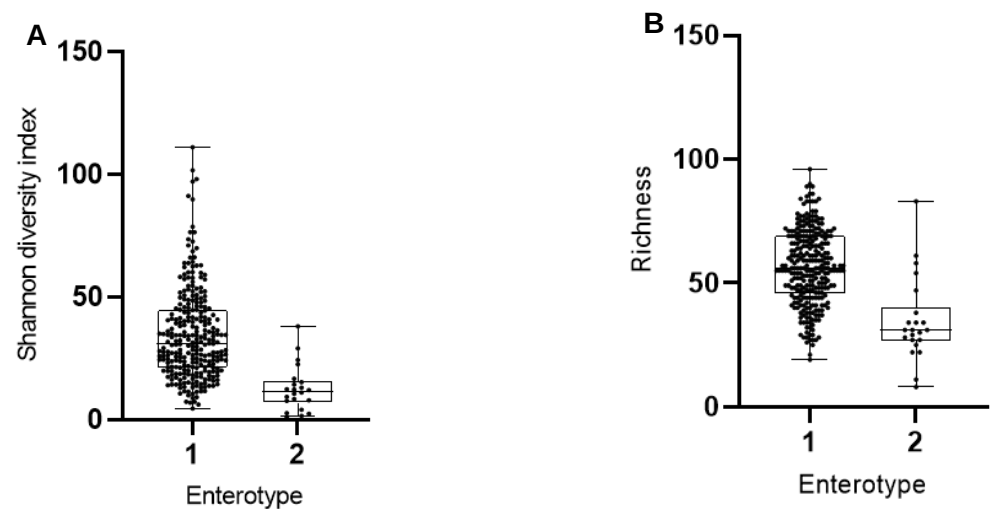


Figure 6D: Alpha diversity: Shannon diversity index according to enterotype (panel A) and richness according to enterotype (panel B).

Participant factors contributing to variation in the gut microbiome that were significant when conducting PERMANOVA were age of participant and the number of older siblings. Age was significant across all taxonomic levels whereas the number of older siblings was only significant at the higher taxonomic levels (supplementary appendix table 38). Therefore, these factors were incorporated into the microbiome model as fixed effects to help enable a full understanding of the role of possible variation in taxa when assessing the differential abundance.

6.4.4 Differential abundance

6.4.4.1 Microbiome composition in relation to infant age

When using multiple linear modelling, as age increased, there was an increase in relative abundance of bacterial families such as *Lachnospiraceae*, *Eubacteriaceae* and *Ruminococcaceae*. Within some of these family levels, specific genera were identified as significantly increasing in abundance with age. In the *Lachnospiraceae* family, these included *Blautia* and *Dorea*, and in the *Ruminococcaceae* family, this was specifically *Feacalibacterium*. However, not all genera were observed in all participants (Figure 6E and supplementary appendix table 39 - supplementary appendix table 44).

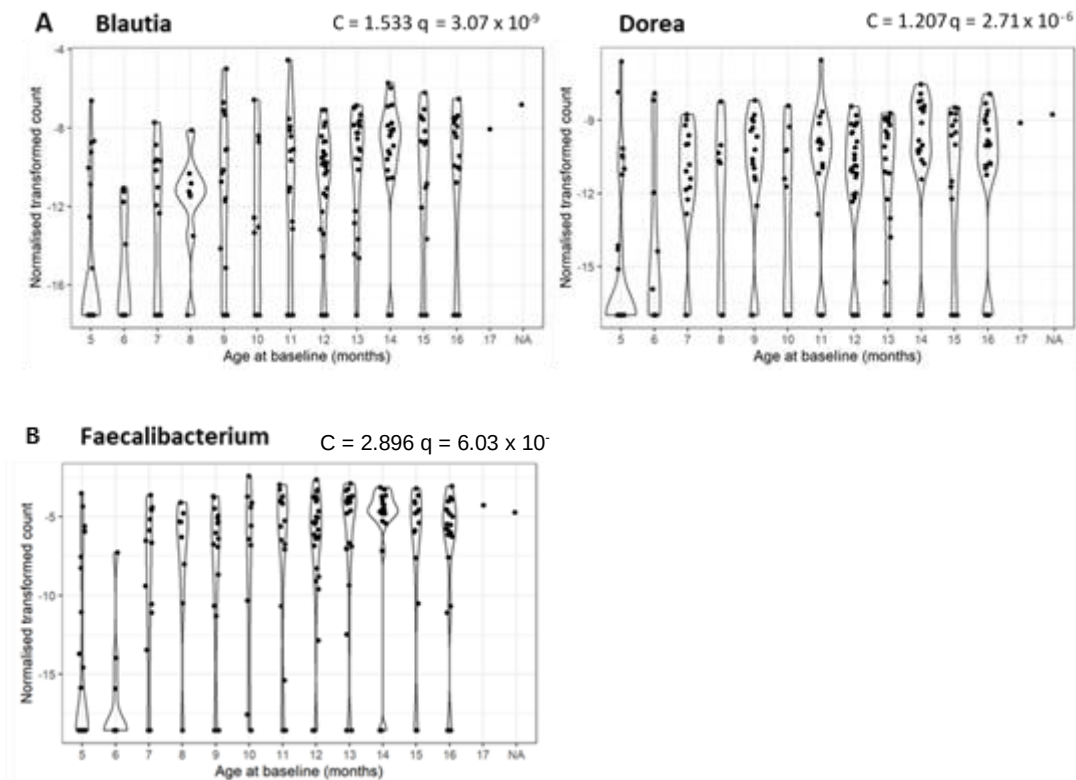


Figure 6E: Bacterial genera increasing in abundance with participant age.

Genus abundance shown as normalised transformed count. Panel A is the *Lachnospiraceae* family (specifically *Blautia* and *Dorea*), panel B is the *Ruminococcaceae* family (specifically *Faecalibacterium*) C = coefficient and q = q value.

When looking further at other genera in *Lachnospiraceae* family such as *Coproccoccus*, *Roseburia* and *Fusicatenibacter*, these appeared to be a lot less prevalent in the younger 5 – 7-month age group. This was also noted with *Oscillibacter* and *Gemmiger* from the *Ruminococcaceae* family (Figure 6F).

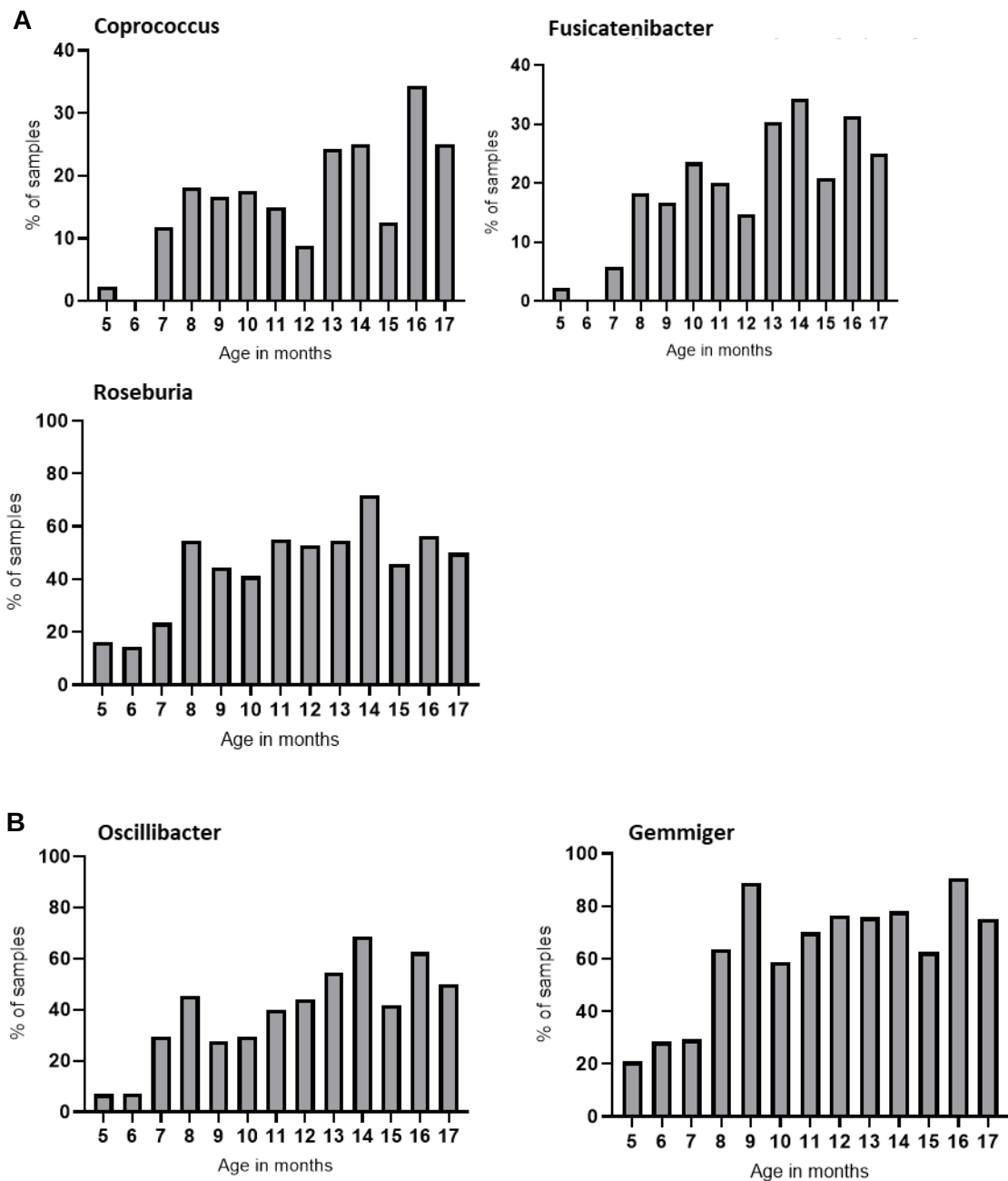


Figure 6F: Bacterial genera increasing in prevalence with participant age.

Genus prevalence shown as percentage of all samples in each age group. Panel A is the *Lachnospiraceae* family (specifically *Coprococcus*, *Fusicatenibacter* and *Roseburia*) and panel B is the *Ruminococcaceae* family (specifically *Oscillibacter* and *Gemmiger*).

Conversely, there was a decrease in relative abundance noted in the *Veillonellaceae* family, specifically the *Veillonella* genus (Figure 6G). *Dialister* was the only other genus identified from this family (supplementary appendix table 41) but there was no decrease in relative abundance or prevalence as age increased (supplementary appendix figure 9).

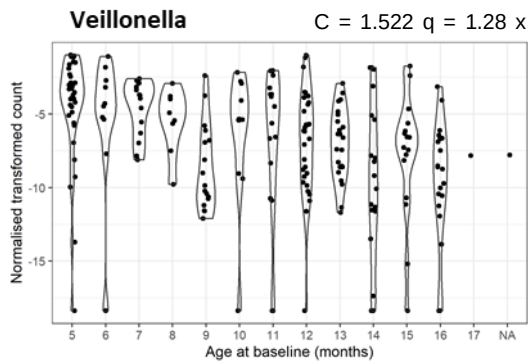


Figure 6G: *Veillonella* abundance decreasing as participant age increasing.
Genus abundance shown as normalised transformed count. C = coefficient and q = q value.

Relative abundance of *Enterobacteriaceae*, *Enterococcaceae*, *Prevotellaceae*, *Sutterellaceae* and *Bifidobacteriaceae* bacterial families did not significantly change with age (Figure 6H).

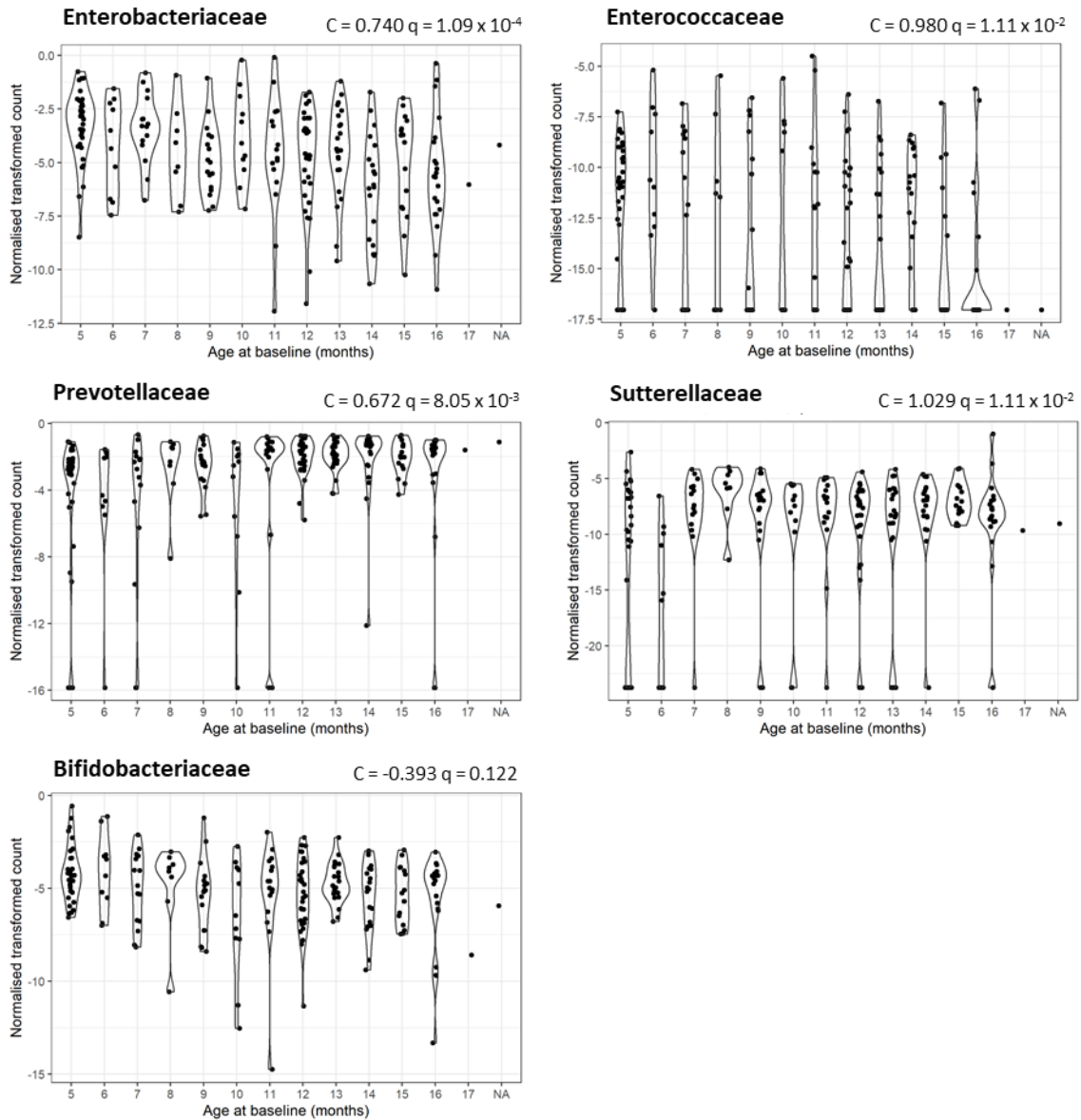


Figure 6H: Bacterial families abundance remaining consistent as participant age increases. Family abundance shown as normalised transformed count. C = coefficient and q = q value.

Within the *Prevotellaceae* family, the *Alloprevotella* genus was prevalent in more samples as age increased whereas the *Prevotella* genus was present in most samples in all age groups. *Bifidobacterium* was the only genus present from the *Bifidobacteriaceae* family and this was present in all samples in all age groups with the exception of one participant (Figure 6I).

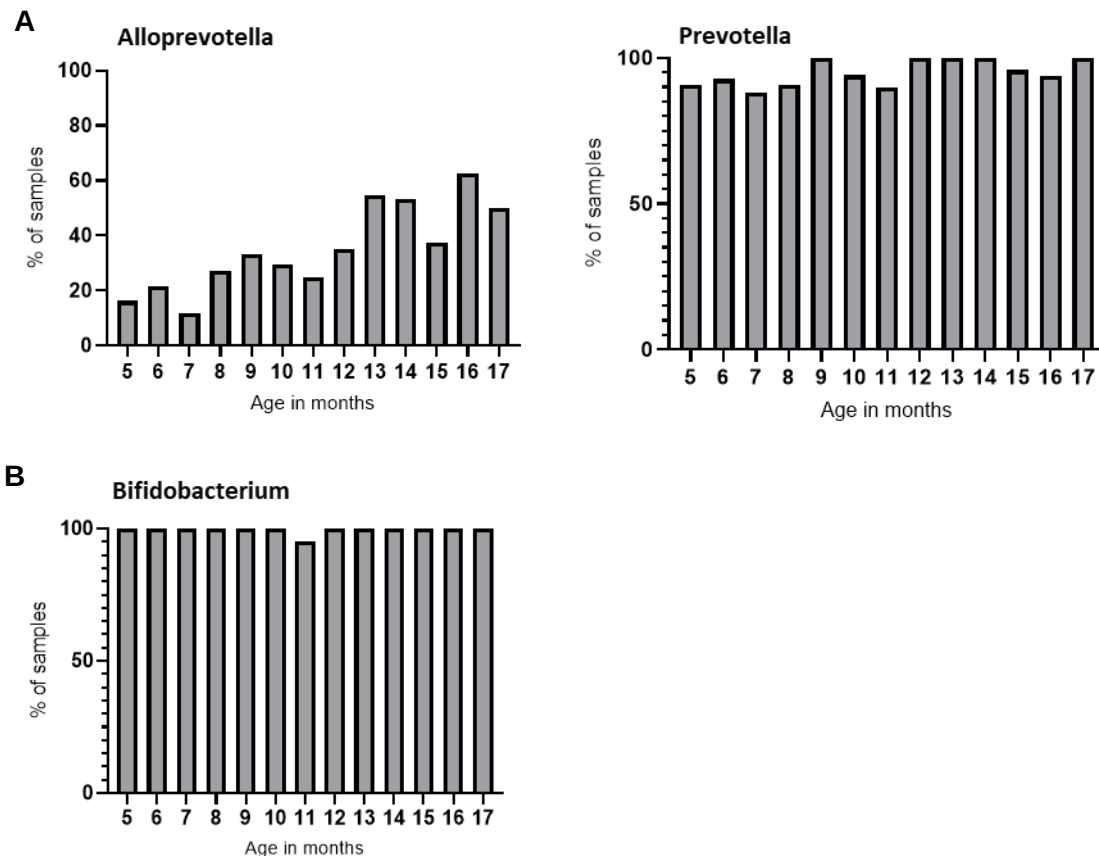


Figure 6I: Prevalence of bacterial genera according to participant age.

Panel A is the Prevotellaceae family and panel B specifically shows the genus Bifidobacterium. Genus prevalence shown as percentage of all samples in each age group.

6.4.4.2 Microbiome composition according to number of older siblings

There were no significant features in the microbiome profile at baseline at any taxonomic level according to the number of older siblings a participant may have had in their household. After adjusting for multiple testing, the p value threshold of 0.05 was not reached when comparing bacterial features across groups with different numbers of siblings.

6.4.4.3 Microbiome composition in relation to NANP responses

There were no significant features in the microbiome profile at baseline at any taxonomic level found to have an impact on antibody responses 28 days following the primary series of vaccinations. After adjusting for multiple testing, the p value threshold of 0.05 was not reached when comparing bacterial features across participants with different levels of NANP-specific IgG responses.

6.4.4.4 Microbiome composition in relation to number of malaria episodes at 12 months

There were no significant features in the microbiome profile at baseline at any taxonomic level found to have an impact on number of clinical malaria episodes following the primary series of vaccinations to 12 months. After adjusting for multiple testing, the p value threshold of 0.05 was not reached when comparing bacterial features across groups with different numbers of malaria episodes.

6.5 Discussion

6.5.1 Gut microbiome characteristics

Two enterotypes were identified using Bray-Curtis distance to assess sample similarity. In existing literature, there have been few studies describing enterotypes in infant gut microbiomes. One of the largest studies assessing these with a distribution of samples worldwide identified four main enterotypes, not only associated with different bacteria but also with different phases of early development. These were dominated by specific bacteria belonging to the phyla *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (227). Enterotype 2 in this study, dominated by *Proteobacteria* alone, with low relative abundance of the other phyla, has not previously been described in the literature in this age group. Of note, *Proteobacteria* has been linked to dysbiosis characterised by metagenomic functional analysis (228-230). Therefore, it would be interesting to investigate these participants further in enterotype 2 with regards to health outcomes and disease burden.

Enterotype assignment did not correlate with any participant factors analysed in this study. However, other potential participant factors that could play a role in enterotypes include recent illnesses, medication and antibiotic use, significant birth history (maternal intrapartum antibiotics to the mother or neonatal antibiotics), sanitation or specific feeding habits prior to the microbiome sampling (day of screening/vaccination) (118). Alternatively, the different enterotypes may be related to intrinsic host genetics given that enterotypes that have been previously identified in literature have differed according to geographical variation (229).

6.5.2 Gut microbiome and participant age

This study encompassed a wide age range of participants between 5-17 months. It is extensively published that the gut microbiome has great variability during this time of development (142, 213) and such changes were demonstrated in this study using multiple linear modelling.

A study assessing 9 and 18-month-old infants found that in both age groups of children, the gut microbiome was dominated by four phyla: *Fimicutes* (64.2%), *Actinobacteria* (24.3%), *Bacteroidetes* (7.7%) and *Proteobacteria* (4.3%). The majority of communities in these samples were from 24 bacterial families. Comparison of the two age groups showed bacterial families that significantly increased over time included *Eubacteriaceae*, *Lachnospiraceae*, *Rikenellaceae*, *Ruminococcaceae* and *Sutterellaceae*. Bacterial families that significantly decreased as age increased were *Actinomycetaceae*, *Bifidobacteriaceae*, *Carnobacteriaceae*, *Clostridiales incertae setis XI*, *Enterobacteriaceae*, *Enterococcaceae*, *Fusobacteriaceae* and *Veillonellaceae* (231). Similar findings have been demonstrated in other studies (142, 232-234) and these findings have been attributed to breastfeeding duration and progression to foods rich in fibre and protein (142, 231). In our study, findings were comparable with the studies mentioned above for the *Lachnospiraceae*, *Ruminococcaceae* and *Veillonellaceae* families (Figure 6G, supplementary tables 8 & 10). However, there was heterogeneity within these bacterial families, where different correlative relationships between age and mean relative abundance were observed at the genus level. These could be attributed to the phenotypic and functional differences of different bacterial genera. It is notable that not all trends outlined in the studies mentioned above were similarly observed in our study. For example, changes in *Sutterellaceae*, *Enterobacteriaceae*, and *Enterococcaceae* were previously shown to correlate with age, but such changes were not observed in our study. Other families, such as *Rikenellaceae*, *Actinomycetaceae*, *Carnobacteriaceae*, *Clostridiales incertae setis XI* and *Fusobacteriaceae*, were not observed in our study (supplementary tables 8 & 10). It should also be recognised that the studies quoting these findings were all conducted

in Europe, sampling infants at different ages, and using smaller sample sizes, therefore, comparability with our results may be limited.

When assessing the prevalence of specific genera from the *Lachnospiraceae* and *Ruminococcaceae* families in our study, there was an increase noted after 7-8 months of age. This would not be expected in our almost exclusively breast-fed cohort as the literature suggests this would be more likely to occur around the time of weaning (142, 231).

The *Prevotellaceae* family, not mentioned in the above studies, was also present in our study. While there was no significant difference noted in relative abundance as age increased, specifically the genus *Alloprevotella* was more prevalent in samples when age increased. *Alloprevotella* has been previously isolated from the human oral cavity, and is a relatively new genus, having been first characterised in 2013. It was initially proposed, separate to *Prevotella*, when phylogenetic analysis of full-length 16S rRNA gene sequences showed sequence divergence and clustering, suggesting a related but novel group at the genus level within the *Prevotellaceae* family (235). From reviewing the literature, this genus has not previously been described as being a part of the infant gut microbiome but described as part of the oral microbiome, where it significantly decreased over time in the age range of 1-4 years (236). In our study, the genus *Prevotella* showed a different trend to *Alloprevotella*, supporting the differences described above, leading to potential impact on function. *Prevotella* was present in nearly all samples at all ages, which is expected in vaginally- delivered children (237, 238), consistent with most participants in our sample. *Prevotella* has previously been noted to be present in higher abundance in children living in West Africa as compared with those living in Europe (224) and this would support the existing hypotheses regarding geographical location and lifestyle may affect colonisation of the gut microbiome after birth. It has also been identified as dominating a potential infant enterotype but not appearing until the second year of life, contributing to the stability and maturation of the infant gut microbiome, which would not be in keeping with our data (227).

The *Bifidobacterium* genus was present in nearly all samples at all ages, which is consistent with vaginally-delivered children (238, 239) and almost all participants being exclusively breast-fed at the time of sampling (231, 240). Changes in the gut microbiome associated with mode of delivery are thought to continue over the first year of life (142) but have been detected in infants up to the age of seven (241). Therefore, we may continue to see these specific changes at a future time point when the participants are older.

6.5.3 Baseline gut microbiome and vaccine immunogenicity

As discussed previously, studies assessing vaccine responses and composition of the gut microbiome in humans have shown conflicting results, with some implicating specific bacterial families or genera with vaccine response, mostly in oral vaccines (144-147). Existing studies conducted with parenteral vaccines (TT/BCG/HBV in infants and the HIV vaccine RV144 in adults) have both suggested correlation of bacterial families as well as specific genera with increased vaccine immunogenicity (150, 151, 223). Specifically, in the infants for TT, HBV and BCG, increased *Bifidobacteria* abundance was associated with increased CD4 memory T-cell responses as well as IgG responses for TT and HBV (150). This was not evident in our study despite *Bifidobacteria* being present in most samples. Potential reasons for this could be that we have assessed the gut microbiome with a different population, looked at a different vaccine, assessed correlation of microbiome composition with only antibody responses (and not T-cell responses), and assessed these responses at one time point. Also, as discussed above, we have sampled in a young age group, where great microbiome composition variability exists (142, 213).

There have been no studies assessing the gut microbiome in relation to malaria vaccines but this is not surprising since there was no licensed malaria vaccine until very recently. Most malaria vaccines are in early stages of clinical development (38) and RTS,S/AS01 is the most advanced malaria vaccine candidate presently (193). No clear immunological correlate of protection has been identified for this vaccine in the target population (242), similar to

R21/Matrix-M, making it difficult to assess the relationship of the gut microbiome with a meaningful immune response.

6.5.4 Baseline gut microbiome and clinical malaria episodes

There have been a small number of studies looking at the association of the gut microbiome and malaria infection. Both animal and human studies have suggested that gut microbiome composition may be associated with protection against malaria (243). Gut microbiome containing higher relative abundance of *Lactobacillus* and *Bifidobacteria* was associated with less severity of disease when infected with various *Plasmodium* species in mice (244) and reduced incidence of *P. falciparum* infection in Malian infants. Increased relative abundance of *Streptococcus* and *Escherichia* were also associated with reduced incidence of malaria parasitaemia, but not of clinical malaria, in this Mali cohort (245). While these genera were all observed in our study, their relative abundance was also not associated with any differences in number of clinical malaria episodes.

6.5.5 Limitations

In this study, due to limitations of time and funding, 16S rRNA genomic sequencing was performed for microbial profiling. This is the main method that has been used to date in most studies determining the composition of gut microbiome to date, however, no standard approach exists and different variable regions may be used for PCR primer binding which may affect characterisation (246). Shotgun metagenomic sequencing is the alternative approach, which would provide more information as it sequences all DNA, not just bacterial gene-specific amplification. This allows for not only identification of low-abundance bacterial genera and a larger number of genera (247), but for other non-bacterial organisms (e.g. viruses and parasites) to be classified. In addition, it avoids issues of amplification bias and can provide functional information of the microbiome (248) such as the functional contribution of each taxon (249), and functional genes in the sample (247). Given the population in this study, this metagenomic sequencing may be of greater value when assessing our hypotheses as we are aware that there may be a higher prevalence of parasites such as malaria, helminths, and

viruses such as cytomegalovirus (CMV), to name but a few. However, shotgun metagenomic sequencing requires deeper sequencing which is costly, as well as requiring larger, more complex reference databases, which are yet to be fully understood and in are development (248, 250).

6.6 Conclusion

In this study, I was able to analyse the gut microbiome of a larger sample of Burkinabe infants, from the same district, than has previously been described in the existing literature.

The existing literature has used smaller sample sizes, populations in both rural and urban settings and has focused on adults and high-income contexts. The composition of the gut microbiome prior to receiving R21/Matrix-M malaria vaccine had no significant effect on NANP-specific IgG responses at 28 days following three vaccinations. Equally, no significant features were highlighted to impact the number of malaria episodes at 12 months following the three malaria vaccinations or in the control group which received three rabies vaccinations. However, the composition of the gut microbiome did significantly change as the age of the participant increased, consistent with existing literature (142, 213).

6.7 Future directions

I hope to continue to look at the gut microbiome in this Burkinabe infant population. One year following the initial sampling described in this study, rectal swabs were taken from participants again when they were administered a booster vaccination. These samples are currently undergoing genomic sequencing. The infants are older and we hope to demonstrate the differences in the gut microbiome, similar to this study, according to age in this population, in this district of Burkina Faso. Similarly, data on individual participant factors have been collected and will be analysed, relative to the microbiome data. A major difference will likely be in the participant's feeding habits, as most children will probably have been weaned at this new time point. While no significant differences were noted in the baseline gut microbiome affecting responses to NANP or number of episodes of malaria experienced, as the participant gets older and the gut microbiome starts to stabilise, there may potentially be bacterial genera or

families that negatively or positively correlate with these factors, similar to that which has been suggested in other vaccine studies (150, 151, 223). We also now have data on NANP responses at later time points and similar to the paediatric study done in Bangladesh (223), it would be sensible to again correlate antibody levels from a later time point with the baseline gut microbiome to assess for any potential associations.

The phase III study assessing the safety and efficacy of R21/Matrix-M malaria vaccine recently began and we hope to assess similar hypotheses with regards to gut microbiome composition, vaccine antibody responses, and clinical malaria episodes in East Africa. This will enable us to also highlight any possible differences in the infant gut microbiome between specific regions in East and West Africa.

7 Conclusions and future work

7.1 Summary of results

7.1.1 Clinical development of R21/Matrix-M

This thesis has discussed the clinical development of R21/Matrix-M through phase I and II trials in the UK and Africa. The safety and efficacy results of R21/Matrix-M are summarised below.

7.1.1.1 Safety

The R21/Matrix-M vaccine has demonstrated an acceptable safety profile in UK adults, Kenyan adults, young children and infants, and Burkinabe infants. There were no SUSARS or SAEs related to vaccinations in any of the trials. In UK adults, there was no significant difference in number of severe AEs between the standard dose of antigen (10µg) and the higher dose of antigen (50µg). In UK and Kenyan adults, pain was the most common local AE reported but when assessing systemic AEs, overall, more were reported per person in the UK than in Kenya (6.1 AEs per person and 0.45 AEs per person respectively). In Kenyan and Burkinabe infants, similar numbers of AEs were reported per participant, according to vaccine adjuvant dose, and fever was the most common AE with four participants experiencing a severe fever (>39.0°C). There was a significant increase in the number of fevers experienced with the higher dose of adjuvant in the Burkinabe infants but overall, numbers of participants experiencing fever were low, with no more than 30% of participants experiencing a fever after any given dose of the higher dose of adjuvant. Most AEs resolved within 24-48 hours. No specific trends of AEs were highlighted when assessing the unsolicited AEs, laboratory AEs or SAEs.

7.1.1.2 Efficacy

Efficacy was initially assessed in a malaria-naïve population in the UK using CHMI. When using the standard vaccination regime of three vaccinations (10µg R21/50µg Matrix-M), 4 weeks apart, vaccine efficacy (VE) of 62.5% was noted. When delaying the third dose, VE of 75% was demonstrated. Furthermore, when participants were boosted with the same vaccine, eight months later, there was VE of 100% and 60% in these groups respectively. VE in a group

that were administered two higher doses of R21 with Matrix-M (50µg) with a fractional third dose of R21 (10µg) was 40%.

The VE seen in the initial CHMI translated to the field where half the antigen dose (5µg R21) was given to the infant population with a low (25µg) or high (50µg) Matrix-M adjuvant dose. VE according to the primary case definition (temperature of $\geq 37.5^{\circ}\text{C}$ and parasitaemia >5000 parasites/µl) was 74% (95% CI 63-82) and 77% (95% CI 67-84) at 6 months following three vaccinations in those groups respectively, and 71% (95% CI 59-79) and 77% (95% CI 67-84) at 12 months. When adjusting for potential confounders such as age, gender and bed net use, VE was very similar, 71% (95% CI 59-79) and 76% (95% CI 65-84) at 12 months in the low or high adjuvant dose groups respectively. Given that in many areas of sub-Saharan Africa, children will suffer from multiple episodes of malaria, it is important to calculate VE against multiple episodes of malaria. This was 70% (95% CI 60-78) in the low adjuvant dose group and 76% (95% CI 66-82) in the high adjuvant dose group.

Twelve months after a booster vaccine dose, which was administered prior to the malaria season and 12 months following the primary series of vaccinations, VE was 71% (95% CI 60-78) in the low adjuvant dose group and 80% (95% CI 72-85) in the higher adjuvant dose group. There was very little difference in efficacy when adjusting for confounders, similar to the first 12 months of follow-up. When looking at VE against multiple episodes of malaria, it was 61% (95% CI 50-69) and 78% (95% CI 71-83) in low and high adjuvant dose groups respectively.

7.1.2 Antibody responses to R21/Matrix-M

NANP-specific-IgG was measured in participants to assess their response to the R21/Matrix-M vaccine. At 28 days following the primary series of vaccinations, responses were induced in all participants receiving the malaria vaccine. In the UK adults, when giving a delayed, fractional third dose of R21/Matrix-M compared with three doses of the standard dose of 10µg, four weeks apart, a significant increase in antibody titres was noted ($p = 0.025$). When the same dose and vaccine regime of R21/Matrix-M (three 10µg vaccinations, four weeks apart) was administered to Kenyan adults, there was no significant difference in response compared

with the UK adults. However, much higher responses were noted in Kenyan infants administered the same dose of vaccine ($p < 0.0001$). In both the phase Ib and the phase IIb trials in Kenya and Burkina Faso, lower and higher adjuvant doses were administered with the R21 antigen due to Matrix-M being a new adjuvant used in a paediatric population. While in Kenya no significant difference was noted in antibody responses when comparing the two adjuvant doses, there was a significantly higher response using the higher adjuvant dose vaccine in the Burkinabe infants ($p < 0.0001$). This difference between populations may have occurred due to sample size numbers; for the higher adjuvant dose, 13 participants in Kenya were analysed compared with 145 participants in Burkina Faso.

Antibody responses did wane in UK adults and Burkinabe infants over time; and following a booster vaccination in the infants, antibody responses were boosted to similar levels as the peak response at 28 days following the primary series of vaccinations.

The reasons for higher antibody responses in the younger population are unclear; as is the variation in the responses noted within the same age range. When looking further at antibody responses in the Burkinabe infant cohort, neither age nor gender appeared to impact antibody responses. When correlating antibody responses with efficacy, with the higher adjuvant dose, a level of NANP-specific IgG was associated with protection. This was 6,618 EU (95% CI 5,565 – 8,397) for the first 12 months following the primary series of vaccinations and 6130 (95% CI, 5347 – 7179) ELISA units for the 12 months following the booster vaccination.

7.1.3 Composition of the gut microbiome and responses to R21/Matrix-M

There were no significant features in the gut microbiome profile at baseline (day of first vaccination) at any taxonomic level found to have an impact on antibody responses at 28 days following the primary series of vaccinations. Similarly, the gut microbiome composition did not affect the number of clinical malaria episodes up to 12 months following the primary series of vaccinations. However, as the age of the participants increased, significant difference in the relative abundance of features at the family and genus level were noted. This may be relevant in the context of assessing immunogenicity and efficacy of booster vaccinations as children

get older. Gut microbiome analysis of these participants is underway on samples taken on the day of booster vaccinations.

7.2 Future work required to assess R21/Matrix-M in an African population

7.2.1 Assessment of safety and efficacy in a phase III trial

To further assess the safety and efficacy of R21/Matrix-M in African infants, a phase III trial is required. It is important to assess if similar vaccine efficacy is achieved in non-seasonal malaria transmission sites, when the vaccines are administered at any time of year. The phase III trial commenced in 2021 across five sites in four countries. These include Nanoro and Dande in Burkina Faso; Bougouni in Mali; Bagamoyo in Tanzania; and Kilifi in Kenya. This will enable assessment in high and low malaria transmission areas, different seasonal and perennial transmission sites, as well as different geographical parts of East and West Africa. As two-thirds of malaria deaths occur in children aged under 5 (2), the age range for assessing the vaccine has also increased from 5-17 months to 5-36 months.

Enrolment is complete in this phase III trial and results are awaited.

7.2.2 Assessment of durability of R21/Matrix-M

In the UK and Burkina Faso studies, antibody responses waned significantly within the year after administration of the primary series of vaccinations. Also, post-hoc pooled analyses conducted using the RTS,S/AS01 malaria vaccine phase III study showed that while efficacy against clinical malaria was 67.6% (95% CI 63.8-71.0) in the first six months following the primary series of vaccinations, it decreased progressively over time to reach 0.1% (95% CI -9.9-9.1) at 30 months following the third dose. In participants receiving a fourth dose of RTS,S/AS01, VE against clinical malaria was 42.9% (95% CI 36.4-48.7) six months after this fourth dose and fell to 14.6% (95% CI 5.8-22.6) by the end of the study (approximately 28 months later) (251). In light of this information, and since the reasons why certain vaccine immune responses are different not only in terms of quality but longevity (118, 121) are unclear, it is important that durability continues to be assessed with R21/Matrix-M. In VAC 076, we saw

that antibody responses waned over one year but were boosted to similar levels as the initial peak response following a booster vaccination. This did also result in continued high-level efficacy at 12 months following the booster vaccination. At this point in time, it is difficult to know if continued yearly boosting will have the same effect with the same dose of vaccine as the children get older. Therefore, this trial has recently been extended for two more years with participants in malaria vaccine groups being further randomised to receive booster vaccinations or not to receive them. The comparison will allow for immunogenicity and efficacy to be assessed in participants over a total of 48 months follow-up, having received four, five or six R21/Matrix-M vaccines.

Information on the duration of protection, as well as the number and timing of booster doses, is key to evaluating the potential rebound phenomenon of higher rates of clinical malaria in the older children that was highlighted in two RTS,S/AS01 studies. The concern is that reducing the instances of malaria impairs naturally-acquired immunity, and if there is not longevity of protection without ongoing booster vaccinations, children who only receive the initial primary series of vaccines and do not receive any boosters may be at increased risk compared with those children who received no vaccines at all. However, one hopes that with some malaria vaccine and environmental exposure, as well as maturation of the immune system as the child becomes older, this would not be the case. A WHO technical consultation in March 2022 concluded that rebound is a real phenomenon but the extent of the rebound does not outweigh the benefits of interventions (252). Understanding the timing and effect of any rebound phenomenon will allow for assessment of risks and benefits of the specific intervention, as well as if specific activities and policy recommendations (e.g. booster vaccination campaigns) should be advocated for. WHO guidance suggests that the best measure of an intervention's effect is the cumulative impact (intervention period plus post-intervention rebound follow-up period) and clinical trials should be designed and funded with this in mind. This should not delay the deployment of highly effective interventions though (252).

In the context of R21/Matrix-M, as protection with vaccines is expected to wane gradually, evaluation of rebound involves ensuring longer term follow-up of participants who have been vaccinated. This longer follow-up needs to be factored into existing trials, as well as future trials, and budgeted accordingly. The plan is to extend the current VAC 076 trial for a further two years after the last vaccine dose and similar follow-up is being considered for the phase III trial.

7.2.3 R21/Matrix-M interference with EPI vaccines

Children aged 5-17 months were our target for the phase II trial. Infants in this age range may receive the measles/rubella (MR) and yellow fever (YF) vaccines at around 9 months of age as part of the Expanded Programme of Immunisation (EPI). It is important to understand if there is any interference with the safety, immunogenicity and efficacy of these vaccines when administering R21/Matrix-M at the same time. When coadministration with EPI vaccines and RTS,S/AS01 was assessed, a favourable safety profile was achieved and seropositivity and seroprotection rates met the expected rate of EPI antigen responses in low income countries (253). It is hoped that the case would be similar with R21/Matrix-M, but this requires assessment. This question was also raised by the WHO when seeking their scientific advice. Evaluation of the interaction between EPI vaccines administered at 9 months of age and R21/Matrix-M will take place in a clinical trial in Mali (VAC 088) imminently.

7.2.4 Assessment of R21/Matrix-M in a younger age group

The EPI is a successful, well-established initiative that ensures the majority of young infants globally have access to childhood immunisations. At age 6, 10, and 14 weeks, infants across Africa receive the pentavalent vaccine and oral polio vaccines at a minimum, with some also receiving the pneumococcal conjugate and rotavirus vaccines also. These young infants are another age group requiring protection against malaria. There are logistical difficulties in reaching this target population for a malaria vaccine. Successful deployment of R21/Matrix-M would ideally be incorporated into the EPI for children. This would avoid multiple healthcare visits for children and their families, and achieve higher coverage. However, it is necessary to

not only understand if R21/Matrix-M is safe, immunogenic and efficacious in this age group, but to also ensure that there is no immune interference or safety concerns when co-administering R21/Matrix-M with the existing current licensed vaccines given in this age group.

Evaluation with EPI vaccines administered at 6, 10 and 14 weeks of age at the same time as R21/Matrix-M will also take place in the clinical trial in Mali (VAC 088) mentioned above.

7.2.5 Assessment of R21/Matrix-M in at risk groups

Malnourished and HIV positive children are important cohorts within the target population. These groups were also raised by the WHO when scientific advice was sought. It is important to assess the safety of administering R21/Matrix-M in such 'risk' groups. Therefore, R21/Matrix-M is due to be evaluated in HIV positive children in a clinical trial in Uganda (VAC 092) later this year. Meanwhile, data have been collected throughout the phase II and phase III trials on nutritional status of participants across the age range and further analysis is ongoing to evaluate the impact of this on immunogenicity and efficacy. No additional safety concerns have been linked to malnourishment as yet.

The other risk group to consider is pregnant women. Malaria infection carries significant risk to the mother, foetus and new born child. With R21/Matrix-M, as we see high antibody responses twenty-eight days following the third vaccine dose, and that immune responses wane over the course of the year, it may be of benefit to target pregnant women as early as possible in their pregnancy through antenatal care. At the same time, monoclonal antibodies (mAbs) may play a role here given the shorter duration of protection needed. An advantage of mAbs over vaccines is that mAbs are effective very soon after administration but conversely given the current cost of mAbs, they may not be a feasible option currently. WHO already recommends vector control and the use of IPTp antenatally (2), therefore, there is a pathway through which the vaccine/mAbs could potentially be delivered. Clinical trials are required to assess the safety and efficacy in pregnant women, and to identify the best time to administer the vaccine or mAbs.

7.2.6 Single-vial formulation of R21/Matrix-M

In the phase II and phase III ongoing trials, R21 is in one vial and Matrix-M is in another. Mixing happens immediately prior to vaccine administration. This adds complexity not only from a storage point of view but also with the preparation and administration steps. Thus, a single-vial formulation would be preferable to help facilitate quicker, more widespread and sustainable deployment of the vaccine. In addition, this would likely reduce the time and resources allocated for vaccine administration (in terms of equipment, costs and training). The Serum Institute of India Pvt. Ltd. (SIPL) have been developing a single-vial formulation which is now currently being tested in Mali, in a phase Ib trial in 5-36-month-old infant and children. This trial will assess the safety and immunogenicity of this single-vial formulation and compare it with the two-vial formulation (VAC 088).

7.3 Future work to assess the immune correlate of protection

As detailed in chapter 5, work has begun on assessing an immune correlate of protection. In the phase II trial, in the malaria vaccine group that received the higher adjuvant dose, NANP-specific IgG levels correlated negatively with number of malaria episodes. In the same group, antibody titres had a significant effect on the hazard ratio (HR) for time to first malaria episode over 12 and 24 months of follow up. For the vaccine group which received the lower adjuvant dose, this correlation was only significant over the second year of follow-up.

More work is needed to understand the immune response to R21/Matrix-M and work is ongoing to assess affinity as well as avidity to the NANP repeat, the full-length and the C- terminus of CSP, antibody isotypes and IgG subclasses as well as cellular responses.

7.4 Future work to assess the impact of the gut microbiome on R21/Matrix-M vaccine response

As discussed in chapter 6 and section 7.1.3, no significant features in the gut microbiome profile at baseline, at any taxonomic level, were found to have an impact on antibody responses at 28 days following the primary series of vaccinations. Similarly, no link was found between the baseline microbiome and the number of clinical malaria episodes up to 12 months

following the primary series of vaccinations. When participants were enrolled they were 5-17 months of age. There was a significant difference in the composition of the gut microbiome as the participants got older, consistent with existing literature (118, 213).

In this age range, the gut microbiome is found to be less diverse and unstable. By the age of 2-3 years, the diversity of the microbiome is thought to increase and is more akin to the microbiome of adults (142). Composition of the gut microbiome has been implicated in directly or indirectly affecting vaccine efficacy and immune responses (140). For example, changes in the gut microbiome following antibiotic administration appear to influence the immune responses to influenza vaccines and oral rotavirus vaccines (152, 153). However, studies evaluating the link between the composition of the gut microbiome and vaccine responses have provided inconsistent results, and thus the understanding of the mechanisms in which the gut microbiome may influence vaccine responses are poorly understood.

Given the age of the participants in this study and the instability and ongoing development of the gut microbiome, it is difficult to be confident that the gut microbiome is not a factor in the immune response to R21/Matrix-M. Also, there was a varied immune response to R21/Matrix-M across the infant age group and the reasons for this are still unknown. We have seen that the antibody levels after a booster dose of R21/Matrix-M, when the infants are 12 months older, are restored to similar levels that were achieved following the primary series. As the participants in our study get older their gut microbiome should become more stable. By the time they receive the booster dose, many participants are over two years of age. It may be helpful to reassess the composition of the gut microbiome after the booster dose, to see if any correlation is observed with vaccine immune responses or vaccine efficacy. Rectal samples taken on the day of the booster vaccination are currently being sequenced with analysis due to take place by the end of the year.

Similar to the infant participants, variations in antibody responses to R21/Matrix-M were noted in the UK and Kenyan adult population. Again, as the microbiome is more stable in adults (118, 213) and geographical location and environment have been linked to changes in gut

microbiome composition (118), it may be helpful to explore if any differences exist in these populations in their gut microbiome that may correlate with vaccine immune responses. If a favourable microbiome profile is found to be linked to enhanced immune responses and/or efficacy, potential interventions could be possible to replicate these profiles in other individuals.

It is important to note though that such interventions may not be able to be translated to a larger population and in a real-world setting. The interventions required may vary between populations according to their genetic and personal characteristics, and they may be costly to implement alone, or in conjunction with vaccine deployment. However, knowledge of these interventions to improve vaccine immune responses and/or efficacy may be helpful in smaller, target populations considered 'high risk' such as those who are immunosuppressed or pregnant individuals.

In this study, NANP- specific IgG levels were used to assess if there was any correlation with bacterial features. A certain antibody level did correlate with protection against malaria in the higher adjuvant dose vaccine group over two years and in the lower adjuvant dose vaccine group in the second year. As it is currently unclear what the correlate of protection for R21/Matrix-M is (further work is being done on this), it may be helpful to assess the gut microbiome and vaccine immune responses when this is known.

7.5 Developing a suitable malaria vaccine for the target population

There are many factors to consider when developing an adequate malaria vaccine for infants in sub-Saharan Africa. The Malaria Vaccine Technology Roadmap outlines that a malaria vaccine needs to be $\geq 75\%$ effective across ≥ 2 years, and booster doses should be administered no more frequently than annually in the target population. Vaccines should be available at low-cost and large scale for the target population and be able to be implemented as quickly as possible, while complementing existing malaria prevention strategies (8, 98).

Over 25 million children are thought to be born each year in regions with moderate to high malaria transmission and this translates into long-term need of more than 100 million doses of

malaria vaccine per year with a 4-dose schedule. With the current recommended malaria vaccine, RTS,S/AS01, vaccine supply is expected to be limited during the first 4-6 years following introduction in 2023 (114).

In the context of R21/Matrix-M, the factors mentioned below have been considered in relation to producing a suitable vaccine.

7.5.1 Safety and efficacy

We aim to show that R21/Matrix-M is safe and efficacious in the African infants and children through our phase II and phase III trials.

7.5.2 Large-scale manufacturing and cost

Following initial development and production of R21 at the University of Oxford, manufacturing of R21/Matrix-M was transferred to SIIPL. This enabled large-scale manufacturing of the vaccine at low-cost, an essential requirement to ensure adequate vaccine supply for the millions of children annually living in malaria endemic regions of Africa. The final cost of the vaccine is currently unknown but it is expected to be in line with the cost of other EPI vaccines. SIIPL have committed to providing more than 200 million doses of R21/Matrix-M vaccine annually once licensed.

7.5.3 Vaccine deployment and implementation

Use of the vaccine needs to be as simple, easy and as quick as possible for healthcare and community workers administering it. Training and education for these individuals are key factors and we are currently canvassing the opinions of local governments and stakeholders across African countries as to the optimal methods for deployment. Considerations such as the vaccine cold chain come into play. The ideal solution would be to provide a vaccine that has thermostability at ambient temperatures, although, given EPI vaccines are transported at a ~4°C cold chain, it is hoped this would be acceptable for a malaria vaccine also. R21/Matrix-M is currently stored at +2 -+ 8°C but ongoing stability is being assessed in higher temperatures as this would facilitate storage and transport. Similarly, the administration of vaccine from a

single-vial formulation would be of benefit to address some of these requirements to ensure smooth deployment and as discussed, in section 7.2.6, evaluation of this formulation is underway.

Methods of deployment need to also be assessed with regards to how the vaccine will reach the target population: who will store, transport, administer, monitor and evaluate the uptake and progress of the vaccine. Local governments may need to provide additional support to programmes already in place, such as the EPI programmes or the National Malaria Control Programme. Communication is ongoing in malaria endemic countries to understand the operational requirements for vaccine deployment.

7.5.4 Engagement of local governments and stakeholders

Local governments and stakeholders need to understand the enormous benefits an effective malaria vaccine can present. These benefits may extend to reduced reliance on healthcare in the short, medium and longer term; better consequences for local, regional and national economies; and overall, a significant improvement in the quality of life of their populations. It may be possible to achieve this through their early involvement in the vaccine development programme, and through education and engagement with vaccine developers, manufacturers and international stakeholders. More robust and frequent communication between all these groups is required to enable sustained social and behavioural change. At the same time, it is important to ensure sufficient funds are allocated to pay for the vaccine, if the vaccine was to be licensed and deployed, as this is a likely barrier to achieving widespread use. However, it is not only the financial implications on local governments that need to be considered. The deployment strategies mentioned above may involve additional funding but also physical resources, training and engagement of communities. Following the phase II data, work is ongoing in seasonal malaria transmission countries to engage various stakeholders, including the Ministries of Health.

7.5.5 Local population approval

Any malaria vaccine needs to be accepted by the population (or their caregivers) who are receiving it. This will maximise uptake and coverage of the vaccine, not only for the initial primary series of vaccinations but for potential boosters also. Misinformation exists around childhood vaccines in general and more recently around the COVID-19 vaccines. Through social science activity (ongoing in the R21/Matrix-M phase III trials), we aim to understand the concerns that people may have regarding the vaccine. Once these issues are understood, education programmes and information campaigns can be appropriately targeted.

7.5.6 Working towards vaccine licensure

Prior to the COVID-19 pandemic, the development of any new vaccine took a long time. The process usually involved an initial research phase often lasting between 1-5 years, followed by preclinical and clinical phases averaging a duration of 15-20 years before licensure could be attained (254). However, the COVID-19 pandemic demonstrated the speed with which vaccines can be developed, tested, approved by regulatory authorities, and then deployed. Clearly, this was only possible through sufficient financial and other resource investment; local, national and international collaborations; public involvement; sharing of information and data; teamworking across geographies and disciplines to maximise expertise across the development process; rapid manufacturing processes; adequate review systems, and relevant stakeholder engagement on a national and international level. There is no reason why the lessons we have learned from our experiences with COVID-19 vaccines cannot be transferred to R21/Matrix-M.

It has taken many years for a malaria vaccine (RTS,S/AS01) to finally achieve recommendation and pre-qualification by the WHO. We are fortunate to have learned from the processes and some of the challenges that RTS,S/AS01 experienced. This has led us to identify the important factors that need to be considered when producing a successful malaria vaccine for licensure. These are not limited to a successful phase III trial assessing safety and efficacy, but also

implementation trials assessing safety, potential increases in severe malaria, and importantly, the operational needs and feasibility of a 4-dose vaccine schedule.

Following the experiences of RTS,S/AS01, we are engaging with the WHO Scientific Advice procedure during further development of R21/Matrix-M. This is to enable early policy recommendations and a prequalification assessment. We have learned that in order for WHO pre-qualification (PQ) to succeed, recommendations for use by the Strategic Advisory Group of Experts on Immunisation (SAGE) and the Malaria Policy Advisory Group (MPAG) are also required. Therefore, we are in the process of communicating with these stakeholders, as well as other stakeholders including European and Indian regulatory authorities, to ensure there is no delay in gaining the various approvals required to achieve successful licensure.

As a result of all of these measures, the development and approvals of R21/Matrix-M should be expedited, to ensure that the ongoing burden of disease, causing significant morbidity and mortality annually, is reduced as soon as possible.

7.5.7 Other malaria prevention strategies

Insecticide-treated nets (ITNs), indoor residual spraying (IRS) and seasonal malaria chemoprevention (SMC) are all successful strategies that currently exist and have been found to be beneficial in reducing malaria burden. An effective malaria vaccine is likely to be of benefit as progress has stalled in reducing malaria cases and deaths (1). A recent trial has demonstrated that when RTS,S/AS01 was administered prior to the malaria season and in combination with SMC, there was a substantially lower incidence of clinical malaria (uncomplicated and severe) as well as deaths from malaria (46). R21/Matrix-M could form a valuable part of the toolkit, along with existing strategies, in working towards the goal of eliminating malaria. As discussed above, the National Malaria Control Programme, may be a key player in delivering malaria vaccines alongside existing prevention interventions.

7.6 Developing R21/Matrix-M as a traveller's vaccine

High-level efficacy of R21/Matrix-M (62.5%-75%) was noted in UK adults when testing efficacy using CHMI. Antibody titres were similar in the UK population when compared with Kenyan adults, despite previous exposure to malaria in Kenyan adults and the vaccine boosting previous immune responses. The efficacy in UK adults was tested 28 days following the last dose of vaccine. Further studies are needed in malaria naïve adults to understand duration of protection, but also those individuals who have prolonged exposure to malaria while living and working in a malaria endemic region. Examples of such individuals include those working in roles within non-governmental organisations or the military. In addition, an effective malaria vaccine for travellers would not only facilitate delivery of aid and healthcare, but may also encourage tourism. Organisations may be more willing to work in malaria endemic regions and in turn invest in these areas, developing the economy. Notwithstanding the obvious benefits to these groups, the priority must be to license a vaccine for those who require it most – infants and children in sub-Saharan Africa, where the highest morbidity and mortality exists. Developing R21/Matrix-M as a traveller's vaccine should be a future goal.

While thinking about this, given that short-term protection may be the requirement for this population, it is important to note that given the success of early mAbs research, a traveller's vaccine may not be required. Or there may be hybrid approaches to be considered such as a priming vaccination regime given at any time for those who travel to endemic areas regularly followed by administration on mAbs immediately prior to travel. As discussed above, further trials are needed to understand more about malaria naïve individuals with prolonged exposure but additionally, trials combining the dual approach of vaccines and mAbs would be of value.

7.7 Achieving elimination

The WHO Global technical strategy for malaria 2016-2030 (GTS) provides a guide to countries working towards malaria control and elimination. In the past 7 years, nine countries have been certified malaria-free, and of those, two were certified as recently as 2021. Ambitious global targets have been set including reducing malaria case incidence and mortality by at least 90%

and eliminating malaria in at least 35 countries by 2030 (255). The Global Malaria Programme coordinates these elimination efforts, but we now have less than eight years to achieve this in a further 24 countries and we are still struggling to achieve malaria control in many. New, innovative methods to prevent malaria are still required, and these may be in the form of second-generation vaccines. New or direct improvements to the current pre-erythrocytic vaccine strategies that exist are required or the addition of blood-stage/transmission-blocking immunity (97).

7.8 Developing R21 further in the context of other malaria vaccine candidates

While RTS,S/AS01 and R21/Matrix-M in combination with existing malaria interventions will help control malaria, a second-generation vaccine is likely to be needed for elimination of malaria in Africa. Lessons learned from existing vaccine candidates in terms of antigen and adjuvant selection, access, formulation, sample sizes, outcome measures, identification of a regulatory acceptable correlate of protection, and specific requirements for regulatory approvals, should help accelerate second-generation vaccine development.

Following administration of a pre-erythrocytic vaccine such as RTS,S/AS01 or R21/Matrix-M, even if minimal sporozoites progress through the liver, blood-stage infection can ensue. Therefore, the combination of partially effective vaccines that can target different stages of the parasite life cycle may overcome the difficulty currently faced in achieving high, durable vaccine efficacy against malaria (256). A multi-stage vaccine may also be more cost-effective and easier to deploy.

There have been a number of studies that have shown encouraging results when pre-erythrocytic and blood-stage antigens have been mixed or co-administered as vaccines, with no safety concerns (257-259). However, to date, success has not been achieved in developing a multi-stage vaccine with promising human efficacy (23, 97). Pre-clinical studies using a model to assess co-administration of transmission-blocking and pre-erythrocytic vaccines

significantly enhanced efficacy of the pre-erythrocytic vaccine, indicating a synergistic interaction (260).

A vaccination strategy involving the combined administration of R21 and PfRH5, a blood stage antigen that has previously demonstrated efficacy against CHMI (261), with Matrix-M is soon to enter phase Ib testing in the Gambia (NCT05357560). It is hoped that this will progress through clinical development to help produce these second-generation vaccines that can increase the chances of elimination. Similarly, a transmission-blocking vaccine candidate, Pfs230D1M, is expected to also enter clinical development with R21 and RH5 imminently, furthering efforts to produce a malaria vaccine targeting all stages of the *P. falciparum* life cycle.

7.9 Concluding remarks

Plasmodium falciparum is a parasite with a complex life cycle. Work on malaria vaccines has been ongoing for many years, with over 140 malaria vaccine candidates entering clinical development phases but not progressing beyond phase I/II trials. However, significant progress has been made recently, with the WHO recommendation for use of RTS,S/AS01 in children from 5 months living in countries in sub-Saharan Africa with moderate to high malaria transmission. Equally, the advancement I have described in this thesis with R21/Matrix-M, offers hope that another malaria vaccine candidate, that can be produced at low-cost and with adequate supply for the target population, will contribute materially to reducing the burden of malaria. This reduction would be enhanced by vaccine use in conjunction with other tools in the malaria elimination package.

If we are able to deliver R21/Matrix-M despite some of the issues considered in this chapter, we can make a positive contribution to the WHO strategic goals of developing a malaria vaccine with a protective efficacy of $\geq 75\%$ over two years, and make greater strides towards malaria control and elimination by 2030.

In recent times, there has also been additional progress made with the development of blood-stage and transmission-blocking vaccines. PfRH5 has demonstrated efficacy in CHMI trials (261), as well as Pfs230D1M in a field trial in Mali (unpublished data, NCT03917654).

The development of a sole, efficacious vaccine to meet the elimination goal will likely require a combination of different antigens involved in different stages of the *P. falciparum* life cycle. Building on the work we have done with R21, if combined with PfRH5 and Pfs230D1M, this could lead to the world's first successful multi-stage malaria vaccine. While a multi-stage vaccine will likely be required for elimination, R21/Matrix-M alone can improve the lives of millions of children affected by malaria.

8 Supplementary appendix

8.1 VAC 072 eligibility criteria

8.1.1 Inclusion criteria

The participant was required to satisfy the following criteria to be eligible to take part:

- Healthy adults aged 18 to 45 years.
- Able and willing (in the Investigator's opinion) to comply with all study requirements.
- Willing to allow the investigators to discuss the volunteer's medical history with their General Practitioner or access this medical history electronically.
- Women only: Must practice continuous effective contraception[£] for the duration of the study.
- Agreement to refrain from blood donation during the course of the study.
- Agree to refrain from blood donation for at least 3 years after the end of their involvement in the study. *
- Written informed consent to participate in the trial.
- Reachable (24/7) by mobile phone during the period between CHMI and completion of antimalarial treatment. *
- Willingness to take a curative anti-malaria regimen following CHMI. *
- For volunteers not living close to the malaria challenge follow-up site (CCVTM, Oxford) agreement to stay in a hotel room close to the trial centre during a part of the study (from at least day 6.5 post mosquito bite until anti-malarial treatment is completed). *
- Answer all questions on the informed consent quiz correctly. *

[£]Female volunteers were required to use an effective form of contraception during the course of the study as there is no information about the effect of these vaccines on a foetus.

8.1.2 Exclusion criteria

- History of clinical malaria (any species).
- Travel to a clearly significantly malaria endemic locality planned for during the study period or within the preceding six months.
- Use of systemic antibiotics with known antimalarial activity within 30 days of CHMI (e.g. trimethoprim-sulfamethoxazole, doxycycline, tetracycline, clindamycin, erythromycin, fluoroquinolones and azithromycin). *
- Receipt of an investigational product in the 30 days preceding enrolment, or planned receipt during the study period.
- Prior receipt of an investigational vaccine likely to impact on interpretation of the trial data as assessed by the investigator.
- Any confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (inhaled and topical steroids are allowed).
- Use of immunoglobulins or blood products within 3 months prior to enrolment.
- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine (e.g. egg products) or malaria infection.
- Any history of anaphylaxis post vaccination.
- History of clinically significant contact dermatitis.
- History of sickle cell anaemia, sickle cell trait, thalassaemia or thalassaemia trait or any haematological condition that could affect susceptibility to malaria infection.

- Pregnancy, lactation or intention to become pregnant during the study.
- Use of medications known to cause prolongation of the QT interval and existing contraindication to the use of Malarone. *
- Use of medications known to have a potentially clinically significant interaction with Riamet and Malarone. *
- Any clinical condition known to prolong the QT interval and existing contraindication to the use of Malarone. *
- History of cardiac arrhythmia, including clinically relevant bradycardia and existing contraindication to the use of Malarone. *
- Clinically significant disturbances of electrolyte balance, e.g., hypokalaemia or hypomagnesaemia.
- Family history of congenital QT prolongation or sudden death and existing contraindication to the use of Malarone. *
- Contraindications to the use of both Riamet and Malarone. *
- History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ).
- History of serious psychiatric condition that may affect participation in the study.
- Any other serious chronic illness requiring hospital specialist supervision.
- Suspected or known current alcohol abuse as defined by an alcohol intake of greater than 25 standard UK units every week.
- Suspected or known injecting drug abuse in the 5 years preceding enrolment.
- Seropositive for hepatitis C virus (antibodies to HCV) at screening (unless has taken part in a prior hepatitis C vaccine study with confirmed negative HCV antibodies prior to participation in that study, and negative HCV RNA PCR at screening for this study).

- Positive family history in both 1st and 2nd degree relatives < 50 years old for cardiac disease. *
- Volunteers unable to be closely followed for social, geographic or psychological reasons.
- Any clinically significant abnormal finding on biochemistry or haematology blood tests, urinalysis or clinical examination. In the event of abnormal test results, confirmatory repeat tests will be requested.
- Any other significant disease, disorder, or finding which may significantly increase the risk to the volunteer because of participation in the study, affect the ability of the volunteer to participate in the study or impair interpretation of the study data.

**Not applicable for volunteers who do not undergo CHMI (Groups 1a and b, 4a and b and 5 who choose not to undergo the option of CHMI).*

8.2 VAC 073 eligibility criteria

8.2.1 Inclusion criteria

The participant was required to satisfy the following criteria to be eligible to take part:

- Healthy adults aged 18 to 45 years (Group 1), healthy child aged 1-5 years (Group 2), healthy infant aged 5 - <12 months (Group 3).
- Able and willing (in the Investigator's opinion) to comply with all study requirements.
- Non-pregnant, non-lactating adult female or adult male.
- Agreement to refrain from blood donation during the study.
- Use of effective method of contraception for duration of study for female participants. For female participants, we will ask them to attend with their family planning records for verification. Effective contraception is defined as a contraceptive method with failure rate of less than 1% per year when used consistently and correctly, in accordance with the product label. Examples of these include: combined oral contraceptives; injectable progestogen; implants of etonogestrel or levonorgestrel; intrauterine device or

intrauterine system; male partner sterilisation at least 6 months prior to the female subject's entry into the study, and the relationship is monogamous; male condom combined with a vaginal spermicide (foam, gel, film, cream or suppository); and male condom combined with a female diaphragm, either with or without a vaginal spermicide (foam, gel, film, cream, or suppository).

- Adults with a Body Mass Index (BMI) 18 to 30 Kg/m²; or young children and infants with Z-score of weight-for-age within $\pm 3SD$.
- Provide written informed consent.
- Plan to remain resident in the study area for 2 years following last dose of vaccination.

8.2.2 Exclusion criteria

- Clinically significant congenital abnormalities as judged by the study clinician.
- Any confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days); immunosuppressant medication within the past 6 months (inhaled and topical steroids are allowed).
- Sickle cell disease.
- Any history of anaphylaxis in relation to vaccination.
- Clinically significant laboratory abnormality as judged by the study clinician.
- Blood transfusion within one month of enrolment.
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned administration of the vaccine candidate.
- Participation in another research study involving receipt of an investigational product in the 30 days preceding enrolment, or planned use during the study period.
- Seropositive for hepatitis B surface antigen (HBsAg) or hepatitis C (HCV IgG).
- Any significant disease, disorder or situation which, in the opinion of the Investigator, may either put the participants at risk because of participation in the trial, or may influence the result of the trial, or the participant's ability to participate in the trial.

8.3 VAC 076 eligibility criteria

8.3.1 Inclusion criteria

The participant was required to satisfy the following criteria to be eligible to take part:

- Aged 5-17 months at the time of first study vaccination.
- Provided written informed consent by parent/guardian.
- Child and parent/guardian resident in the study area villages and anticipated to be available for vaccination and follow-up for 2 years following last dose of vaccination.

8.3.2 Exclusion criteria

The participant could not be part of the study if any of the following applied:

- Clinically significant skin disorder (psoriasis, contact dermatitis etc.), immunodeficiency, cardiovascular disease, respiratory disease, endocrine disorder, liver disease, renal disease, gastrointestinal disease, neurological illness.
- Weight-for-age Z score of less than –3 or other clinical signs of malnutrition.
- History of allergic reaction, significant IgE-mediated event, or anaphylaxis to immunisation.
- History of allergic disease or reactions likely to be exacerbated by any component of the vaccines.
- Clinically significant laboratory abnormality as judged by the study clinician.
- Blood transfusion within one month of enrolment.
- Administration of immunoglobulins and/or any blood products within the three months preceding the planned administration of the vaccine candidate.
- Previous vaccination with experimental malaria vaccines.
- Participation in another research study involving receipt of an investigational product in the 30 days preceding enrolment, or planned use during the study period.
- Current participation in another clinical trial, or within 12 weeks of this study.
- Known maternal HIV infection (no testing was be done by the study team).

- Any confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (For corticosteroids, this meant prednisolone, or equivalent, ≥ 0.5 mg/kg/day. Inhaled and topical steroids were allowed).
- Any significant disease, disorder or situation which, in the opinion of the Investigator, may either put the participants at risk because of participation in the trial, or may influence the result of the trial, or the participant's ability to participate in the trial.

8.4 Grading criteria of AEs

8.4.1 VAC 072 and VAC 073 adults

Supplementary appendix table 1: Severity grading criteria for local AEs. *erythema and swelling ≤ 3 mm is an expected consequence of skin puncture and will therefore not be considered an adverse event.

Adverse Event	Grade	Intensity
Pain at injection site	1	Pain that is easily tolerated
	2	Pain that interferes with daily activity
	3	Pain that prevents daily activity
Erythema at injection site*	1	$>3 - \leq 50$ mm
	2	$>50 - \leq 100$ mm
	3	>100 mm
Swelling at injection site	1	$>3 - \leq 20$ mm
	2	$>20 - \leq 50$ mm
	3	>50 mm

Supplementary appendix table 2: Severity grading criteria for systemic AEs

GRADE 0	None
GRADE 1	Mild: Transient or mild discomfort (<48 hours); no medical intervention/therapy required
GRADE 2	Moderate: Mild to moderate limitation in activity - some assistance may be needed; no or minimal medical intervention/therapy required
GRADE 3	Severe: Marked limitation in activity, some assistance usually required; may require medical intervention/therapy

8.4.2 VAC 073 young children and infants

Supplementary appendix table 3: Severity grading criteria for local AEs

Pain at injection site	0	Absent
	1	Minor reaction to touch
	2	Cries/protests on touch
	3	Cries when limb is moved/spontaneously painful
Swelling at injection site (Record greatest surface diameter in mm)	0	0
	1	<5
	2	5-20
	3	>20

Supplementary appendix table 4: Severity grading criteria for systemic AEs

Fever (Record temperature in °C)	0	<37.5°C
	1	37.5-38.0°C
	2	>38-39.0°C
	3	>39.0°C
Irritability/Fussiness	0	Behaviour as usual
	1	Crying more than usual, no effect on normal activity Crying more than usual, interferes with normal activity
	2	Crying that cannot be comforted, prevents normal activity
	3	
Drowsiness	0	Behaviour as usual

	1	Drowsiness easily tolerated
	2	Drowsiness that interferes with normal activity
		Drowsiness that prevents normal activity
	3	
Loss of appetite	0	Appetite as usual
	1	Eating less than usual/ no effect on normal activity
		Eating less than usual/ interferes with normal activity
	2	Not eating at all
	3	

8.4.3 VAC 076 infants

Supplementary appendix table 5: Severity grading criteria for local AEs

Pain at injection site	0	Absent
	1	Minor reaction to touch
	2	Cries/protests on touch
	3	Cries when limb is moved/spontaneously painful
Swelling at injection site (Record greatest surface diameter in mm)	0	0
	1	<5
	2	5-20
	3	>20
Redness/discoloration at injection site (Record greatest surface diameter in mm)	0	0
	1	<5

	2	5-20
	3	>20

Supplementary appendix table 6: Severity grading criteria for systemic AEs

Fever (Record temperature in °C)	0	<37.5°C
	1	37.5-38.0°C
	2	>38-39.0°C
	3	>39.0°C
Irritability/Fussiness	0	Behaviour as usual
	1	Crying more than usual, no effect on normal activity Crying more than usual, interferes with normal activity
	2	Crying that cannot be comforted, prevents normal activity
	3	
Drowsiness	0	Behaviour as usual
	1	Drowsiness easily tolerated
	2	Drowsiness that interferes with normal activity Drowsiness that prevents normal activity
	3	
Loss of appetite	0	Appetite as usual
	1	Eating less than usual/ no effect on normal activity Eating less than usual/ interferes with normal activity

	2	Not eating at all
	3	

8.5 Blood sampling for laboratory AEs

8.5.1 VAC 072

8.5.1.1 All participants in all groups following primary series of vaccinations

Supplementary appendix table 7: Schedule of blood sampling for participants in groups 1-5.

Haematology included a full blood count, and biochemistry consisted of sodium, potassium, urea, creatinine, albumin and liver function tests. Magnesium was also measured at screening and C-1 (day before CHMI). Viral serology included HBsAg, HCV Ab and HIV. B-HCG serum was only performed at screening and on the day before malaria challenge. Urinary β -HCG was performed on day of vaccinations. C-1 visit was only applicable to those undergoing CHMI. All participants in groups 4b and 5 did not have the day 3 visit.

Event	Screening	VAC 1			VAC 2		VAC 3			C-1
Timeline (days)		0	3	7	28	35	56/168	63/175	84/196	
Haematology	X	X	X	X	X	X	X	X	X	X
Biochemistry	X	X	X	X	X	X	X	X	X	X
Viral serology	X									
B-HCG serum	X									X

8.5.1.2 All participants who opted to have a booster vaccination in groups 1, 2 and 3

Supplementary appendix table 8: Schedule of blood sampling for participants having a booster vaccination.

Haematology included a full blood count, and biochemistry consisted of sodium, potassium, urea, creatinine, albumin and liver function tests.

Event	Booster VAC		
Timeline (days)		7	28
Haematology	X	X	X
Biochemistry	X	X	X

8.5.1.3 All participants who took part in the CHMI including control groups

Supplementary appendix table 9: Schedule of clinic attendances post CHMI for groups 2a, 3a and 6 for VAC 072A.

Haematology included a full blood count, and biochemistry consisted of sodium, potassium, urea, creatinine, albumin, magnesium and liver function tests.

End-points (EP) will commence 24hrs following start of treatment at 24hr intervals																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
Treatment may be started at any point along the time-line																										Tx		EP+ 1		EP+ 2		C+3 5		C+9 0																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
Timeline (days in relation to CHMI)	C+ 0	C+6. 5	C+ 7	C+7. 5	C+ 8	C+8. 5	C+ 9	C+9. 5	C+1 0	C+10. 5	C+1 1	C+11. 5	C+1 2	C+12. 5	C+1 3	C+13. 5	C+1 4	C+14. 5	C+1 5	C+1 6	C+1 7	C+1 8	C+1 9	C+2 0	C+2 1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
PCR for malaria (mL)		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Haematology included a full blood count, and biochemistry consisted of sodium, potassium, urea, creatinine, albumin, magnesium and liver function tests.

End-points (EP) will commence 24hrs following start of treatment at 24hr intervals												
C+ 0.5	C+ 11	C+1 1.5	C+ 12	C+1 2.5	C+ 13	C+1 3.5	C+ 14	C+1 4.5	C+ 15	T x	EP +1	EP +2
										X		
2	2	2	2	2	2	2	2	2	2		2	2
										2 +		
										3 +		
							30					
2	2	2	2	2	2	2	32	2	2	5	2	2

8.5.2 VAC 073

Supplementary appendix table 11: Schedule of blood sampling for all participants in VAC 073.

Haematology included a full blood count, and biochemistry consisted of sodium, potassium, urea, creatinine and ALT. Viral serology included HBsAg, HCV Ab and HIV.

Event	Screening	VAC 1			VAC 2			VAC 3			Booster	
Timeline (days)		0	2	7	28	30	35	56	58	63	421	456
Haematology	X	X	X		X	X	X	X	X	X	X	X
Biochemistry	X	X	X		X	X	X	X	X	X	X	X
Viral serology	X											
Malaria PCR	X	X										
Haemoglobinopathy screening	X											

8.5.3 VAC 076

Supplementary appendix table 12: Schedule of blood sampling for all participants in VAC 076.

Haematology included a full blood count, and biochemistry consisted of bilirubin, creatinine, AST, ALT and glucose. Booster vaccinations took place prior to the malaria season in June 2021, one year after the primary series of vaccinations.

Event	Screening	VAC 1		VAC 2	VAC 3			Booster			
Timeline (days)		0	7	28	56	84	236	421	B28	B168	B365
Haematology	X	X		X		X	X	X	X	X	X
Biochemistry	X	X		X		X	X	X	X	X	X
Malaria blood film	X						X	X		X	X

8.6 Extract from VAC 072 vaccine dilutions SOP - 10µg R21 mixed with 50µg Matrix-M

Calculations to prepare one dose

		R21	MM
To prepare a mixture of R21 with Matrix-M in volume of	A =	550 µl	
Product concentration in original vial	B =	50 µg/ml	375 µg/ml
Volume of original product required	C =	330 µl	220 µl
Mixture contains (B x C) / 1000	D=	16.5 µg	82.5 µg
Mixture contains concentrations of (D / A) x 1000	E =	30 µg/ml	150 µg/ml
Intended dose for administration	F =	10 µg	50 µg
Volume to be extracted (<u>per dose</u>) (F / E) x 1000	G=	330 µl*	
Doses in extracted volume (G x E) / 1000	H=	9.9 µg	49.5 µg

* Calculations rounded to 2 sf

Equipment

- 1 x vial R21
- 1 x vial Matrix-M
- 1 x 2ml sterile nitrogen filled vial for dilution (or similar)
- 2 x 0.5 ml insulin syringe with 29G needle attached
- 1 x 1ml sterile syringe
- 1 x Intramuscular needle (23G 1" or 1 ¼")
- 3 x alcohol swabs
- 1 x sterile dressing
- 1 x Injection tray
- PPE – Gloves, apron, goggles
- Sharps bin

A. Preparation

1. Clean work surfaces.

2. Prepare equipment.
3. Collect 1 vial of R21 vaccine from the freezer and record details on the vaccine record form and vaccine log.
4. Collect 1 vial of Matrix-M from the fridge and record details on the vaccine record and vaccine log.
5. Place the R21 and Matrix-M vials on the work surface.
6. Wear protective eyewear, disposable apron and clean disposable gloves.
7. Hold the R21 vial in gloved hand to encourage thawing of the frozen vaccine.

B. Mixing of R21 with Matrix-M

1. Remove the flag label from the R21 vials and attach it to the vaccine record.
2. Remove the cap from the R21 vial and from the 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 0.5 ml insulin syringe with needle attached and ensuring the needle is filled, draw up a total of **330 µl** of **R21** vaccine (*≈ 33 units on the syringe*) from the R21 vial. *(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS***

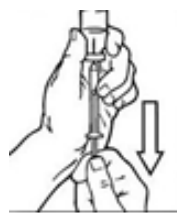


Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **330 µl** of **R21** vaccine into the vial (fig 2).

DO NOT INJECT AGAIN OR PULL BACK ON THE SYRINGE.

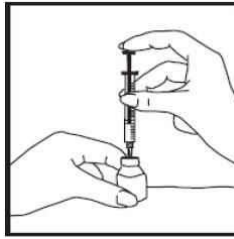


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vials in accordance with local policy.
6. Remove the flag label from the original Matrix-M vial and attach it to the vaccine record.
7. Remove the cap from the Matrix-M vial, clean the top with an alcohol swab and allow to dry.
8. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **220 µl** of **Matrix-M**. (*≈ 22 units on the syringe*).

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*

9. Inject the **220 µl** of **Matrix-M** into the vial containing 330 µl of R21 with a slow single depression of the syringe.

DO NOT INJECT AGAIN OR PULL BACK ON THE SYRINGE.

10. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Extracting final volume of R21 mixed with Matrix-M

1. Using a fresh 1 ml syringe and an intramuscular needle, insert the needle into the vial and invert. Very gently draw back and reinject three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.



Figure 3

2. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixture.
3. Leaving the needle in the vial (with syringe still attached) place on the vaccine tray ready for administration.
4. Administer the vaccine intramuscularly according SOP VC002. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with a sterile dressing.
5. Dispose needle, syringe and 2ml vial in accordance with local policy.

8.7 Extract from VAC 072 vaccine dilutions SOP - 50µg R21 mixed with 50µg Matrix-M

Calculations to prepare one dose

		R21	MM
To prepare a mixture of R21 with Matrix-M in volume of	A =	1360 µl	
Product concentration in original vial	B =	50 µg/ml	375 µg/ml
Volume of original product required	C =	1200 µl	160 µl
Mixture contains (B x C) / 1000	D=	60 µg	60 µg
Mixture contains concentrations of (D / A) x 1000	E =	44 µg/ml*	44 µg/ml*
Intended dose for administration	F =	50 µg	50 µg
Volume to be extracted (<u>per dose</u>) (F / E) x 1000	G=	1100 µl*	
Doses in extracted volume (G x E) / 1000	H=	48.4 µg	48.4 µg

* Calculations rounded to 2 sf

Equipment to prepare one dose

4 x vials R21
1 x vial Matrix-M
1 x 2ml sterile nitrogen filled vial for dilution (or similar) (One per dose)
1 x 0.5 ml insulin syringe with 29G needle attached
2 x 2 ml syringe
2 x Intramuscular needle (23G 1" - 1 ¼")
6 x alcohol swabs
1 x sterile dressing
1 x Injection tray
PPE – Gloves, apron, goggles
Sharps bin

A. Preparation

1. Clean work surfaces.
2. Prepare the equipment.
3. Collect 4 vials of R21 vaccine from the freezer.
4. Record details on the vaccine record form and vaccine log.
5. Collect 1 vial of Matrix-M from the fridge
6. Record details on the vaccine record and vaccine log.
7. Place the R21 and Matrix-M vials on the work surface.
8. Wear protective eyewear, disposable apron and clean disposable gloves.
9. Hold one R21 vial in gloved hand to encourage thawing of the frozen vaccine, once defrosted, follow the same procedure with the other 3 vials.

B. Mixing of R21 with Matrix-M to obtain concentration required

1. Remove the flag labels from the R21 vials and attach them to the vaccine record.
2. Remove the cap from the R21 vials and from the 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 2 ml syringe and a 23G needle and ensuring both the needle and the hub of the needle are filled, draw up a total volume of **1200 µl** of **R21** vaccine (1.2 ml) from the R21 vials (fig. 1). Once the needle has been removed from a vial, do not use that vial again.



Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **1200 µl** of **R21** vaccine into the vial (fig 2). Once fully depressed withdraw the needle slightly to ensure the tip is not below the level of the now diluted vaccine and release the plunger thus allowing the pressure in the vial to be released. **DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.**

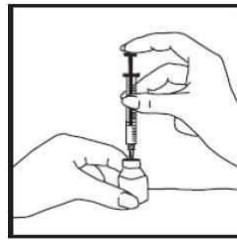


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vials in accordance with local policy.
6. Remove the flag label from the original Matrix-M vial and attach it to the vaccine record.
7. Remove the cap from the Matrix-M vial.
8. Clean the top of the Matrix-M vial with an alcohol swab and allow to dry.
9. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **160 µl** of **Matrix-M**. (*≈ 16 units on the syringe*).

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*

10. Inject the **160 µl** of **Matrix-M** into the vial containing 1200 µl of R21 with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

11. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Extracting final volume of R21 mixed with Matrix-M

1. Using a fresh 2 ml syringe and an intramuscular needle, insert the needle into the vial and invert. Very gently draw back and reinject three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.

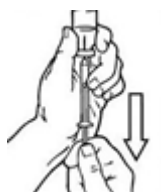


Figure 3

2. Ensuring both the hub of the needle and needle are filled, draw up **1100 µl** (1.1 ml) from vial containing the final mixture.
3. Leaving the needle into the vial (with syringe still attached) place on the vaccine tray ready for administration.
4. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
5. Dispose used needle, syringe and 2ml vial in accordance with local policy.

8.8 Extract from VAC 072 vaccine dilutions SOP - 2µg R21 mixed with 50µg Matrix-M

Calculations to prepare two doses (Allows to administer only one)

Dilution to 1/5 of R21 vaccine		
(A) To prepare dilution of R21 vaccine in volume of	A =	720 µl
(B) Dilution required	B =	1/5
(C) Volume of neat R21 vaccine required (B x A)	C =	140 µl*
(D) Volume of diluent required 720 µl – (B x A)	D =	580 µl
(E) Product concentration in original vial	E =	50 µg/ml

(F) Final concentration after dilution of R21 (E x B)	F =	10 µg/ml
(G) Intended dose for administration	G =	2 µg

Mixture of Diluted R21 with Matrix-M			
		R21	MM
(A) To prepare a final dilution of R21 with Matrix-M in volume of	A	1200 µl	
(B) Concentration of diluted vaccine and adjuvant	B	10 µg/ml	375 µg/ml
(C) Volume of diluted R21 and Matrix-M required	C	720 µl	480 µl
(D) Mixture contains (C x B) / 1000	D	7.2 µg	180 µg
(E) Mixture contains concentrations of (D / A) x 1000	E =	6 µg/ml	150 µg/ml
(F) Intended dose for administration	F =	2 µg	50µg
(G) Volume to be extracted per dose (F / E) x 1000	G=	330 µl*	
(H) Doses in extracted volume (G x E) / 1000	H=	1.98 µg	49.5 µg

* Calculations rounded to 2 sf

Equipment for two volunteers

- 1 x vial R21
- 1 x vial Matrix-M
- 1 x 0.9% saline for injection (5 or 10 ml)
- 1 x 2ml sterile nitrogen filled vial for dilution (or similar)
- 2 x 0.5 ml insulin syringe with 29G needle attached

1 x 1 ml insulin syringe with 29G needle attached
2 x 1 ml sterile syringe (1 per volunteer)
2 x Intramuscular needle (23G 1" - 1 ¼") (1 per volunteer)
4 x alcohol swabs
2 x sterile dressing (1 per volunteer)
2 x Injection tray (1 per volunteer)
PPE – Gloves, apron, goggles
Sharps bin

A. Preparation

1. Clean work surface.
2. Prepare the equipment
3. Collect one vial of R21 vaccine from the freezer.
4. Record details on the vaccine record form and vaccine log.
5. Collect one vial of Matrix-M from the fridge.
6. Record details on the vaccine record and vaccine log.
7. Place the R21 and Matrix-M vials on the work surface.
8. Wear protective eyewear, disposable apron and clean disposable gloves.
9. Hold the R21 vial in gloved hand to encourage thawing of the frozen vaccine.

B. Dilution of R21 to 1/5 solution

1. Remove the flag label from the original R21 vial and attach it to the vaccine record.
2. Remove the cap from the R21 vial and 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 0.5ml insulin syringe with needle attached and ensuring the needle is filled, draw up **140 µl** of **R21** vaccine (*≈ 14 units on the syringe*) from the R21 vial.
(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS)

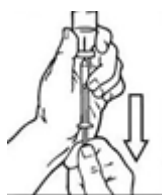


Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **140 µl** of **R21** vaccine into this vial (fig 2).

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

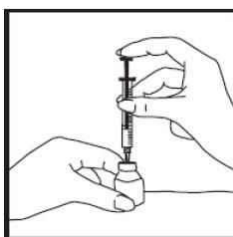


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vial in accordance with local policy.
6. Using a fresh 1 ml insulin syringe/needle and ensuring both the hub of the needle and needle are filled, draw up **580 µl** of **0.9% saline**. (*≈ 58 units on the syringe*).
7. Inject the **580 µl** of **saline** into the vial containing the 140 µl of R21 with a slow single depression of the syringe. Once fully depressed withdraw the needle slightly to ensure the tip is not below the level of the now diluted vaccine and release the plunger thus allowing the pressure in the vial to be released.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

8. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Mixing of diluted R21 with Matrix-M

1. Remove the flag label from the original Matrix-M vial and attach it to the vaccine record
2. Remove the cap from the Matrix-M vial.
3. Clean the top of the Matrix-M vial with an alcohol swab and allow to dry.
4. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **480 µl** of **Matrix-M**. (*≈ 48 units on the syringe*).

(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)

5. Inject the **480 µl** of **Matrix-M** into the Adelphi vial containing 720 µl of diluted R21 with a slow single depression of the syringe. Once fully depressed withdraw the needle slightly to ensure the tip is not below the level of the now diluted vaccine and release the plunger thus allowing the pressure in the vial to be released.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

6. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

D. Extracting final volume of diluted R21 mixed with Matrix-M

1. Using a fresh 1 ml syringe and an intramuscular needle, insert the needle into the vial and invert. Very gently draw back and reinject the mixed vaccine three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.

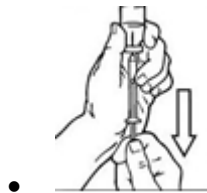


Figure 3

2. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixed dilution.
3. Leaving the needle in the vial (with syringe still attached) place on the vaccine tray ready for administration.
4. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
5. Once administered keep the vial on the work surface ready to extract the next dose, otherwise dispose used needle, syringe and 2ml vial in accordance with local policy

STEPS to prepare a second dose:

6. Clean the top of the vial with an alcohol swab and allow to dry.
7. Using a fresh 1 ml syringe and an intramuscular needle, insert the needle into the vial and invert. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixed dilution.
8. Leave the needle in the vial until ready to administer the vaccine.
9. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
10. Dispose used needle, syringe and 2ml vial in accordance with local policy.

Supplementary appendix table 13: Total NANP IgG as measured by ELISA.

Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to day 84 or 196, 28 days following the primary series of vaccinations in the VAC 072 trial. Dunn's multiple comparisons test used to calculate p values

Group	Number of participants	Day 84/196 GMT (EU) [95% CI]	Range (EU)
1/3	26	1389 [1031-1871]	419-8076
2	14	2661 [1771-3998]	635-5863
4	10	1009 [316-3221]	35-6128
5	7	3980 [2500-6337]	1658-9334

Supplementary appendix table 14: Comparison of geometric mean titres (GMT) at day 84 or 196, 28 days following the primary series of vaccinations in the VAC 072 trial. Dunn's multiple comparisons test used to calculate p values

Group comparison	Significant?	P value
2 vs 1/3	No	0.120
2 vs 4	No	0.516
2 vs 5	No	>0.999
1/3 vs 4	No	>0.999
1/3 vs 5	Yes	0.025
4 vs 5	No	0.112

Supplementary appendix table 15: Total NANP IgG as measured by ELISA.

Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to B28, 28 days following the booster vaccination.

Group	Number of participants	B28 GMT (EU) [95% CI]	Range (EU)
2	3	7705 [1614-36791]	4656-15601
3	6	2375 [997-5657]	971-11299

Supplementary appendix table 16: Total NANP IgG as measured by ELISA.

Adults aged 18-45, received R21c as part of the VAC 053 and VAC 065 clinical trials or R21 as part of the VAC 072 clinical trial. Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to day 84, 28 days following the primary series of vaccinations. Mann-Whitney used to calculate p value of 0.41

10µg R21/50µg Matrix-M	Number of participants	GMT (EU) [95% CI]	Range (EU)
R21c	18	1561 [1122-2174]	416-3938
R21	26	1389 [1031-1871]	419-8077

8.9 Extract from VAC 073 vaccine dilutions SOP - $5\mu\text{g}$ R21 mixed with $25\mu\text{g}$ Matrix-M

Calculations to prepare one dose

Mixture of R21 with Matrix-M		R21	MM
(A) To prepare a mixture of R21 with Matrix-M in volume of	A =	300 μl	
(B) Product concentration in original vial	B =	50 $\mu\text{g/ml}$	375 $\mu\text{g/ml}$
(C) Volume of original product required	C =	180 μl	120 μl
(D) Mixture contains (B x C) / 1000	D =	9 μg	45 μg
(E) Mixture contains concentrations of (D / A) x 1000	E =	30 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$

Dilution 1/2 of the R21 with Matrix-M mixture		R21	MM
(A) To prepare a final dilution of R21 with Matrix-M in volume of	A =	600 μl	
(B) Dilution required	B =	1/2	
(C) Volume of mixture required (A x B)	C =	300 μl	
(D) Volume of diluent required 600 μl – (A x B)	D =	300 μl	
(E) Product concentration before dilution	E =	30 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$

(F) Final concentration after dilution of R21 (E x B)	F =	15 µg/ml	75 µg/ml
(G) Intended dose for administration	G =	5 µg	25 µg
(H) Volume to be extracted (<u>per dose</u>) (G / F) x 1000	H=	330 µl*	
(I) Doses in extracted volume (H x F) / 1000	I=	4.95 µg	24.75 µg

* Calculations rounded to 2 sf

Equipment

1 x vial R21
 1 x vial Matrix-M
 1 x 0.9% saline for injection (5 or 10 ml)
 1 x 2ml sterile nitrogen filled vial for dilution (or similar)
 3 x 0.5 ml insulin syringe with 29G needle attached (or similar insulin syringe with needle attached)
 1 x 1 ml sterile syringe
 1 x Intramuscular needle (size according to local procedures for intramuscular injections)
 3 x alcohol swabs
 1 x Sterile dressing
 1 x Sterile gauzes
 1 x Injection tray
 PPE – Gloves, apron, goggles
 Sharps bin

A. Preparation

1. Clean work surface.
2. Prepare the equipment:
3. Collect 1 vial of R21 vaccine from the freezer and record details on the vaccine log.
4. Collect 1 vial of Matrix-M from the fridge and record details on the vaccine log.
5. Place the R21 and Matrix-M vials on the work surface.
6. Wear protective eyewear, disposable apron and clean disposable gloves.
7. Hold the R21 vial in gloved hand to encourage thawing of the frozen vaccine

B. Mixing of R21 with Matrix-M

1. Remove the flag label from the original R21 vial and attach it to the Vaccine Mixing and Dilution Record.
2. Remove the cap from the R21 vial and 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 0.5ml insulin syringe with needle attached and ensuring the needle is filled, draw up **180 µl** of **R21** vaccine (*≈ 18 units on the syringe*) from the R21 vial.

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*



Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **180 µl** of **R21** vaccine into the vial (fig 2).

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

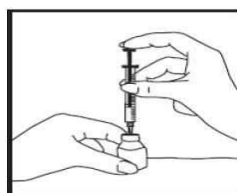


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vial in accordance with local policy.
6. Remove the flag label from the original Matrix-M vial and attach it to the Vaccine Mixing and Dilution Record.
7. Remove the cap from the Matrix-M vial.
8. Clean the top of the Matrix-M vial with an alcohol swab and allow to dry.
9. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **120 µl** of **Matrix-M**. (*≈ 12 units on the syringe*).

(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these

by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)

10. Inject the **120 µl** of **Matrix-M** into the vial containing 180 µl of R21 with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE

11. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Dilution of the mixture to 1/2 solution

1. Using a 0.5 ml insulin syringe/needle and ensuring the needle is filled, draw up **300 µl** of **0.9% saline**. (*≈ 30 units on the syringe*).
2. Inject the **300 µl** of **saline** into the vial containing the 300 µl of R21 mixed with Matrix-M with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

3. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

D. Extracting final volume of diluted R21 mixed with Matrix-M

1. Open sterile gauze and place on vaccine tray
2. Using a fresh 1 ml syringe and an intramuscular needle, and keeping the cap of the needle on top of sterile gauze on vaccine tray, insert the needle into the vial and invert. Very gently draw back and reinject the mixed vaccine three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.

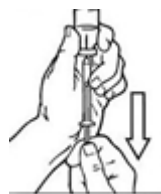


Figure 3

3. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixed dilution.

4. Using a **single-handed** scoop technique, re-sheath the needle (fig. 4).

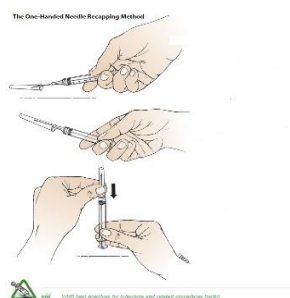


Figure 4

5. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
6. Dispose used needle, syringe and 2ml vial in accordance with local policy.

8.10 Extract from VAC 073 vaccine dilutions SOP - $5\mu\text{g}$ R21 mixed with $50\mu\text{g}$ Matrix-M

Calculations to prepare one dose

Dilution of R21 vaccine		
(A) To prepare dilution of R21 vaccine in volume of	A =	360 μl
(B) Dilution required	B =	1/2
(C) Volume of neat R21 vaccine required (B x A)	C =	180 μl
(D) Volume of diluent required 360 μl – (B x A)	D =	180 μl
(E) Product concentration in original vial	E =	50 $\mu\text{g/ml}$
(F) Final concentration after dilution of R21 (E x B)	F =	25 $\mu\text{g/ml}$

Mixture of Diluted R21 with Matrix-M			
		R21	MM
(A) To prepare a final dilution of R21 with Matrix-M in volume of	A =	600 µl	
(B) Concentration of diluted vaccine and adjuvant	B =	25 µg/ml	375 µg/ml
(C) Volume of diluted R21 and Matrix-M required	C =	360 µl	240 µl
(D) Mixture contains (C x B) / 1000	D =	9 µg	90 µg
(E) Mixture contains concentrations of (D / A) x 1000	E =	15 µg/ml	150 µg/ml
(F) Intended dose for administration	F =	5 µg	50µg
(G) Volume to be extracted per dose (F / E) x 1000	G=	330 µl*	
(H) Doses in extracted volume (G x E) / 1000	H=	4.95 µg	49.5 µg

* Calculations rounded to 2 sf

Equipment

1 x vial R21
 1 x vial Matrix-M
 1 x 0.9% saline for injection (5 or 10 ml)
 1 x 2ml sterile nitrogen filled vial for dilution (or similar)
 3 x 0.5 ml insulin syringe with 29G needle attached (or similar insulin syringe with needle attached)
 1 x 1 ml sterile syringe
 1 x Intramuscular needle (size according to local procedures for intramuscular injections)
 3 x alcohol swabs
 1 x Sterile dressing
 1 x Injection tray
 PPE – Gloves, apron, goggles
 Sharps bin

A. Preparation

1. Clean work surface.
2. Prepare the equipment:
3. Collect 1 vial of R21 vaccine from the freezer and record details on the vaccine log.
4. Collect 1 vial of Matrix-M from the fridge and record details on the vaccine log.
5. Place the R21 and Matrix-M vials on the work surface.
6. Wear protective eyewear, disposable apron and clean disposable gloves.
7. Hold the R21 vial in gloved hand to encourage thawing of the frozen vaccine

B. Dilution of R21 to 1/2 solution

1. Remove the flag label from the original R21 vial and attach it to the Vaccine Mixing and Dilution Record.
2. Remove the cap from the R21 vial and 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 0.5ml insulin syringe with needle attached and ensuring the needle is filled, draw up **180 µl** of **R21** vaccine (*≈ 18 units on the syringe*) from the R21 vial.
*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*



Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **180 µl** of **R21** vaccine into this vial (fig 2).

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

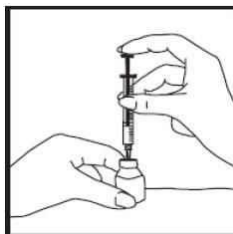


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vial in accordance with local policy.
6. Using a fresh 0.5 ml insulin syringe/needle and ensuring both the hub of the needle and needle are filled, draw up **180 µl** of **0.9% saline**. (*≈ 18 units on the syringe*).
7. Inject the **180 µl** of **saline** into the vial containing the 180 µl of R21 with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

8. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Mixing of diluted R21 with Matrix-M

1. Remove the flag label from the original Matrix-M vial and attach it to the Vaccine Mixing and Dilution Record.
2. Remove the cap from the Matrix-M vial.
3. Clean the top of the Matrix-M vial with an alcohol swab and allow to dry.
4. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **240 µl** of **Matrix-M**. (*≈ 24 units on the syringe*).

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS***

5. Inject the **240 µl** of **Matrix-M** into the Adelphi vial containing 360 µl of diluted R21 with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

6. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

D. Extracting final volume of diluted R21 mixed with Matrix-M

1. Using a fresh 1 ml syringe and an intramuscular needle, insert the needle into the vial and invert. Very gently draw back and reinject the mixed vaccine three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.

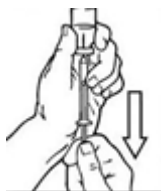


Figure 3

2. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixed dilution.
3. Leaving the needle in the vial (with syringe still attached) place on the vaccine tray ready for administration.
4. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
5. Dispose used needle, syringe and 2ml vial in accordance with local policy.

8.11 Extract from VAC 073 vaccine dilutions SOP - 10µg R21 mixed with 50µg Matrix-M

Calculations to prepare one dose

		R21	MM
To prepare a mixture of R21 with Matrix-M in volume of	A =	550 µl	
Product concentration in original vial	B =	50 µg/ml	375 µg/ml
Volume of original product required	C =	330 µl	220 µl

Mixture contains (B x C) / 1000	D=	16.5 µg	82.5 µg
Mixture contains concentrations of (D / A) x 1000	E =	30 µg/ml	150 µg/ml
Intended dose for administration	F =	10 µg	50 µg
Volume to be extracted (<u>per dose</u>) (F / E) x 1000	G=	330 µl*	
Doses in extracted volume (G x E) / 1000	H=	9.9 µg	49.5 µg

* Calculations rounded to 2 sf

Equipment

1 x vial R21
 1 x vial Matrix-M
 1 x 2ml sterile nitrogen filled vial for dilution (or similar)
 2 x 0.5 ml insulin syringe with 29G needle attached (or similar insulin syringe with needle attached)
 1 x 1 ml sterile syringe
 1 x Intramuscular needle (size according to local procedures for intramuscular injections)
 3 x alcohol swabs
 1 x Sterile dressing
 1 x Injection tray
 PPE – Gloves, apron, goggles
 Sharps bin

A. Preparation

1. Clean work surface.
2. Prepare the equipment:
3. Collect 1 vial of R21 vaccine from the freezer and record details on the vaccine log.
4. Collect 1 vial of Matrix-M from the fridge and record details on the vaccine log.
5. Place the R21 and Matrix-M vials on the work surface.
6. Wear protective eyewear, disposable apron and clean disposable gloves.

7. Hold the R21 vial in gloved hand to encourage thawing of the frozen vaccine

B. Mixing of R21 with Matrix-M to obtain concentration required

1. Remove the flag label from the R21 vials and attach them to the Vaccine Mixing Record.
2. Remove the cap from the one of the R21 vials and from the 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 0.5ml insulin syringe with needle attached and ensuring the needle is filled, draw up **330 µl** of **R21** vaccine (*≈ 33 units on the syringe*) from the first R21 vial.

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*

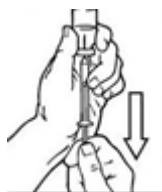


Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **330 µl** of **R21** vaccine into the vial (fig 2).

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

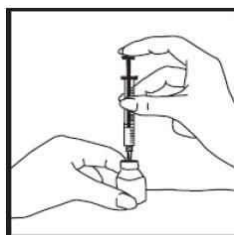


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vial in accordance with local policy.
6. Remove the flag label from the original Matrix-M vial and attach it to the Vaccine Mixing Record.
7. Remove the cap from the Matrix-M vial, clean the top with an alcohol swab and allow to dry.

8. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **220 µl** of **Matrix-M**. (*≈ 22 units on the syringe*).

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*

9. Inject the **220 µl** of **Matrix-M** into the vial containing 330 µl of R21 with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

10. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Extracting final volume of diluted R21 mixed with Matrix-M

1. Using a fresh 1 ml syringe and an intramuscular needle, insert the needle into the vial and invert. Very gently draw back and reinject three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.

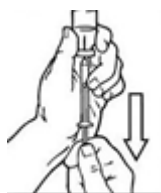


Figure 3

2. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixture.
3. Leaving the needle in the vial (with syringe still attached) place on the vaccine tray ready for administration.
4. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
5. Dispose used needle, syringe and 2ml vial in accordance with local policy.

Supplementary appendix table 17: Total NANP IgG as measured by ELISA.

Adults aged 18-45, young children aged 1-5 and infants 5 - < 12 months. Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to day 84, 28 days following primary series of vaccinations.

Dose of R21/Matrix-M	Group	Age group	Number of participants	GMT (EU) [95% CI]	Range (EU)
10µg R21/50µg Matrix-M	1	Adults	18	1392 [984-1969]	308-4239
10µg R21/50µg Matrix-M	2b	Young children	16	7739 [4819-12428]	1687-32673
10µg R21/50µg Matrix-M	3b/d	Infants	18	11271 [6995-18160]	1634-30861
5µg R21/25µg Matrix-M	2a	Young children	3	1985 [17-238319]	387-16631
5µg R21/25µg Matrix-M	3a/c	Infants	17	7219 [4684-11126]	1634-30861
5µg R21/50µg Matrix-M	3e	Infants	15	13280 [7366-23942]	2715-86352

Supplementary appendix table 18: Total NANP IgG as measured by ELISA.

Adults aged 18-45, young children aged 1-5 and infants 5 - < 12 months. Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to day 421, one year following primary series of vaccinations.

Dose of R21/Matrix-M	Group	Age group	Number of participants	GMT (EU) [95% CI]	Range (EU)
10µg R21/50µg Matrix-M	1	Adults	15	356 [218-583]	55-1173
10µg R21/50µg Matrix-M	2b	Young children	16	588 [474-1277]	63-2888
10µg R21/50µg Matrix-M	3b/d	Infants	18	1780 [1093-2897]	524-31294
5µg R21/25µg Matrix-M	2a	Young children	3	244 [14-4343]	68-648
5µg R21/25µg Matrix-M	3a/c	Infants	16	778 [474-1277]	141-2814
5µg R21/50µg Matrix-M	3e	Infants	14	1144 [570-2295]	169-9344

Supplementary appendix table 19: Total NANP IgG as measured by ELISA.

Infants are aged 5 - < 12 months. Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to day 456, 28 days following the booster vaccination.

Dose of R21/Matrix-M	Group	Age group	Number of participants	GMT (EU) [95% CI]	Range (EU)
10µg R21/50µg Matrix-M	3b/d	Infants	18	6661 [4558-9734]	1947-34273
5µg R21/25µg Matrix-M	3a/c	Infants	16	6044 [3742-9763]	904-21956
5µg R21/50µg Matrix-M	3e	Infants	14	7958 [4662-13584]	2175-34301

Supplementary appendix table 20: Total NANP IgG as measured by ELISA.

Infants are aged 5 - < 12 months. Geometric mean titres (GMT) and range readings are in ELISA units (EU) and correspond to day 84, 28 days following the primary series and day 456, 28 days following the booster vaccination. Mann-Whitney used to calculate p values.

Dose of R21/Matrix-M	Group	Age group	Day 84 GMT (EU) [95% CI]	B28 GMT (EU) [95% CI]	P value
10µg R21/50µg Matrix-M	3b/d	Infants	11271 [6995-18160]	6661 [4558-9734]	0.09
5µg R21/25µg Matrix-M	3a/c	Infants	7219 [4684-11126]	6044 [3742-9763]	0.63
5µg R21/50µg Matrix-M	3e	Infants	13280 [7366-23942]	7958 [4662-13584]	0.19

8.12 Extract from VAC 076 vaccine dilutions SOP - $5\mu\text{g}$ R21 mixed with $25\mu\text{g}$ Matrix-M

Calculations to prepare one dose

Mixture of R21 with Matrix-M		R21	MM
(A) To prepare a mixture of R21 with Matrix-M in volume of	A =	300 μl	
(B) Product concentration in original vial	B =	50 $\mu\text{g/ml}$	375 $\mu\text{g/ml}$
(C) Volume of original product required	C =	180 μl	120 μl
(D) Mixture contains (B x C) / 1000	D =	9 μg	45 μg
(E) Mixture contains concentrations of (D / A) x 1000	E =	30 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$

Dilution 1/2 of the R21 with Matrix-M mixture		R21	MM
(A) To prepare a final dilution of R21 with Matrix-M in volume of	A =	600 μl	
(B) Dilution required	B =	1/2	
(C) Volume of mixture required (A x B)	C =	300 μl	
(D) Volume of diluent required 600 μl – (A x B)	D =	300 μl	
(E) Product concentration before dilution	E =	30 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$

(F) Final concentration after dilution of R21 (E x B)	F =	15 µg/ml	75 µg/ml
(G) Intended dose for administration	G =	5 µg	25 µg
(H) Volume to be extracted (<u>per dose</u>) (G / F) x 1000	H=	330 µl*	
(I) Doses in extracted volume (H x F) / 1000	I=	4.95 µg	24.75 µg

* Calculations rounded to 2 sf

Equipment

1 x vial R21
 1 x vial Matrix-M
 1 x 0.9% saline for injection (5 or 10 ml)
 1 x 2ml sterile nitrogen filled vial for dilution (or similar)
 3 x 0.5 ml insulin syringe with 29G needle attached (or similar insulin syringe with needle attached)
 1 x 1 ml sterile syringe
 1 x Intramuscular needle (size according to local procedures for intramuscular injections)
 3 x alcohol swabs
 1 x Sterile dressing
 1 x Sterile gauzes
 1 x Injection tray
 PPE – Gloves, apron, goggles
 Sharps bin

A. Preparation

1. Clean work surface.
2. Prepare the equipment:
3. Collect 1 vial of R21 vaccine from the freezer and record details on the vaccine log.
4. Collect 1 vial of Matrix-M from the fridge and record details on the vaccine log.
5. Place the R21 and Matrix-M vials on the work surface.
6. Wear protective eyewear, disposable apron and clean disposable gloves.

7. Hold the R21 vial in gloved hand to encourage thawing of the frozen vaccine

B. Mixing of R21 with Matrix-M

1. Remove the flag label from the original R21 vial and attach it to the Vaccine Mixing and Dilution Record.
2. Remove the cap from the R21 vial and 2 ml sterile nitrogen filled vial, clean the tops with an alcohol swab and allow to dry.
3. Using a 0.5ml insulin syringe with needle attached and ensuring the needle is filled, draw up **180 µl** of **R21** vaccine (*≈ 18 units on the syringe*) from the R21 vial.

*(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)*



Figure 1

4. With the 2ml sterile vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **180 µl** of **R21** vaccine into the vial (fig 2).

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

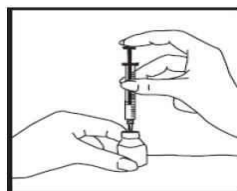


Figure 2

5. Remove and dispose of the needle/syringe. Dispose of the used R21 vaccine vial in accordance with local policy.
6. Remove the flag label from the original Matrix-M vial and attach it to the Vaccine Mixing and Dilution Record.
7. Remove the cap from the Matrix-M vial.
8. Clean the top of the Matrix-M vial with an alcohol swab and allow to dry.
9. Using a fresh 0.5ml insulin syringe/needle and ensuring the needle is filled, draw up **120 µl** of **Matrix-M**. (*≈ 12 units on the syringe*).

(NB: Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle into the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1) **DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS**)

10. Inject the **120 µl** of **Matrix-M** into the vial containing 180 µl of R21 with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE

11. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

C. Dilution of the mixture to 1/2 solution

1. Using a 0.5 ml insulin syringe/needle and ensuring the needle is filled, draw up **300 µl** of **0.9% saline**. (*≈ 30 units on the syringe*).
2. Inject the **300 µl** of **saline** into the vial containing the 300 µl of R21 mixed with Matrix-M with a slow single depression of the syringe.

DO NOT RE-INJECT AGAIN OR PULL BACK ON THE SYRINGE.

3. Remove and dispose of the syringe/needle and gently swirl the vial for 5 seconds to aid mixing.

D. Extracting final volume of diluted R21 mixed with Matrix-M

1. Open sterile gauze and place on vaccine tray
2. Using a fresh 1 ml syringe and an intramuscular needle, and keeping the cap of the needle on top of sterile gauze on vaccine tray, insert the needle into the vial and invert. Very gently draw back and reinject the mixed vaccine three times to ensure a good mix (fig. 3).

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS.

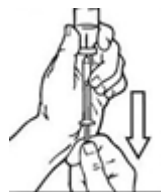


Figure 3

3. Ensuring both the hub of the needle and needle are filled, draw up **330 µl** (0.33 ml) from vial containing the final mixed dilution.

4. Using a **single-handed** scoop technique, re-sheath the needle (fig. 4). Attach a label to the syringe with the participant trial number.

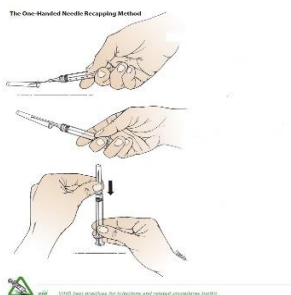


Figure 4

5. Administer the vaccine. When administering the vaccine do not change needles or attempt to draw back on the needle once inserted into the muscle, this will create an unwanted dead space which may lead to variations in the dose administered. Cover the injection site with and sterile dressing.
6. Dispose used needle, syringe and 2ml vial in accordance with local policy.

8.13 Extract from VAC 076 vaccine dilutions SOP - *5µg R21 mixed with 50µg Matrix-M*

Calculations to prepare one dose:

	R21 Vial	Matrix-M Vial
Dose in mcg per 0.5ml (To be injected)	5	50
Targeted fill volume per vial(mL) @ SIPL	0.65	0.5
Concentration (µg/mL)	20	200
Formulation Batch size (L)	50	10

Mixture of R21 with Matrix-M			
		R21	MM
(A) To prepare a mixture of R21 with Matrix-M in volume of	A =	1 ml	
(B) Concentration of diluted vaccine and adjuvant	B =	20 µg/ml	200 µg/ml
(C) Volume of R21 and Matrix-M required	C =	0.5 ml	0.5 ml

(D) Mixture contains (C x B)	D =	10 µg	100 µg
(E) Mixture contains concentrations of (D / A)	E =	10 µg/ml	100 µg/ml
(F) Intended dose for administration	F =	5 µg	50µg
(G) Volume to be extracted per dose (F / E)	G=	0.5 ml	
(H) Doses in extracted volume (G x E)	H=	5 µg	50 µg

Equipment

1 x vial R21
 1 x vial Matrix-M
 2 x 1 ml sterile syringes
 3 x 23G 1" (25 mm) needles
 2 x alcohol swabs
 1 x Injection tray
 PPE – Gloves, apron, goggles
 Sharps bin

A. Preparation and collection of the kit

1. Kits must be maintained in a fridge or cool box between +2 and +8°C until ready to use.
2. The vaccine preparation team must be sure that the vaccine administrator is readily available to administer the vaccine before starting preparation.
3. Clean work surface.
4. Prepare the equipment.
5. Check participant number in the randomization letter and check that participant has been randomized to R21/Matrix M group. This check must be done by at least two members of the staff.
6. Complete blinding label with participant study number.
7. Collect kit containing R21 and Matrix-M from the fridge and record details on the accountability log and 'VAC076 Extension - Vaccination Record'
8. Wear protective eyewear, disposable apron and clean disposable gloves.

B. Mixture of R21 with Matrix-M

1. Remove the cap from the R21 and Matrix-M vials. Wipe the top of the vials with a single use alcohol swipe and **allow to dry**.

2. Using a 1 ml syringe and a 23G (25 mm) needle attached and ensuring the hub of the syringe and the needle are filled, draw up **0.5 ml** of **R21** vaccine from the R21 vial.

***N.B.** Where very small volumes are being used it is likely that air bubbles will appear in the syringe. By keeping the needle in the vial, it will be possible to remove these by tapping and drawing back and forth. (Fig. 1)*

DO NOT REMOVE THE NEEDLE FROM THE VIAL DURING THIS PROCESS

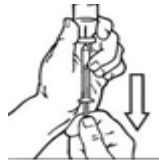


Figure 1

3. With the Matrix-M vial sitting on the work surface and using a single depression of the syringe, slowly and gently inject the **0.5 ml** of **R21** vaccine into the vial containing the Matrix-M (fig 2). Once fully depressed, equalise vial pressure before removing the needle from the vial: withdraw the needle slightly to ensure the tip is **NOT** below the level of the now mixed vaccine (Fig. 3) and withdraw 0.5 ml of air.

DO NOT RE-INJECT AGAIN.

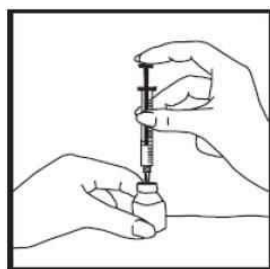


Figure 2

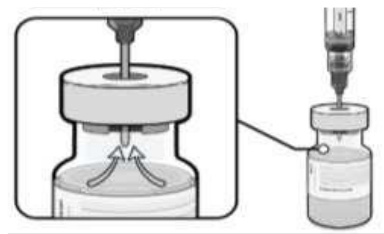


Figure 3

4. Remove and dispose of the needle/syringe and gently invert the vial 5 times to aid mixing. Do NOT shake.
5. Put the used R21 to one side to avoid confusion.

C. Extracting final volume of R21 mixed with Matrix-M

1. Using a fresh 1 ml syringe and a 23G (25mm) needle, insert the needle into the vial and invert (fig. 4). Ensuring both the **hub of the needle and needle are filled**, draw up **0.57 ml** from vial containing the mixture.

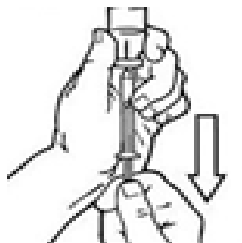


Figure 4

2. Reinvert the vial to an upright position and remove the needle/syringe from the vial.
3. Remove the needle and replace with a fresh 23G (25 mm) needle, **leave cap on**.
4. Gently measure to a volume of **0.5 ml**.
5. Attach blinding label, check ID number on the label against the ID number in the Vaccination record and place syringe in a box/tray.
6. Complete Section II of the 'VAC076 Extension - Vaccination Record'
7. Hand over box/tray containing vaccine and 'VAC076 Extension - Vaccination record' to the vaccine administrator. Vaccine must be administered as soon as possible and never later than 30 minutes after the vaccine was removed from the fridge.
8. Complete 'VAC076 Extension - Randomization and Vaccine preparation form'.
9. Remove the label from the original R21 and Matrix-M vial and attach it to the 'VAC076 Extension - Randomization and Vaccine preparation form'.
10. Keep the 'VAC076 Extension - Randomization and Vaccine preparation form' in a locked cabinet only accessible to 'unblinded' staff.
11. The 'VAC076 Extension – Vaccination record' does not contain unblinded information and may be filed with the rest of the participant documents.

Supplementary appendix table 21: Comparison of demographics and characteristics of participants who were lost to follow-up and those who were not.

This includes all participants who received the booster vaccination at 12 months following the primary series of vaccinations. Numbers are number of participants (%). Group 1 received 5µg R21/25µg Matrix-M™, group 2 received 5µg R21/50µg Matrix-M™ and group 3, the control group, received Rabivax-S. ^ITN – insecticide- treated net, £SMC - seasonal malaria chemoprevention, %A round of SMC is 3 doses of treatment received per month. *P value obtained from a logistic regression of lost to follow up against each baseline characteristic

	Predictive of withdrawal	Withdrawn	Not withdrawn
	P value*	n=31 (%)	n=378 (%)
Randomised group	0.584		
1		8 (6.1)	124 (93.9)
2		12 (8.8)	125 (91.2)
3		11 (7.9)	129 (92.1)
Sex	0.08		
Male		20 (10.0)	181 (90.1)
Female		11 (5.3)	197 (94.7)
Age	0.185		
Mean (SD)		11.5 (3.9)	12.5 (2.8)
Adequate ITN^ use	0.478		
Yes		28 (7.8)	337 (92.3)
No		2 (4.7)	41 (95.4)
SMC£	0.225		
At least one round%		27 (7.1)	352 (92.9)
None		4 (13.3)	26 (86.7)

Supplementary appendix table 22: Time to first episode of malaria meeting primary case definition of clinical malaria episode, from 14 days to 6 and 12 months following primary series (three vaccinations).

Group 1 received 5µg R21/25µg MM, Group 2 received 5µg R21/50µg MM and Group 3 received Rabinax-S. Primary analysis was based on a modified intention-to-treat population. Primary case definition of clinical malaria is presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ AND *P. falciparum* parasites density > 5000 asexual forms/µL. Cox proportional hazards model used to calculate hazard ratio (HR). Vaccine efficacy is calculated by 1-HR and expressed as a percentage. *Cox proportional hazards model, adjusted for sex, age category (5-9 months, 10-12 months and >12 months), adequate insecticide treated net use and receiving at least one round (3 doses in one month) of SMC.

	Case definition according to parasitaemia	Time point post primary series of vaccinations	Number with at least one episode of clinical malaria (%)	Unadjusted efficacy		Adjusted efficacy*	
				% (95% CI)	P-value	% (95% CI)	P-value
Group 1	Primary >5000 parasites/µl	6 months	43/146 (29.5)	74 (63-82)	<0.0001	74 (63-82)	<0.0001
		12 months	50/146 (34.3)	71 (59-79)	<0.0001	70 (58-79)	<0.0001
		24 months	82/132 (34.3)	66 (55-74)	<0.0001	66 (55-74)	<0.0001
Group 2	Primary >5000 parasites/µl	6 months	38/146 (26.0)	77 (67-84)	<0.0001	75 (64-83)	<0.0001
		12 months	39/146 (26.7)	77 (67-84)	<0.0001	75 (64-83)	<0.0001

	Case definition according to parasitaemia	Time point post primary series of vaccinations	Number with at least one episode of clinical malaria (%)	Unadjusted efficacy		Adjusted efficacy*	
				% (95% CI)	P-value	% (95% CI)	P-value
		24 months	70/137 (51.1)	75 (66-81)	<0.0001	76 (68-83)	<0.0001
Groups 1 and 2 combined	Primary >5000 parasites/μl	6 months	81/292 (27.7)	75 (67-81)	<0.0001	75 (66-82)	<0.0001
		12 months	89/292 (30.5)	74 (65-80)	<0.0001	73 (64-79)	<0.0001
		24 months	152/269 (56.5)	71 (63-77)	<0.0001	71 (63-78)	<0.0001
Group 3 (control group)	Primary >5000 parasites/μl	6 months	105/147 (71.4)				
		12 months	106/147 (72.1)				
		24 months	128/140				

				Unadjusted efficacy		Adjusted efficacy*	
	Case definition according to parasitaemia	Time point post primary series of vaccinations	Number with at least one episode of clinical malaria (%)	% (95% CI)	P-value	% (95% CI)	P-value
			(91.4)				

Supplementary appendix table 23: Time to first episode of malaria meeting case definitions of clinical malaria episode, from 14 days to 12 months following booster vaccination.

Group 1 received 5µg R21/25µg Matrix-M™, group 2 received 5µg R21/50µg Matrix-M™ and group 3 received Rabivax-S. Primary case definition of clinical malaria is presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ AND *P. falciparum* parasites density > 5000 asexual forms/µL. Secondary case definition of clinical malaria is presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ and/ or history of fever within the last 24 hours AND *P. falciparum* parasites density > 0 asexual forms/µL. Cox proportional hazards model used to calculate hazard ratio (HR). Vaccine efficacy is calculated by 1-HR and expressed as a percentage. *Cox proportional hazards model, adjusted for sex, age category (5-9 months, 10-12 months and >12 months), adequate insecticide treated net use and receiving at least one round (3 doses in one month) of SMC.

	Case definition according to parasitaemia	Time point post booster	Number with at least one episode of clinical malaria (%)	Unadjusted efficacy		Adjusted efficacy*	
				% (95% CI)	P-value	% (95% CI)	P-value
Group 1	Primary >5000 parasites/µl	12 months	67/132 (50.8)	71 (60-78)	<0.0001	70 (59-78)	<0.0001
	Secondary >0 parasites/µl	12 months	72/132 (54.6)	71 (61-78)	<0.0001	70 (60-78)	<0.0001
Group 2	Primary >5000 parasites/µl	12 months	54/137 (39.4)	80 (72-85)	<0.0001	80 (72-85)	<0.0001
	Secondary >0 parasites/µl	12 months	65/137 (47.5)	77 (69-83)	<0.0001	77 (69-83)	<0.0001
Group 3 (control group)	Primary >5000 parasites/µl	12 months	121/140 (86.4)				
	Secondary >0 parasites/µl	12 months	124/140 (88.6)				

Supplementary appendix table 24: Time to first episode of malaria meeting secondary case definition of clinical malaria episode, from 14 days to 6 and 12 months following primary series (three vaccinations).

Group 1 received 5µg R21/25µg MM, Group 2 received 5µg R21/50µg MM and Group 3 received Rabivax-S. Primary analysis was based on a modified intention-to-treat population. Secondary case definition of clinical malaria is presence of axillary temperature $\geq 37.5^{\circ}\text{C}$ and/ or history of fever within the last 24 hours AND *P. falciparum* parasites density > 0 asexual forms/µl. Cox proportional hazards model used to calculate hazard ratio (HR). Vaccine efficacy is calculated by 1-HR and expressed as a percentage. *Cox proportional hazards model, adjusted for sex, age category (5-9 months, 10-12 months and >12 months), adequate insecticide treated net use and receiving at least one round (3 doses in one month) of SMC.

	Case definition according to parasitaemia	Time point	Number with at least one episode of clinical malaria (%)	Unadjusted efficacy		Adjusted efficacy*	
				% (95% CI)	P-value	% (95% CI)	P-value
Group 1	Secondary >0 parasites/µl	6 months	53/146 (36.3)	73 (63-81)	<0.0001	74 (64-81)	<0.0001
		12 months	61/145 (42.1)	70 (60-78)	<0.0001	71 (60-79)	<0.0001
		24 months	89/132 (67.4)	67 (56-75)	<0.0001	66 (68-83)	<0.0001
Group 2	Secondary >0 parasites/µl	6 months	43/146 (29.5)	79 (70-85)	<0.0001	78 (69-85)	<0.0001
		12 months	43/146 (29.5)	80 (72-86)	<0.0001	80 (71-86)	<0.0001

	Case definition according to parasitaemia	Time point	Number with at least one episode of clinical malaria (%)	Unadjusted efficacy		Adjusted efficacy*	
				% (95% CI)	P-value	% (95% CI)	P-value
		24 months	81/137 (59·1)	75 (67-81)	<0.0001	76 (68-83)	<0.0001
Groups 1 and 2 combined	Secondary >0 parasites/μl	6 months	96/292 (32·8)	76 (69-82)	<0.0001	76 (69-82)	<0.0001
		12 months	104/291 (35·7)	75 (68-81)	<0.0001	75 (67-81)	<0.0001
		24 months	170/269 (63·2)	71 (56-75)	<0.0001	72 (64-78)	<0.0001
Group 3 (control group)	Secondary >0 parasites/μl	6 months	118/147 (80·2)				
		12 months	120/147 (81·6)				
		24 months	131/140				

	Case definition according to parasitaemia	Time point	Number with at least one episode of clinical malaria (%)	Unadjusted efficacy		Adjusted efficacy*	
				% (95% CI)	P-value	% (95% CI)	P-value
			(93·6)				

Supplementary appendix table 25: Calculation of case rate per 1000 person-years and vaccine efficacy (unadjusted) against all malaria episodes.

Case incidence rates for all episodes were calculated using negative binomial regression over 6 months following booster vaccination (June – December 2020) for groups 1 to 3(207). Wald Test p value comparison to group 3 was used (control group).

Group	Person-years at risk	Events	Rate per 1000 person-years (95% CI)	Protective Efficacy % (95% CI)	Wald Test P-value
1	66.0	90	1362.7 (1108.3-1675.4)	64.8 (55.5-72.1)	<0.0001
2	68.5	45	656.5 (490.2-879.3)	78.4 (72.0-83.4)	<0.0001
3	70.0	249	3554.7 (3139.5-4024.8)		

Supplementary appendix table 26: Calculation of case rate per 1000 person-years and vaccine efficacy (unadjusted) against all malaria episodes.

Case incidence rates for all episodes were calculated using negative binomial regression over 6 -12 months (January – June 2020 and 2021) following booster vaccination for groups 1 to 3(207). Wald Test p value comparison to group 3 was used (control group).

Group	Dry season time point	Person-years at risk	Events	Rate per 1000 person-years (95% CI)	Protective Efficacy % (95% CI)	Wald Test P-value
1	Post primary series	65.1	25	384.0 (259.5-568.3)	44.8 (-2.44-70.2)	0.060
2	Post primary series	67.8	15	221.3 (133.4-367.1)	68.1 (36.1-84)	0.001
3	Post primary series	69.2	48	693.2 (522.4-919.9)		
1	Post booster	56.3	40	709.9 (520.7-967.8)	56.2 (35.4-70.3)	<0.0001
2	Post booster	56.8	30	528.4 (369.4-755.7)	66.8 (49.1-78.4)	<0.0001
3	Post booster	59.2	95	1603.6 (1311.5-1960.8)		
1	Combined	121.5	65	535.2 (419.7-682.5)	52.5 (31.3-67.2)	<0.0001
2	Combined	124.6	45	361.3 (269.7-483.9)	67.2 (51.0-78.1)	<0.0001
3	Combined	128.5	143	1113.0 (944.7-1311.2)		

Supplementary appendix table 27: Impact of gender on number of malaria episodes.

Group 1 received 5µg R21/25µg MM, Group 2 received 5µg R21/50µg MM and Group 3 received Rabinax-S. Mann-Whitney used to calculate p-value

Group	Male (mean)	Female (mean)	P value
1	0.397	0.6150	0.111
2	0.542	0.250	0.046
3	2.333	2.379	0.754

Supplementary appendix table 28: Comparisons of number of fevers recorded at highest grade between groups after each dose.

Group 1 received 5µg R21/25µg MM, Group 2 received 5µg R21/50µg MM and Group 3, the control group, received Rabivax-S. Day 28 is 28 days following first vaccination and Day 84 is 28 days following third vaccination. There were 150 participants in each group who received the first dose of the vaccination series. One hundred and forty-nine participants in Group 1, 147 participants in Group 2 and 149 participants in Group 3 received a second dose. One hundred and forty-six participants in Group 1, 147 participants in Group 2 and 149 participants in Group 3 received a third dose. Fisher's exact test used to calculate p values

	First dose	Second dose	Third dose	Booster dose
Group 1 vs 2	0.03	0.0002	0.01	0.05
Group 1 vs 3	0.84	0.03	0.72	0.02
Group 2 vs 3	0.01	<0.0001	0.003	<0.0001

Supplementary appendix table 29: Unsolicited Adverse Events in Group 1 (5µg R21/25µg Matrix-M).

These participants received up to 4 doses of 5µg R21/25µg Matrix-M: 3 doses given 4 weeks apart and a booster dose approximately 12 months later. Numbers are number of participants. Unsolicited adverse events were collected for 28 days following each dose of vaccination. All terms are coded according to MedDRA preferred term. Severity grading of AEs was based on activity/medical intervention/therapy required as described in chapter 2. All unsolicited AEs were mild or moderate in severity and assessed as having no relationship to vaccine.

AE category	Dose 1	Dose 2	Dose 3	Booster	Total
Amoebiasis	6	7	13	14	40
Bronchiolitis	8	3	4	3	18
Bronchitis	0	3	0	0	3
Conjunctivitis	4	3	2	4	13
Cough	1	1	0	0	2
Dermatitis	4	4	6	3	17
Diarrhoea	5	2	0	0	7
Ear Infection	4	6	4	0	14
Eczema	0	0	2	0	2
Fungal Skin Infection	0	1	1	1	3
Furuncle	0	1	1	0	2
Gastroenteritis	3	5	10	8	26
Influenza	0	2	0	0	2
Laryngitis	0	1	0	0	1
Malaria	13	1	11	0	25
Oral Candidiasis	4	4	1	0	9
Pharyngitis	26	18	15	7	66
Pneumonia	3	1	1	3	8
Prurigo	0	1	4	7	12
Rash Pustular	2	5	4	0	11
Rhinitis	11	12	9	10	42
Staphylococcal Skin Infection	2	2	1	5	10

Subcutaneous Abscess	1	0	0	0	1
Thermal burn	0	0	0	1	1
Tinea Infection	0	0	1	0	1
Urinary Tract Infection	0	1	0	0	1
Wound	0	1	1	0	2
Total	97	85	91	66	339

Supplementary appendix table 30: Unsolicited Adverse Events in Group 2 (5µg R21/50µg Matrix-M).

These participants received up to 4 doses of 5µg R21/50µg Matrix-M: 3 doses given 4 weeks apart and a booster dose approximately 12 months later. Numbers are number of participants. Unsolicited adverse events were collected for 28 days following each dose of vaccination. All terms are coded according to MedDRA preferred term. Severity grading of AEs was based on activity/medical intervention/therapy required as described in chapter 2. All unsolicited AEs were mild or moderate in severity and assessed as having no relationship to vaccine. *There was one participant with Bronchiolitis classified as severe.

AE category	Dose 1	Dose 2	Dose 3	Dose 4	Total
Amoebiasis	10	2	11	18	41
Bronchiolitis*	4	5	3	4	16
Bronchitis	0	1	0	0	1
Conjunctivitis	6	2	4	5	17
Cough	5	0	0	0	5
Dermatitis	9	4	5	5	23
Dermatitis Diaper	0	1	0	0	1
Diarrhoea	2	0	1	0	3
Ear Infection	1	5	8	1	15
Eczema	0	0	1	0	1
Fungal Skin Infection	1	1	2	0	4
Furuncle	0	2	1	0	3
Gastroenteritis	9	4	7	7	27
Hordeolum	1	0	0	0	1
Influenza	2	2	1	0	5
Malaria	9	3	5	0	17
Oral Candidiasis	1	1	1	1	4
Parotitis	0	1	0	0	1
Pharyngitis	25	27	16	1	69
Pneumonia	4	2	2	1	9
Prurigo	1	4	4	5	14
Rash	1	0	0	0	1
Rash Pustular	1	3	4	0	8
Rhinitis	6	4	7	12	29
Staphylococcal Skin Infection	1	2	2	4	9
Subcutaneous Abscess	1	0	0	0	1
Thermal Burn	0	1	0	0	1
Tinea Infection	0	1	0	0	1
Urinary Tract Infection	0	0	2	0	2
Wound	0	1	1	1	3
Total	100	79	88	65	332

Supplementary appendix table 31: Unsolicited Adverse Events in Group 3 (Rabivax-S).

These participants received up to 4 doses of the control vaccine (Rabivax -S): 3 doses given 4 weeks apart and a booster dose approximately 12 months later. Numbers are number of participants. Unsolicited adverse events were collected for 28 days following each dose of vaccination. All terms are coded according to MedDRA preferred term. Severity grading of AEs was based on activity/medical intervention/therapy required as described in chapter 2. All unsolicited AEs were mild or moderate in severity and assessed as having no relationship to vaccine.

AE category	Dose 1	Dose 2	Dose 3	Dose 4	Total
Amoebiasis	3	13	9	10	35
Bronchiolitis	6	1	4	4	15
Bronchitis	0	1	0	0	1
Conjunctivitis	6	4	2	3	15
Cough	3	0	0	0	3
Dermatitis	1	4	7	5	17
Dermatitis Diaper	1	1	0	0	2
Diarrhoea	1	0	2	0	3
Ear Infection	1	6	10	0	17
Eczema	0	1	0	1	2
Furuncle	1	0	0	0	1
Gastroenteritis	4	7	9	10	30
Influenza	1	2	0	0	3
Keratitis	1	0	0	0	1
Malaria	7	5	23	1	36
Oral Candidiasis	4	3	2	0	9
Parotitis	0	0	1	0	1
Pharyngitis	22	20	17	6	65
Pneumonia	5	2	1	0	8
Prurigo	0	2	5	5	12
Rash Pustular	1	0	5	0	6
Rhinitis	14	7	8	14	43
Staphylococcal Skin Infection	0	2	0	12	14
Subcutaneous Abscess	0	0	1	0	1
Thermal burn	0	0	0	1	1
Wound	1	0	1	0	2
Total	83	81	107	72	343

Supplementary appendix table 32: Laboratory adverse events following the primary series of vaccinations.

Numbers are number of participants. For each participant, the event has been counted only once at its highest grading recorded over the 7 days. Group 1 received 5µg R21/25µg MM, Group 2 received 5µg R21/50µg MM and Group 3, the control group, received Rabivax-S. Day 28 is 28 days following first vaccination and Day 84 is 28 days following third vaccination. There were 150 participants in each group who received the first dose of the vaccination series. One hundred and forty-nine participants in Group 1, 147 participants in Group 2 and 149 participants in Group 3 received a second dose. One hundred and forty-six participants in Group 1, 147 participants in Group 2 and 149 participants in Group 3 received a third dose. Grading of laboratory adverse events was assessed using the most up to date Division of AIDS (DAIDs) Table for Grading the Severity of Adult and Paediatric Adverse Events. All were assessed as not related to vaccination.

Grade	Mild				Moderate			
	Screening	Day 28	Day 84	Total	Screening	Day 28	Day 84	Total
AE								
Anaemia								
Group 1	1	1	0	2	6	1	15	22
Group 2	0	0	2	2	14	5	20	39
Group 3	2	0	0	2	4	5	10	19
Leucocytosis								
Group 1	0	0	0	0	0	1	0	1
Group 2	0	0	0	0	0	0	0	0
Group 3	0	0	0	0	0	0	0	0

Supplementary appendix table 33: Geometric mean titre (GMT) of total NANP IgG as measured by ELISA (A). Comparisons of NANP at specified timepoints (B)

Group 1 received 5µg R21/25µg MM and Group 2 received 5µg R21/50µg MM. Day 84 is 28 days after the third vaccination, Day 236 is 6 months after third vaccination, Day 421 is 12 months after third vaccination and B28 is 28 days after the booster vaccination. The booster vaccination was administered at approximately 12 months following third vaccination. Mann-Whitney test used to calculate p values.

A

Group	GMT (EU) [95% CI]			
	Day 84	Day 236	Day 421	B28
Group 1	6122 [5161-7289]	1769 [1475-2122]	746 [565-984]	6529 [5230-8151]
Group 2	11438 [9985-13102]	3256 [2772-3824]	1616 [1320-1979]	10170 [9016-11472]
Group 1 vs 2	p < 0.0001	p < 0.0001	p < 0.0001	p = 0.0006

B

	D84 vs D236	D236 vs D421	D84 vs B28
Group 1	p < 0.0001	p < 0.0001	p = 0.15
Group 2	p < 0.0001	p < 0.0001	p = 0.19

Supplementary appendix table 34: Correlation of NANP-specific IgG response data with malaria episodes.

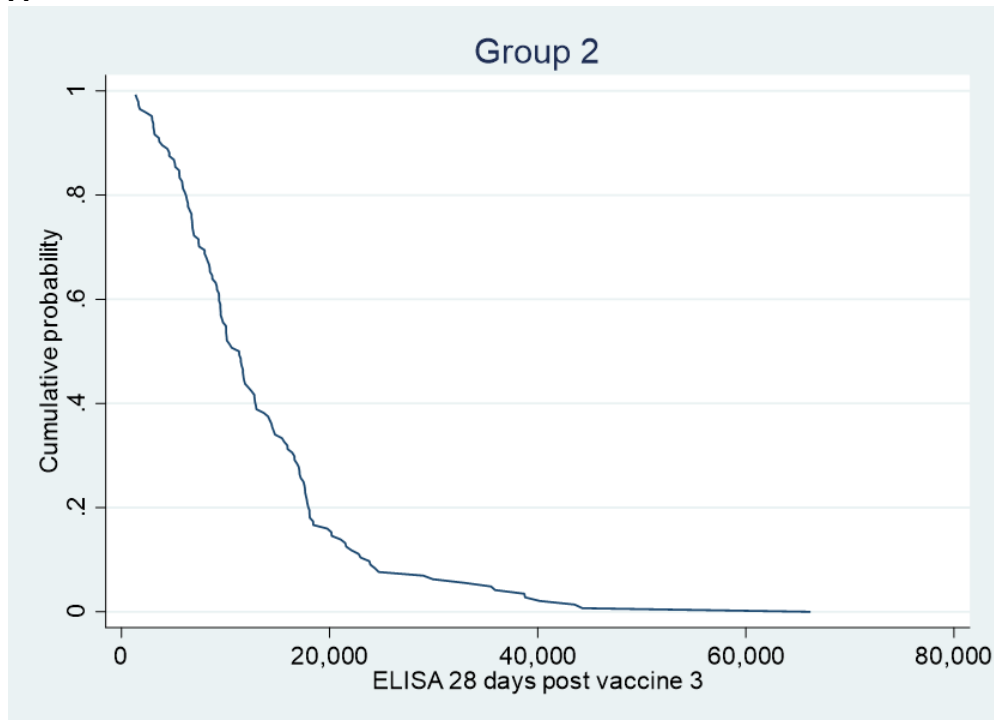
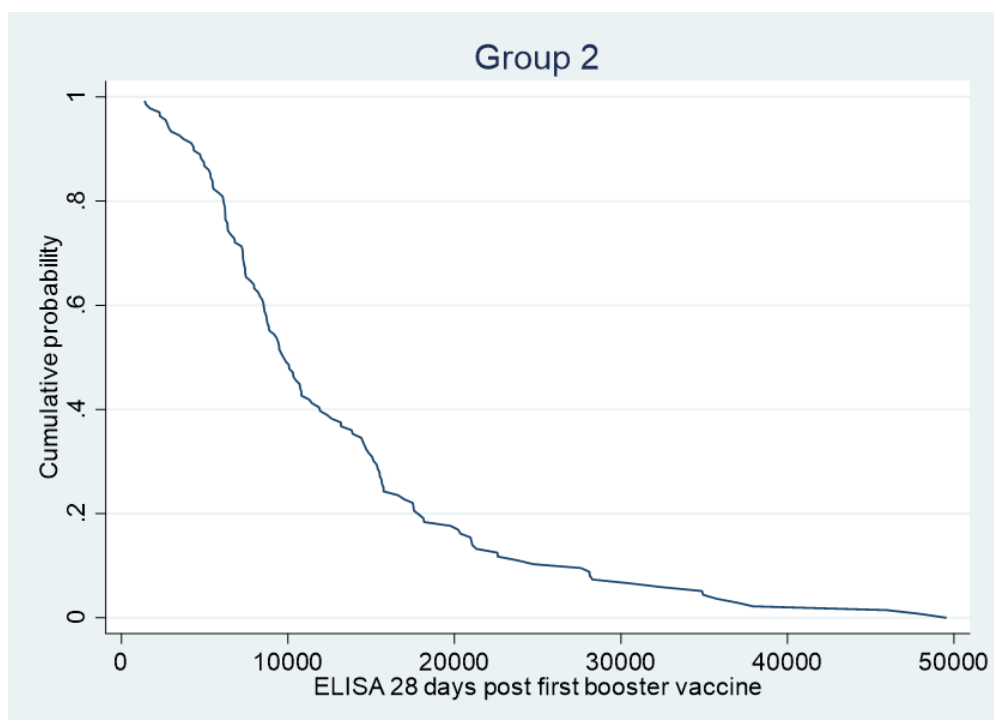
For year 1, antibody responses were measured by ELISA at 28 days following the primary series of vaccinations and at least one episode of malaria according to the primary case definition. For year 2, antibody responses were measured by ELISA at 28 days following the booster vaccination. A represents group 1 who received 5µg R21/25µg Matrix-M for all their vaccinations and B represents group 2 who received 5µg R21/50µg Matrix-M. *Calculated from a linear regression model, ^Group also included in model.

A

Year 1				Year 2		
	n	Mean (log ELISA)	SD (log ELISA)	n	Mean (log ELISA)	SD (log ELISA)
At least 1 malaria case	49	3.8	0.27	66	3.7	0.55
No malaria cases	94	3.8	0.32	65	3.9	0.56
	Difference in means*	95% CI*	P-value*	Difference in means*	95% CI*	P-value*
	0.04	-0.07 to 0.14	0.514	0.2	-0.03 to 0.35	0.099
P-value for Spearman's rank correlation between number of malaria episodes and immune response (Spearman's rho [95% CI])						
0.210 (-0.11 [-0.28 - 0.07])				0.0011 (-0.28 [-0.44 - -0.12])		
HR for log (ELISA) when included in Cox model for time to first episode of malaria^	95% CI	P-value	HR for log (ELISA) in Cox model^	95% CI	P-value	
0.72	0.29 to 1.76	0.470	0.79	0.58 to 1.06	0.119	

B

Year 1				Year 2		
	n	Mean (log ELISA)	SD (log ELISA)	n	Mean (log ELISA)	SD (log ELISA)
At least 1 malaria case	39	3.9	0.28	54	3.9	0.30
No malaria cases	106	4.1	0.33	82	4.1	0.31
	Difference in means*	95% CI*	P-value*	Difference in means*	95% CI*	P-value*
	0.21	0.10 to 0.33	0.0004	0.12	0.01 to 0.22	0.032
P-value for Spearman's rank correlation between number of malaria episodes and immune response (Spearman's rho [95% CI])						
0.0001 (-0.32 [-0.45 - -0.19])				0.0198 (-0.20 [-0.34 - -0.06])		
HR for log (ELISA) when included in Cox model for time to first episode of malaria^	95% CI	P-value	HR for log (ELISA) in Cox model^	95% CI	P-value	
0.23	0.09 to 0.54	0.001	0.41	0.18 to 0.93	0.034	

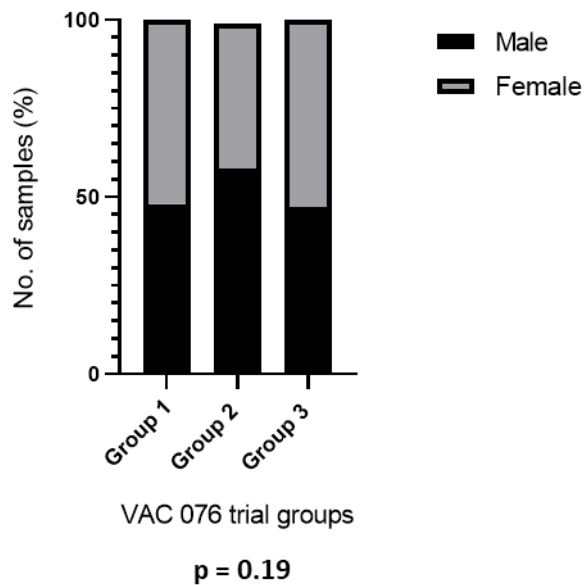
A**B**

Supplementary appendix figure 1: Reverse cumulative distribution to identify a threshold as correlate of protection in group 2 who received 5 μ g R21/50 μ g Matrix-M.

A is calculated using the NANP antibody levels at 28 days following primary series and the correlate of protection equates to 6618 ELISA units (95% CI 5565-8397). B is at 28 days following booster vaccination and the correlate of protection equates to 6130 ELISA units (95% CI 5547-7179).

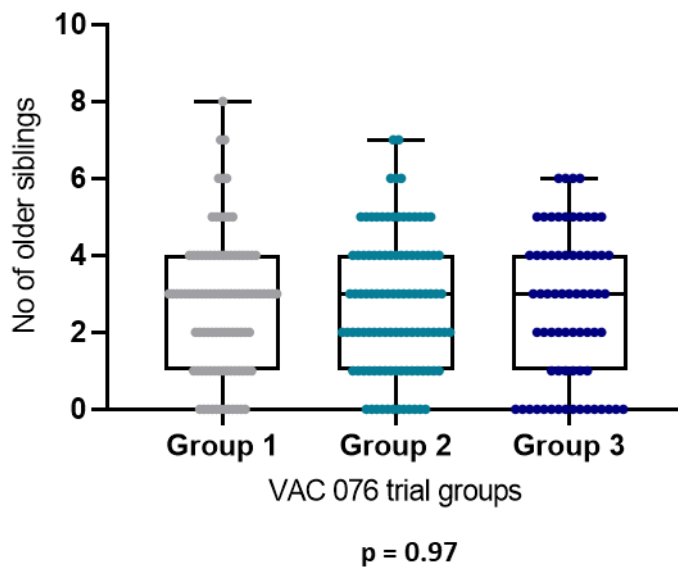
8.15 Confounder analysis

8.15.1 Gender



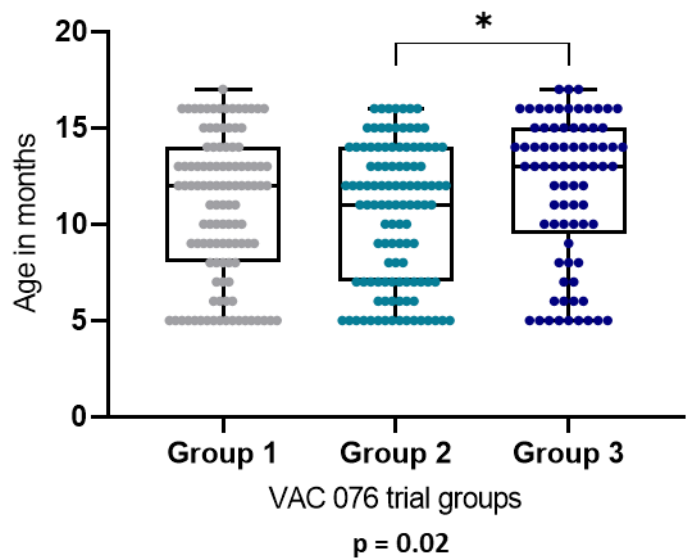
Supplementary appendix figure 2: Analysing gender of participants according to group.
Chi-square used to calculate p-value.

8.15.2 Number of older siblings in household



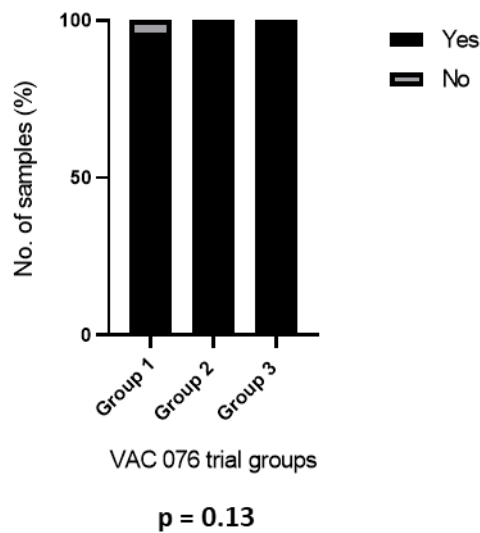
Supplementary appendix figure 3: Analysing number of older siblings in households of participants according to group.
Kruskal-Wallis used to calculate p-value.

8.15.3 Age



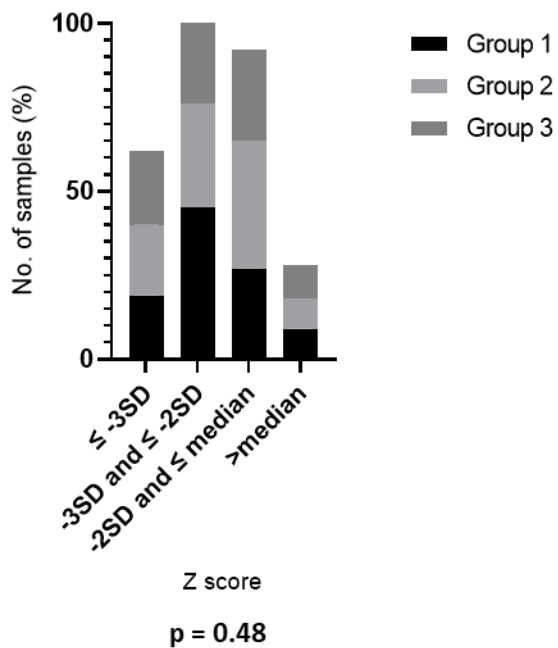
Supplementary appendix figure 4: Analysing age of participants according to group.
Kruskal-Wallis with Dunn's multiple comparisons test used to calculate p-value.

8.15.4 Children breast-fed at baseline



Supplementary appendix figure 5: Analysing children breast-fed at baseline according to group.
Fisher's exact used to calculate p-value

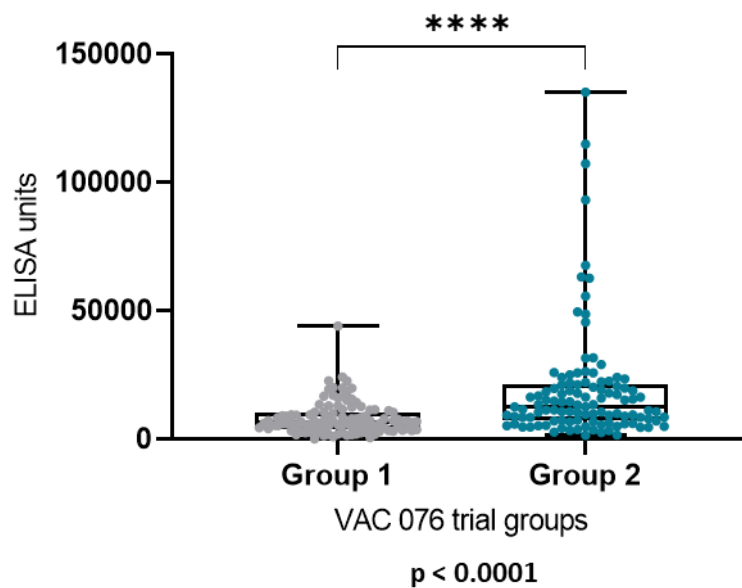
8.15.5 Z-score



Supplementary appendix figure 6: Analysing Z score (nutritional status) of participants according to group.

Chi-square used to calculate p-value

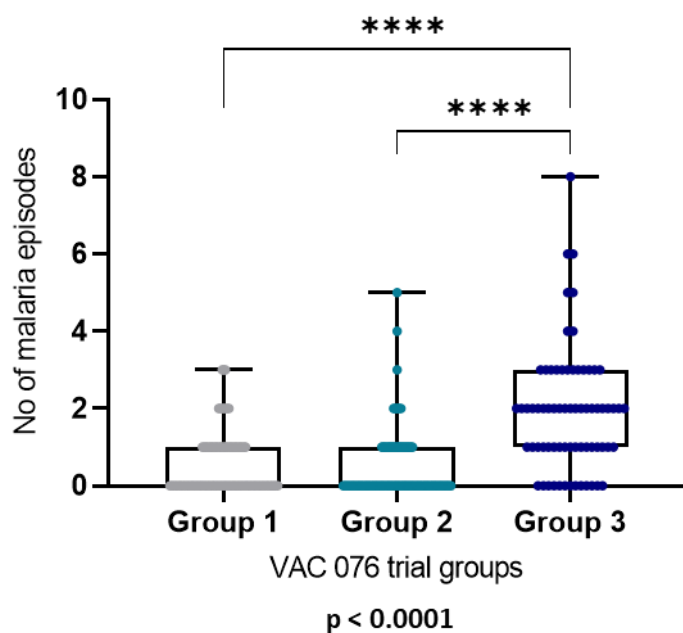
8.15.6 NANP-specific IgG at 28 days following primary series of vaccinations



Supplementary appendix figure 7: Analysing NANP-specific IgG of participants, 28 days after three malaria vaccines (R21/Matrix-M) were received according to group.

Mann-Whitney used to calculate p-value

8.15.7 Number of malaria episodes at 12 months following primary series of vaccinations



Supplementary appendix figure 8: Analysing number of malaria episodes participants experienced according to group.

Kruskal-Wallis with Dunn's multiple comparisons test used to calculate p-value

8.16 Microbiome profile

Supplementary appendix table 35: Mean relative abundance (%) of all phyla identified as part of the microbiome profile at baseline of all participants according to group.

	Group 1	Group 2	Group 3
Firmicutes	37.86592017	37.74921579	38.06618255
Bacteroidetes	34.86691	34.86691	34.86691
Actinobacteria	8.493635	8.493635	8.493635
Proteobacteria	16.60626	16.60626	16.60626
Spirochaetes	0.2399203	0.2399203	0.2399203
Tenericutes	0.233920794	0.233920794	0.233920794
Fusobacteria	1.555122309	1.555122309	1.555122309
Candidatus Saccharibacteria	0.020638239	0.020638239	0.020638239
Deinococcus-Thermus	0.005343411	0.005343411	0.005343411
Verrucomicrobia	0.059584603	0.059584603	0.059584603
Lentisphaerae	0.003144593	0.003144593	0.003144593
Cyanobacteria/Chloroplast	0.005569539	0.005569539	0.005569539
Elusimicrobia	0.033053024	0.033053024	0.033053024
Chloroflexi	0.004575148	0.004575148	0.004575148
Synergistetes	0.000865939	0.000865939	0.000865939
Candidate division WPS-1	0.000333567	0.000333567	0.000333567
Planctomycetes	0.000390263	0.000390263	0.000390263
Deferribacteres	0.000108121	0.000108121	0.000108121

	Group 1	Group 2	Group 3
BRC1	0.000294	0.000294	0.000294
Gemmatimonadetes	0.000667014	0.000667014	0.000667014
Armatimonadetes	0.00035	0.00035	0.00035

8.17 Alpha-diversity

Supplementary appendix table 36: ANOVA used for significance testing assessing individual participant factors against the Shannon diversity index.
Significant values are considered as $p < 0.05$.

Participant factor	p value
Age at baseline	<0.0001
Gender	0.68
Z score	0.04
Number of older siblings	0.04
Weaned at baseline	0.0002
Number of malaria episodes (12 months)	0.11
NANP-specific IgG value at Day 84	0.42

8.18 Multiple linear regression

Supplementary appendix table 37: Model designed with all terms that were significant by ANOVA and enterotype.

Significance testing of terms used in model (Shannon diversity index ~ age at baseline + enterotype + Z score + number of older siblings. Wald test used to calculate p value.

Term	p value
Age at baseline	2.78E-10
Enterotype	6.63E-7
Z score	0.39
Number of older siblings	0.09

8.19 Permutational multivariate analysis of variance (PERMANOVA)

Supplementary appendix table 38: PERMANOVA of significant participant factors at each taxonomic level.

Significant values are considered as $p < 0.05$.

	Age at baseline	Number of older siblings	Gender	Z score	Weaned at baseline	Number of malaria episodes (12 months)	NANP-specific IgG value at Day 84
Phylum	0.001	0.007	0.977	0.302	0.425	0.559	0.286
Class	0.001	0.033	0.905	0.184	0.172	0.767	0.207
Order	0.001	0.028	0.934	0.193	0.094	0.621	0.166
Family	0.001	0.087	0.757	0.118	0.307	0.429	0.413
Genus	0.001	0.113	0.357	0.059	0.299	0.408	0.387

8.20 Multiple linear modelling

Supplementary appendix table 39: Summary of number of significant features at the genus level in both multiple linear models according to age at baseline, number of older siblings in household, antibody responses to R21/Matrix-M at 28 days following the third vaccination (NANP ELISA day 84) and number of clinical malaria episodes according to the primary case definition at 12 months.

Model 1: NANP-specific IgG levels Day 84 + age at baseline + number of older siblings + phylum; random effect: enterotype. Model 2: total number of malaria episodes at 12 months + age at baseline + number of older siblings + phylum; random effect: enterotype).

Term	Number of significant genera in both models
Age at baseline	41
Number of older siblings	1
NANP ELISA at day 84	0
Number of clinical malaria episodes	0

Supplementary appendix table 40: Summary of number of significant features at the family level in both multiple linear models according to age at baseline, number of older siblings in household, antibody responses to R21/Matrix-M at 28 days following the third vaccination (NANP ELISA day 84) and number of clinical malaria episodes according to the primary case definition at 12 months.

Model 1: NANP-specific IgG levels Day 84 + age at baseline + number of older siblings + phylum; random effect: enterotype. Model 2: total number of malaria episodes at 12 months + age at baseline + number of older siblings + phylum; random effect: enterotype).

Term	Number of significant families in both models
Age at baseline	14
Number of older siblings	0
NANP ELISA at Day 84	0
Number of clinical malaria episodes	0

Supplementary appendix table 41: Significant features at the genus level in relation to age at baseline in model 1

(NANP-specific IgG levels Day 84 + age at baseline + number of older siblings + phylum; random effect: enterotype). 216 participant samples were analysed in this model. P values were adjusted for multiple testing using the Benjamini-Hochberg method.

Genus	Number of participants genus present in	% of total participants genus present in	Adjusted p value
Alloprevotella	77	35.7	2.14E-06
Anaerostipes	111	51.4	2.14E-06
Anaerotruncus	37	17.1	0.04830693
Anaerovorax	119	55.1	0.04039696
Atopobium	80	37	0.000377393
Bacteroidales_unclassified	21	9.7	0.024533479
Bacteroidetes_unclassified	17	7.9	0.048992128
Blautia	147	68.1	3.07E-09
Brachyspira	68	31.5	0.001007846
Butyricoccus	72	33.3	0.001059087
Catenibacterium	46	21.3	0.016597525
Christensenella	21	9.7	0.034526814
Clostridiales_unclassified	97	44.9	1.40E-05
Clostridium.IV	64	29.6	0.047452664
Clostridium.XIVa	180	83.3	5.84E-10
Collinsella	185	85.6	0.002390416
Coprococcus	36	16.7	0.001233841
Deltaproteobacteria_unclassified	26	12	0.04675409

Genus	Number of participants genus present in	% of total participants genus present in	Adjusted p value
Dialister	177	81.9	0.000844404
Dorea	153	70.8	2.71E-06
Eggerthella	34	15.7	0.034526814
Enterococcus	122	56.5	0.008397955
Escherichia.Shigella	216	100	0.000103306
Eubacterium	158	73.1	2.05E-06
Faecalibacterium	162	75	6.03E-11
Firmicutes_unclassified	33	15.3	0.013133476
Fusicatenibacter	42	19.4	0.000972782
Gemmiger	135	62.5	1.22E-08
Haemophilus	122	56.5	0.00095703
Holdemanella	49	22.7	0.00456079
Intestinimonas	90	41.7	7.15E-09
Lachnospiraceae_unclassified	170	78.7	7.27E-10
Odoribacter	41	19	0.001770065
Oscillibacter	83	38.4	6.17E-08
Porphyromonadaceae_unclassified	91	42.1	0.001370862
Prevotella	204	94.4	0.00785607
Roseburia	102	47.2	3.14E-06
Ruminococcaceae_unclassified	80	37	0.001365578

Genus	Number of participants genus present in	% of total participants genus present in	Adjusted p value
Ruminococcus	33	15.3	0.025754575
Ruminococcus2	120	55.6	1.33E-10
Senegalimassilia	123	56.9	0.024533479
Succinivibrio	44	20.4	0.028250037
Sutterella	169	78.2	0.004777076
Veillonella	203	94	1.28E-05
Veillonellaceae_unclassified	44	20.4	0.001365578

Supplementary appendix table 42: Significant features at the family level in relation to age at baseline in model 1

(NANP-specific IgG levels Day 84 + age at baseline + number of older siblings + phylum; random effect: enterotype). 216 participant samples were analysed in this model. P values were adjusted for multiple testing using the Benjamini-Hochberg method.

Family	Number of participants family present in	% of total participants family present in	Adjusted p value
Anaeroplasmataceae	27	12.5	0.028455348
Brachyspiraceae	68	31.5	0.001857055
Bacteroidales_unclassified	21	9.7	0.028455348
Christensenellaceae	21	9.7	0.043419735
Clostridiales_unclassified	97	44.9	3.07E-05
Enterobacteriaceae	216	100	0.000109099
Enterococcaceae	122	56.5	0.011057868
Eubacteriaceae	158	73.1	4.01E-06
Firmicutes_unclassified	33	15.3	0.016324405
Lachnospiraceae	208	96.3	0.011057868
Pasteurellaceae	124	57.4	0.004905507
Prevotellaceae	204	94.4	0.008049867
Ruminococcaceae	191	88.4	7.18E-09
Sutterellaceae	182	84.3	0.011057868

Supplementary appendix table 43: Significant features at the genus level in relation to age at baseline in model 2

(Total number of malaria episodes at 12 months + age at baseline + number of older siblings + phylum; random effect: enterotype). 204 participant samples were analysed in this model. P values were adjusted for multiple testing using the Benjamini-Hochberg method.

Genus	Number of participants genus present in	% of total participants genus present in	Adjusted p value
Alloprevotella	71	34.8	6.24E-06
Anaerostipes	104	51	1.49E-06
Anaerovorax	114	55.9	0.030783894
Atopobium	77	37.7	0.000450161
Bacteroidales_unclassified	21	10.3	0.030783894
Blautia	136	66.7	4.69E-09
Brachyspira	62	30.4	0.001066236
Butyricicoccus	67	32.8	0.000632287
Catenibacterium	45	22.1	0.02571245
Christensenella	20	9.8	0.034346674
Clostridiales_unclassified	89	43.6	1.30E-05
Clostridium.IV	61	29.9	0.032459564
Clostridium.XIVa	169	82.8	7.14E-10
Collinsella	172	84.3	0.001751775
Coprococcus	32	15.7	0.00141233
Dialister	165	80.9	0.000632287
Dorea	141	69.1	3.34E-06

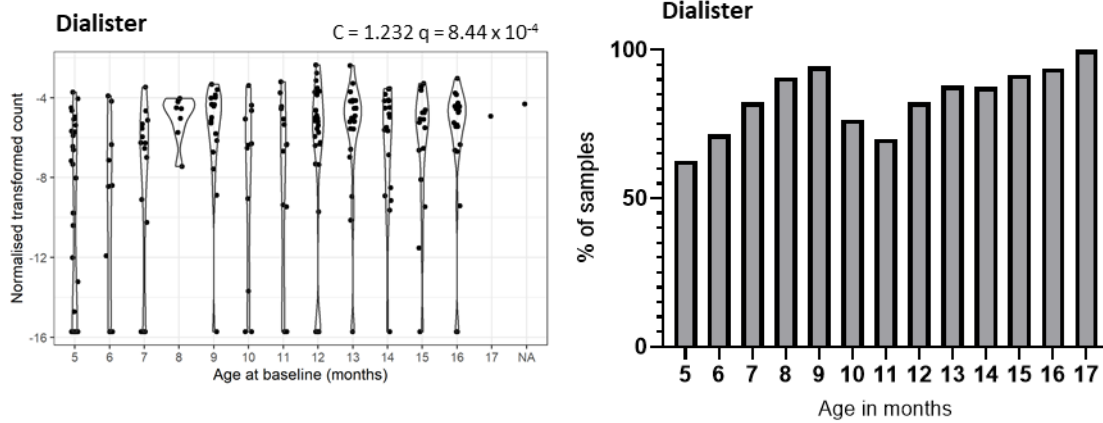
Genus	Number of participants genus present in	% of total participants genus present in	Adjusted p value
Enterococcus	115	56.3	0.007873994
Escherichia.Shigella	204	100	0.000238469
Eubacterium	147	72.1	1.71E-06
Faecalibacterium	150	73.5	1.35E-10
Firmicutes_unclassified	28	13.7	0.01168628
Fusicatenibacter	39	19.1	0.001584948
Gemmiger	124	60.8	1.72E-08
Haemophilus	112	54.9	0.000864068
Holdemanella	44	21.6	0.003917313
Intestinimonas	84	41.2	1.14E-08
Lachnospiraceae_unclassified	159	77.9	7.14E-10
Odoribacter	37	18.1	0.001920422
Oscillibacter	77	37.7	4.75E-08
Porphyromonadaceae_unclassified	87	42.6	0.003040552
Prevotella	192	94.1	0.010396207
Roseburia	95	46.6	1.77E-06
Ruminococcaceae_unclassified	74	36.3	0.000718284
Ruminococcus	30	14.7	0.020342121
Ruminococcus2	110	53.9	1.35E-10
Senegalimassilia	112	54.9	0.011113371

Genus	Number of participants genus present in	% of total participants genus present in	Adjusted p value
Streptococcus	201	98.5	0.044411147
Succinivibrio	39	19.1	0.030783894
Sutterella	155	76	0.004188642
Veillonella	191	93.6	2.42E-05
Veillonellaceae_unclassified	41	20.1	0.001584948

Supplementary appendix table 44: Significant features at the family level in relation to age at baseline in model 2

(Total number of malaria episodes at 12 months + age at baseline + number of older siblings + phylum; random effect: enterotype). 204 participant samples were analysed in this model. P values were adjusted for multiple testing using the Benjamini-Hochberg method.

Family	Number of participants family present in	% of total participants family present in	Adjusted p value
Anaeroplasmataceae	24	11.8	0.027417454
Bacteroidales_unclassified	21	10.3	0.03861006
Brachyspiraceae	62	30.4	0.002058236
Christensenellaceae	20	9.8	0.03861006
Clostridiales.Incertae.Sedis.XIII	119	58.3	0.03861006
Clostridiales_unclassified	89	43.6	2.66E-05
Desulfovibrionaceae	99	48.5	0.03861006
Enterobacteriaceae	204	100	0.000239803
Enterococcaceae	115	56.4	0.010711631
Eubacteriaceae	147	72.1	3.71E-06
Firmicutes_unclassified	28	13.7	0.015845913
Lachnospiraceae	196	96.1	0.00628718
Pasteurellaceae	114	55.9	0.005724627
Prevotellaceae	192	94.1	0.010711631
Ruminococcaceae	180	88.2	2.19E-08
Sutterellaceae	169	82.8	0.009052106



Supplementary appendix figure 9: Dialister abundance and prevalence remaining consistent as participant age increases.

Genus abundance shown as normalised transformed count and prevalence shown as percentage of all samples in each age group. C = coefficient and q = q value. Q value is synonymous to the adjusted p value. P values were adjusted for multiple testing using the Benjamini-Hochberg method.

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