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Assessment of the Safety, Immunogenicity and Efficacy of Novel Blood-Stage Malaria Vaccines

By:

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Abstract

Despite decades of research and the availability of effective medications, malaria remains a significant global health issue. The vast majority of infections are caused by two species: *Plasmodium falciparum* and *Plasmodium vivax*. There is currently no licensed malaria vaccine but an effective vaccine is widely considered necessary to maintain the progress towards eradication, particularly given the increasing issues of insecticide and antimalarial resistance developing. The manifestations of malaria disease are caused by the blood-stage of the parasite against which endemic populations, who are exposed to multiple episodes of malaria, develop some degree of natural immunity. It is therefore considered that vaccines against the blood-stage may mimic the immunity seen in these individuals. This thesis describes three early-phase clinical trials for blood-stage malaria vaccines, all carried out in healthy volunteers at the Jenner Institute in Oxford.

The first of these was a Phase Ia trial of a novel *P. vivax* blood-stage vaccine, ChAd63/MVA PvDBP. This is the only blood-stage vivax vaccine to reach clinical trial and was safe and immunogenic, with functional activity of the antibodies induced by vaccination demonstrated *in vitro*.

The second trial was a Phase I/IIa trial of a candidate *P. falciparum* vaccine, FMP2.1/AS01B. Vaccine efficacy was assessed by blood-stage controlled human malaria infection (CHMI) using a model developed for this trial. Although the vaccine did not demonstrate any efficacy, the CHMI model was highly reproducible.

The final Phase Ia trial examined novel *P. falciparum* vaccines, ChAd63/MVA RH5. This is the first clinical trial in which purified IgG demonstrated inhibition of growth of *P. falciparum in vitro* in all strains tested.

These studies have demonstrated the potential for developing an effective vaccine against blood stage vivax and falciparum malaria as well as the potential for using the CHMI model for proof-of-concept efficacy testing of novel malaria vaccines.

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Publications resulting from this work

To date, one manuscript based on the work in this thesis has been published, one is in press and one is in preparation.

1. Payne, R.O., K.H. Milne, S.C. Elias, N.J. Edwards, A.D. Douglas, R.E. Brown, S.E. Silk, S. Biswas, K. Miura, R. Roberts, T.W. Rampling, N. Venkatraman, S.H. Hodgson, G.M. Labbe, F.D. Halstead, I.D. Poulton, F.L. Nugent, H. de Graaf, P. Sukhtankar, N.C. Williams, C.F. Ockenhouse, A.K. Kathcart, A.N. Qabar, N.C. Waters, L.A. Soisson, A.J. Birkett, G.S. Cooke, S.N. Faust, C. Woods, K. Ivinson, J.S. McCarthy, C.L. Diggs, J. Vekemans, C.A. Long, A.V. Hill, A.M. Lawrie, S. Dutta, and S.J. Draper, *Demonstration of the Blood-Stage Plasmodium falciparum Controlled Human Malaria Infection Model to Assess Efficacy of the P. falciparum Apical Membrane Antigen 1 Vaccine, FMP2.1/AS01.* J Infect Dis, 2016. **213**(11): p. 1743-51.

2. Payne, R. O., P.M. Griffin, J.S. McCarthy, and S.J. Draper, *Plasmodium vivax Controlled Human Malaria Infection – Progress and Prospects.* Trends in Parasitology, in press: http://dx.doi.org/10.1016/j.pt.2016.11.001.

3. Payne R.O., S.E. Silk, S.C. Elias, K.H. Milne, D. Llewellyn, T.A. Rawlinson, R. Shakri, G.M. Labbé, J. Jin, N.J. Edwards, I.D. Poulton, R. Roberts, S.C. de Cassan, A. Nicosia, S. Moyle, E. Berrie, A.V. S. Hill, A.M. Lawrie, C.E. Chitnis and S.J. Draper, *A chimpanzee adenovirus- and MVA-vectored vaccine against the Plasmodium vivax Duffy-binding protein is safe and immunogenic in adults*. Manuscript in preparation.

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Abbreviations

ACT	Artemisinin-based combination therapy
AE	Adverse event
ALT	Alanine transaminase
AMA1	Apical membrane antigen 1
ANC	Absolute neutrophil count
APC	Antigen presenting cell
AR	Adverse reaction
ARDS	Acute respiratory distress syndrome
ASC	Antibody secreting cell
AU	Arbitrary unit/ antibody unit
CBF	Clinical Biomanufacturing Facility
CCVTM	Centre for Clinical Vaccinology and Tropical Medicine
CFCA	Calibration free concentration analysis
ChAd63	Chimpanzee adenovirus 63
ChAd63 PvDBP	Recombinant chimpanzee adenovirus 63 encoding region II of the *Plasmodium vivax* Duffy-binding protein
ChAd63 RH5	Recombinant chimpanzee adenovirus 63 encoding *Plasmodium falciparum* reticulocyte-Binding Protein Homologue 5
CHMI	Controlled human malaria infection
CI	Chief Investigator
CMV	Cytomegalovirus
CRF	Clinical research facility/ case report form
CS/CSP	Circumsporozoite protein
CV	Coefficient of variation
CVA	Chorioallantois vaccinia Ankara
CYRPA	Cysteine-rich protective antigen
DARC	Duffy antigen receptor for chemokines
DBL	Duffy-binding like
DBP	Duffy-binding protein
DiCo	Diversity-Covering *P. falciparum* AMA1 vaccine candidate
DNA	Deoxyribonucleic acid
EBA	Erythrocyte binding antigens
EBL	Erythrocyte-binding ligand
EBV	Epstein-Barr virus

ECG	Electrocardiogram
eCRF	Electronic case report form
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FCS	Foetal calf serum
FDA	United States Food and Drug Administration
G6PD	Glucose-6-phosphate dehydrogenase
GIA	Growth inhibition activity
GMO	Genetically modified organism
GMP	Good manufacturing practice
GP	General Practicioner
GPA	glycophorin A
GSK	GlaxoSmithKline
HBsAg	Hepatitis B surface antigen
HCG	Human Chorionic Gonadotrophin
HCV	Hepatitis C virus
Hgb	Haemoglobin
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HTLV	Human T-cell lymphotropic virus
IB	Investigator's brochure
ICGEB	International Centre for Genetic Engineering and Biotechnology
ICH	International Conference on Harmonisation
IFAT	Immunofluorescence antibody test
IFN-γ	Interferon gamma
IM	Intramuscular
IMP	Investigational medicinal product
IMX313	Chimeric version of the oligomerisation domain from chicken complement inhibitor C4bp
ITN	Insecticide-treated net
IV	Intravenous
LDH	Lactate dehydrogenase
LLD	Lower limit of detection
LLN	Lower limit of normal

LLQ	Lower limit of quantification
LPS	Lipopolysaccharide
LSC	Local Safety Committee
LSM	Local safety monitor
LSP	Long synthetic peptide
mAb	Monoclonal antibody
mBC	Memory B cell
MDG	Millenium development goal
MedDRA	Medical Dictionary for Regulatory Activities
ME-TRAP	Multiple epitopes and thrombospondin-related adhesion protein
µg	Microgram
MGSA	Melanoma growth stimulating activity
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare products Regulatory Agency
MSC	Microbiological safety cabinet
MSP1	Merozoite surface protein 1
MVA	Modified vaccinia virus Ankara
MVA PvDBP	Modified vaccinia virus Ankara encoding region II of the *Plasmodium vivax* Duffy-binding protein
MVI	Malaria Vaccine Initiative
NaSCN	Sodium thiocyanate
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIH	National Institutes of Health
NIHR	National Institute for Health Research
OD	Optical density
OVC	Oxford Vaccine Centre
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PfRH	*Plasmodium falciparum* reticulocyte-binding protein homologue
PfRH5/ RH5	Reticulocyte-Binding Protein Homologue 5
PfRIPR	PfRh5-interacting protein
pfu	plaque-forming units
PI	Principal Investigator

PMR	Parasite multiplication rate
POC	Proof-of-concept
PvCSP	*Plasmodium vivax* circumsporozoite protein
PvDBP	*Plasmodium vivax* Duffy-binding protein
PvDBP_RII	*Plasmodium vivax* Duffy-binding protein region II
Pvs25	*Plasmodium vivax* 25 kDa oocyst/ookinete surface protein
QC	Quality control
QP	Qualified person
qPCR	Quantitative polymerase chain reaction
QIMR	Queensland Institute of Medical Research
R&D	Research and Development
RBC	Red blood cells
RBL/RBP	Reticulocyte binding-like proteins
RBM	Roll Back Malaria
RBP	Reticulocyte-binding protein
RON2	Merozoite rhoptry neck complex protein
RPMI	Cell culture media developed at Roswell Park Memorial Institute
rPvCS	Recombinant *Plasmodium vivax* circumsporozoite protein
RSV	Respiratory syncytial virus
SA	Sialic acid
SAE	Serious adverse event
Sal I	Salvador I *Plasmodium vivax* strain
SAR	Serious adverse reaction
SCID	Severe Combined Immunodeficiency
SCORE	Systematic Coronary Risk Evaluation
SFU	Spot-forming units
SmPC	Summary of Product Characteristics
SOP	Standard operating procedure
SUSAR	Suspected unexpected serious adverse reaction
UK	United Kingdom
ULN	Upper limit of normal
UNICEF	United Nations Children's Fund
UNDP	United Nations Development Programme
USA	United States of America
TBV	Transmission-blocking vaccine

TLR	Toll-like receptor
TOPS	The over-volunteering prevention service
tPA	Tissue plasminogen activator
TRAP	Thrombospondin-related adhesion protein
ULN	Upper limit of normal
VLP	Virus-like particle
vp	viral particle
WBC	White blood cells
WHO	World Health Organisation
WIRB	Western Institutional Review Board
WRAIR	Walter Reed Army Institute of Research
WTCRF	Wellcome Trust Clinical Research Facility

Chapter One:

Introduction

1.1 Background

There are five *Plasmodium* species that cause human malaria, by far the most common being *P. falciparum,* which is also the species which causes the vast majority of mortality relating to malaria. *P. vivax* is the second most common species to cause human malaria. The remaining three species, *P. ovale*, *P. malariae* and *P. knowlesi,* cause a much lower proportion of cases worldwide, with *P. knowlesi* only relatively recently recognised as a human malaria, although primarily a zoonosis (1). *Plasmodium* species are apicomplexan protozoan parasites transmitted by the female *Anopheles* mosquito which injects sporozoites into the human host when it takes a blood meal. The Apicomplexa phylum is so named because of the specialised apical complex of organelles within this group of parasites which is essential for invasion (2).

According to the World Malaria Report 2015 there were an estimated 438,000 deaths annually worldwide. Globally, there are thought to be around 3.2 billion people at risk of malaria, with sub-Saharan African populations at highest risk of acquiring malaria: approximately 90% of deaths are estimated to occur in the World Health Organisation (WHO) African Region. The number of global cases of malaria has decreased from an estimated 262 million in 2000 to an estimated 214 million cases in 2015. Of the 106 countries with ongoing malaria transmission, 33 have achieved significant progress towards elimination, estimating fewer than 1000 malaria cases in 2015 compared with 13 countries in 2000 (3). The decrease in malaria cases, particularly in Africa, has been observed since concerted efforts have been made within the framework of the United Nations Millennium Development Goals (MDGs) and with the Roll Back Malaria (RBM) initiative. However, challenges to the success of current strategies to combat malaria (such as insecticide treated nets (ITNs), indoor residual spraying, and antimalarial drugs) include: the development of resistance of *Anopheles* mosquitoes to certain insecticides; the development of resistance of malaria parasites to chemotherapeutic agents (4); the absence of a gametocidal drug suitable for mass administration (5); and the risk of re-importation of malaria into geographic regions previously cleared of malaria using environmental elimination measures.

The RBM Partnership was launched in 1998 by the WHO, the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP) and the World Bank. A major goal of the RBM Partnership is to support the development of a vaccine against malaria as a key future strategy for reducing mortality from malaria. The development of an effective vaccine may indeed be necessary for the greater goal of global eradication of malaria (6). The recently updated Malaria Vaccine Technology Roadmap calls for the development of a vaccine against *P. falciparum* and *P. vivax* by 2030, that will have protective efficacy of at least 75% against clinical malaria, suitable for administration to appropriate at-risk groups and development of vaccines to reduce malaria transmission suitable for administration in mass campaigns (7).

1.2 *Plasmodium falciparum* malaria

The vast majority of cases of falciparum malaria occur in sub-Saharan Africa. There were an estimated 187 (132 – 259) million clinical cases of *P. falciparum* malaria in sub-Saharan Africa in 2015. It is estimated that 663 (542 – 753) million cases have been averted in Africa since the introduction of control measures in 2000, the most effective of which has been the widespread use of ITNs which is thought to account for 68 (62 – 73)% of the averted cases (8). Despite these improvements, malaria still remains the biggest killer of the parasitic diseases, with many challenges to the goal of eradication.

1.2.1 The lifecycle of *P. falciparum* malaria

The *P. falciparum* parasite is injected into the human host (when the female *Anopheles* mosquito takes a blood meal) in the form of sporozoites. The number of sporozoites injected varies widely, but has been estimated as an average of 15 in the case of falciparum malaria (9). Once injected, the majority of sporozoites make their way from the dermis through the bloodstream to the host's liver, although they may also be transported in the lymphatic system (10). In the liver the sporozoites invade hepatocytes and form liver schizonts. These are cells full of mitotically dividing parasites (2). This process occurs over 6 – 7 days, at the end of which

merosomes are budded from the cell and enter the hepatic sinusoids (11). When these merosomes eventually rupture, thousands of merozoite-stage parasites are released into the bloodstream, moving from the 'pre-erythrocytic' stage of the parasite life cycle to the 'erythrocytic' stage (Figure 1-1). Merozoites invade erythrocytes, where they develop into ring stages, trophozoites and then into schizonts. These rupture, releasing merozoites into the bloodstream to begin a new cycle. Unlike *P. vivax*, the *P. falciparum* parasite can invade erythrocytes of all ages, enabling it to achieve far higher levels of parasitaemia. Cycles of replication occur every 48 hours and are responsible for the clinical manifestations of malaria infection (11). Some trophozoites do not develop into schizonts, but instead develop into gametocytes, the sexual-stage of the *Plasmodium* parasite. These develop over a period of 10 – 12 days and are essential for the ongoing transmission of malaria (12).

Inside the mosquito the sporogonic cycle takes place. The microgamete exflagellates and enters the macrogamete to form a zygote within the mosquito midgut. The zygotes become motile (ookinetes) and penetrate the midgut wall where they develop into oocysts. These rupture and release sporozoites which migrate to the mosquito salivary glands, ready to be injected when the mosquito takes another blood meal (13).

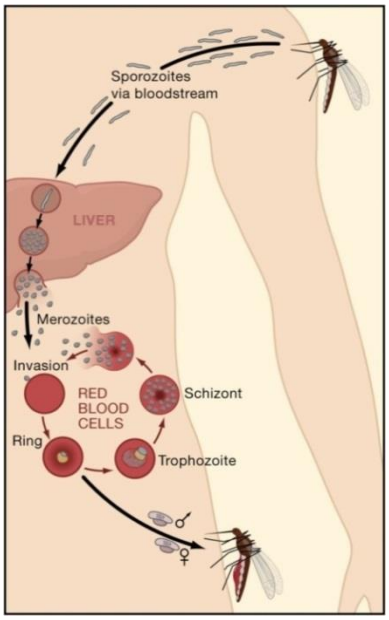


Figure 1-1: Life cycle of Plasmodium falciparum.

Reprinted from Cell, Volume 124, Cowman and Crabb, Invasion of red blood cells by malaria parasites, 755-66., Copyright (2006), with permission from Elsevier

Plasmodium merozoite invasion of erythrocytes is a complex process. Merozoites are released into the bloodstream when schizonts rupture and then make contact with red blood cells. The malaria parasite utilises a complex secretory system, and contains multiple secretory organelles, including micronemes and rhoptries. Merozoites are usually only free in the circulation for less than a minute, but may take several minutes to make a successful contact (14, 15). After an initial interaction with the erythrocyte, the merozoite reorients, so that its apical end faces the surface of the cell. A tight junction is formed between the merozoite and erythrocyte, and this then moves from the apical to the posterior end of the merozoite. During this movement ligands involved in the invasion process are removed, and the erythrocyte membrane subsequently encases the merozoite, creating a parasitophorous vacuole around the invading parasite along with the rest of the rhoptry contents (proteins and lipids). This separates the parasite from the host-cell cytoplasm, providing a hospitable environment for development (2, 16). The micronemal parasite ligands (erythrocyte binding antigens, EBAs, or Duffy-binding proteins, DBPs) and rhoptry ligands (reticulocyte binding-like proteins, RBLs or RBPs) are two families of antigens that are functionally conserved across *Plasmodium* species and are thought to be involved in the tight attachment step between the parasite and new host red blood cell. It appears that parasites need at least one member of each of these families to invade erythrocytes.

P. falciparum has multiple erythrocyte binding proteins, including EBA-175, EBA-140, EBA-181 and EBL-1. The *P. falciparum* RBL proteins are referred to as reticulocyte-binding protein homologues (PfRH), and there have been six identified currently – namely, PfRH1, PfRH2a, PfRH2b, PfRH3, PfRH4 and PfRH5 (2). These proteins contribute towards multiple invasion pathways for *P. falciparum*, and therefore redundancy, which may be advantageous for host immune invasion and contribute to the ability of the parasite to invade erythrocytes of all ages (2, 17, 18).

1.2.2 Clinical manifestations of *Plasmodium falciparum* malaria

The symptoms and pathology attributable to *P. falciparum* occur during the erythrocytic (blood) stage of infection; the pre-erythrocytic stage is asymptomatic. Children under five years of age are the most severely affected; accounting for 70% of deaths from this infection. Pregnant women are also at increased risk, and malaria causes indirect mortality from abortion and intrauterine growth retardation (19). The reason mortality decreases after the age of five is due to the acquisition of natural immunity in children living in endemic areas, which decreases the risk of death and severe disease. Those who are not repeatedly exposed to malaria infection lose their immunity and remain at risk. Repeated, fairly constant exposure occurs in populations with 'stable' transmission. In these populations the entomological inoculation rate is >10/year and natural immunity is acquired in early childhood. In areas where there is unstable transmission, with wide fluctuations in the intensity of malaria transmission (entomological inoculation rate <5/year), populations do not develop natural immunity and both children and adults are at risk of severe disease. These populations are at particular risk of malaria epidemics if there is a sudden increase in the inoculation rate, with high incidence of malaria in all age groups and high rates of severe malaria if those infected are not promptly and effectively treated (3).

The initial symptoms of malaria are non-specific, and often described as 'flu-like', with fever, headaches, myalgia and malaise. If untreated, malaria will progress to cause more severe disease. Children are particularly susceptible to severe anaemia and hypoglycaemia, whereas adults are more likely to develop pulmonary oedema, acute kidney injury and jaundice. Cerebral malaria (causing coma) and acidosis occur in all age groups (19). The features of severe malaria are shown in Table 1.1.

Clinical features of severe malaria	Laboratory and other findings
• Impaired consciousness (including unrousable coma) • Prostration, i.e. generalised weakness so that the patient is unable to sit, stand or walk without assistance • Multiple convulsions: more than two episodes within 24h • Deep breathing and respiratory distress (acidotic breathing) • Acute pulmonary oedema and acute respiratory distress syndrome • Circulatory collapse or shock, systolic blood pressure < 80mm Hg in adults and < 50mm Hg in children • Acute kidney injury • Clinical jaundice plus evidence of other vital organ dysfunction • Abnormal bleeding	• Hypoglycaemia (< 2.2mmol/l or < 40mg/dl) • Metabolic acidosis (plasma bicarbonate < 15mmol/l) • Severe normocytic anaemia (haemoglobin < 5g/dl, packed cell volume < 15% in children; <7g/dl, packed cell volume < 20% in adults) • Haemoglobinuria • Hyperlactataemia (lactate > 5mmol/l) • Renal impairment (serum creatinine > 265µmol/l) • Pulmonary oedema (radiological) *High parasitaemia is a risk for increased mortality from malaria but the relationship is complex and varies depending on transmission levels. In low-transmission areas, mortality from acute falciparum malaria begins to increase with a parasitaemia >2.5%, whereas in areas of higher transmission much higher parasite densities may be well tolerated. Parasitaemia > 20% is associated with a high risk in any epidemiological context.*

Table 1.1: Overview of severe malaria manifestations.

These may occur in isolation or, more commonly, in combination (20).

1.2.3 Treatment of *P. falciparum* malaria

The recommended treatment of falciparum malaria is based on the severity of disease. Uncomplicated cases should be treated with an oral Artemisinin combination therapy (ACT) such as artemether and lumefantrine or artesunate and amodiaquine, except in pregnant women in their first trimester. Quinine + clindamycin therapy is recommended in this group. Severe falciparum malaria should be treated with intravenous (IV) or intramuscular (IM) artesunate for at least 24 hours and until they can tolerate oral medication. Once a patient has received at least 24 hours of IV or IM artesunate, and is able to tolerate oral medication, treatment should be completed with 3 days of an ACT (21).

1.3 *Plasmodium vivax* malaria

Plasmodium vivax is the most common non-falciparum malaria. It is the most geographically widespread malaria because the parasite is able to survive in colder climates and at higher altitudes than *P. falciparum*. Infection with *P. vivax* accounted for approximately 50% of global malaria cases outside of Africa in 2015, the majority of which occurred in South-East Asia (3).

The global incidence of vivax malaria is very difficult to quantify as detection of *P. vivax* infections is more difficult, due to lower parasitaemias than are seen with *P. falciparum* malaria. The WHO estimate that there were 13.8 million cases globally in 2015 (3) but others have previously put the estimated figure much higher at 132 – 391 million cases/year and 2.6 billion people at risk of infection (13). Having long been thought of as a 'benign' malaria, it has more recently been recognised as a cause of significant morbidity with severe and even fatal manifestations (22). There were an estimated 1400 – 14,900 deaths due to *P. vivax* in 2015, the vast majority of which occurred outside sub-Saharan Africa (3).

Control of *P. vivax* is challenging, with some re-emergence seen in areas where eradication has previously been achieved. This is due to multiple factors including relapses, difficulty detecting asymptomatic infection, resistance to antimalarials and a lack of understanding of parasite biology (23). Recent calls for control and 'eradication' of malaria worldwide (24) have focused attention on this neglected disease and the need for development of an effective *P. vivax* vaccine to be used alongside current control methods (7, 25).

1.3.1 The *Plasmodium vivax* Lifecycle

The process of parasite invasion from the skin to the liver is the same in *P. vivax* as for *P. falciparum*. However, not all *P. vivax* parasites that enter the liver develop into tissue schizonts; some develop into hypnozoites, a dormant form of the parasite not seen in *P. falciparum* infections, that can cause relapse of disease weeks to months (or rarely even years) later (Figure 1-2).

Similar to *P. falciparum*, after around 7 days the tissue schizont ruptures, releasing merozoites into the bloodstream to begin the erythrocytic cycle. Unlike *P. falciparum*, which invades normocytes, *P. vivax* preferentially invades reticulocytes. This difference is thought to be due to the RBP proteins expressed by the vivax parasite. *P. vivax* was initially thought to express two reticulocyte binding proteins, PvRBP1 and PvRBP2 which bind specifically to reticulocytes (2). However, since the *P. vivax* genome sequence became available it has been demonstrated that there are in fact many more than two members in the *Pvrbp* gene family, with 8 genes predicted to be protein-coding (2 of these correspond to the genes encoding the originally discovered PvRBP1 and PvRBP2 proteins, *Pvrbp1a* and *Pvrbp2c*) (26). These proteins only form a committed attachment with reticulocytes and not normocytes (2, 16, 18, 27). This preference for reticulocytes limits the parasitaemia seen with *P. vivax* as reticulocytes only make up 1-2% of circulating erythrocytes (13). The equivalent family of proteins in *P. falciparum* is PfRH1-5.

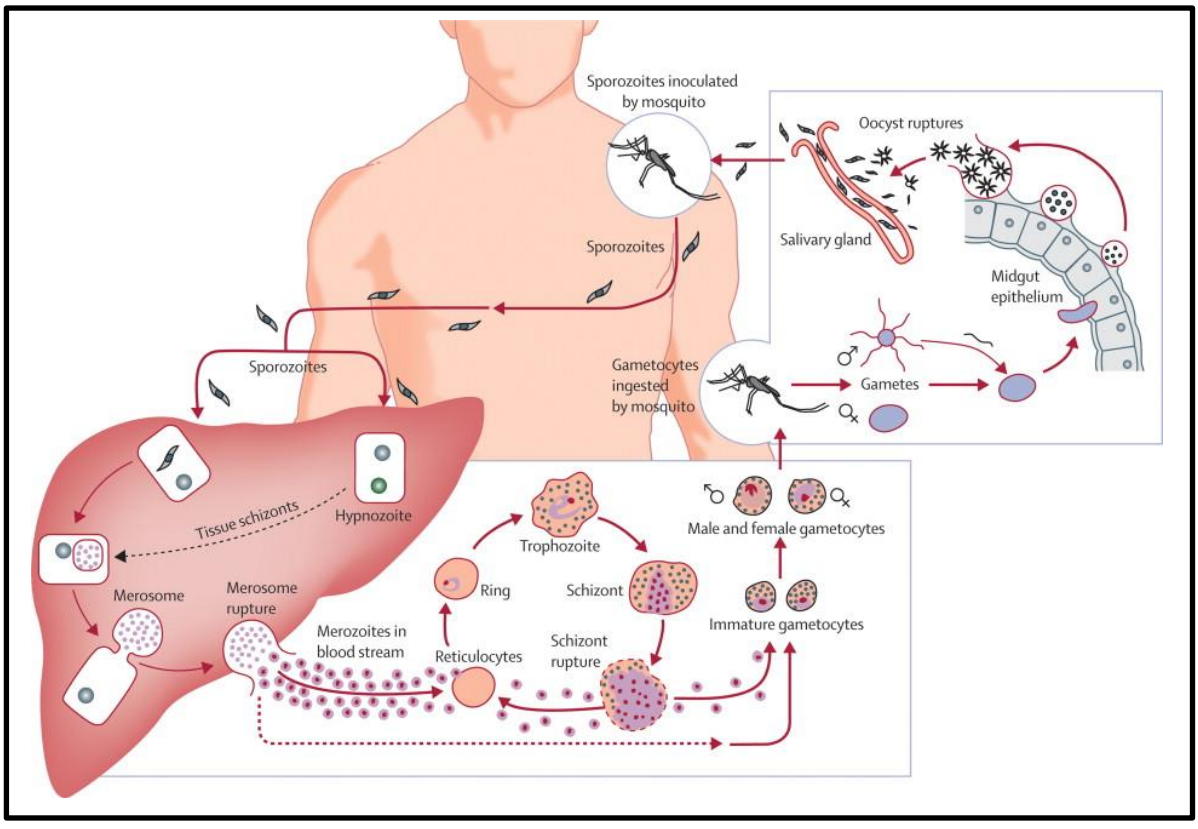


***Figure 1-2: Life-cycle of Plasmodium* vivax**

(Reprinted from The Lancet Infectious Diseases, Volume 9, Issue 9, Mueller et al., Key gaps in the knowledge of Plasmodium vivax, a neglected human malaria parasite., Pages 555 ‑566, Copyright (2009), with permission from Elsevier).

Invasion of erythrocytes requires the formation of a moving junction between the merozoite and erythrocyte, involving direct interaction between apical ligands of the Duffy-binding like (DBL) protein family and erythrocyte receptors. The DBL protein in *P. vivax* is the Duffy-binding protein (PvDBP) which is located in the micronemes and released to the merozoite surface, along with other ligands, when required for erythrocyte invasion. This delayed release is thought to be a mechanism for evading the host immune system (28, 29). The receptor-binding domain of *P. vivax* Duffy-binding protein (PvDBP) maps to a conserved cysteine-rich region, referred to as region II (PvDBP_RII), and an interaction between this ligand and the Duffy antigen receptor for chemokines (DARC) on erythrocytes is required for invasion. DARC is a chemokine receptor, also known as Fy glycoprotein or CD234. Individuals lacking the Duffy blood group antigen are resistant to blood-stage *P. vivax* infection. The high levels of Duffy blood group negativity in much of sub-Saharan Africa has essentially led to the disappearance of *P. vivax* from most of the continent, and has arisen independently in Papua New Guinea (16).

The formation of the sexual-stages and development within the mosquito in *P. vivax* is similar again to that of *P. falciparum*, but gametocytes are present earlier in infection, appearing as or before clinical symptoms develop (13). This enables transmission to occur before the onset of symptoms, and therefore before any treatment is given, which has important implications for control and elimination.

1.3.2 Clinical Manifestations of *P. vivax* infection

The majority of *P. vivax* infections occur in South and South-East Asia, with children under 5 years of age being the most at-risk group (30). In endemic areas repeated exposure leads to the development of immunity, and in these populations asymptomatic infection may occur. Natural immunity is a complex process, with humoral immunity playing an important role. Binding-inhibitory antibodies to PvDBP_RII have been associated with natural protection against *P. vivax* blood-stage infection (31). There are many other processes linked to infection

and disease from *P. vivax*, however, including strain-specific virulence (22) and differences in the Duffy blood-group antigen (Fy). The Duffy blood-group antigen has two distinct alleles which result from a single point mutation in the N terminal region, Fy^a and Fy^b. The ancestral allele is believed to be Fy^b, but the Fy^a allele looks to be advancing to fixation in many populations in Asia. PvDBP_RII binding has been shown to be significantly lower to Fy^a than Fy^b, with the Fy^b phenotype associated with a higher risk of infection and disease. This is not fully understood but may be due to differences in electrostatic charge between the positively charged PvDBP_RII and negatively charged N-terminal region of Fy or differences in Fy sulfation. PvDBP_RII-specific antibody titres are not significantly different between the phenotypes, suggesting that naturally-acquired immunity may be more effective in populations where Fy^a is the predominant allele (32).

1.3.2.1 Symptoms of P. vivax malaria

Features of *P. vivax* malaria include fever (which may be periodical), headache, myalgia, gastrointestinal disturbance, abdominal pain and cough. Symptoms alone cannot differentiate *P. vivax* malaria from *P. falciparum* malaria (33). Most infections are uncomplicated but severe disease can occur. Disease severity is assessed using the severity scoring developed by the WHO for falciparum malaria (21) as a severity score specific for vivax malaria has not been developed but the severe manifestations are similar.

1.3.2.2 Severe Manifestations and Mortality

Having long been considered a 'benign' malaria, there is increasing evidence that *P. vivax* causes significant morbidity and some mortality. Splenic rupture has long been accepted as a complication of vivax malaria (34) but more recently severe manifestations and complications have been recognised. Children aged 0-5 years appear to be at highest risk in endemic countries, with more cases of severe malaria associated with *P. vivax* mono-infection than *P. falciparum* mono-infection reported in this age group (35). The most common severe manifestation is anaemia, but other severe manifestations including coma, acute respiratory

distress syndrome (ARDS), abnormal bleeding, hepatic dysfunction and renal dysfunction are reported (22, 35-38).

Severe malarial anaemia is most profound in young children in endemic regions and the mechanisms for this are not fully understood. In Papua New Guinea severe anaemia (haemoglobin <5g/dL) has been reported more commonly in children admitted with severe vivax malaria than severe falciparum malaria (39). In the study by Kochar *et al.* in Bikaner, India, severe anaemia was seen in 75% of children presenting with severe vivax malaria compared with 81% of those presenting with severe falciparum malaria. However, in the 0-5 years age group, 75% of those severe vivax had severe anaemia whereas this was only the case for 26% of those with severe falciparum malaria. Thrombocytopenia is also commonly seen and can be severe, with the need for platelet transfusion reported (35).

Respiratory distress has been reported as more commonly occurring in young children (aged less than 2 years) with vivax malaria than falciparum (35, 38), and there have been several reports of ARDS secondary to vivax, including fatal cases (22, 33, 36, 40, 41).

Cerebral malaria (including status epilepticus and coma) is reported in several studies (37, 42), although it occurs much less frequently than in falciparum malaria (33) and has a better outcome in terms of mortality.

Mortality from vivax malaria was originally recognised decades ago when *P. vivax* was used in malaria therapy for the treatment of neurosyphilis. It was recognised that certain strains were more virulent and had higher mortality rates in these patients. S.F. Kitchen's description of vivax malaria as 'benign' in the Boyd malariology text in 1949, which described death due to vivax alone as rare, had a substantial impact on the field. The reduction in vivax malaria research in the 1950s, which continued until very recently, meant there was little information to contradict this belief (22). Several studies have now reported vivax-associated mortality, with similar case fatality rates in those presenting to hospital with severe vivax malaria as is seen with severe falciparum (35, 38, 43). An autopsy series carried out in Manaus concluded

that in 13 of the 17 patients studied, *P. vivax* was the cause of death or a significant contribution to decompensation of a pre-existing condition, leading to death (44).

Differences in presentation and in the populations most affected by vivax is likely to be due to several confounding factors including comorbidities (for example, malnutrition), strain-specific virulence, host genetic factors (for example Fy^b blood group antigen), parasite resistance to antimalarials and differences in the collection and reporting of data.

1.4.3 Relapse

Unlike *P. falciparum*, *P. vivax* has a dormant liver-stage known as a hypnozoite which enables it to relapse and cause multiple episodes of disease following a single infection (45, 46). Relapse is a major cause of morbidity, particularly in young children, and contributes significantly to on-going vivax transmission. Relapse periodicity is very variable, occurring within weeks (particularly in 'tropical zones') to several months later in temperate zones. Not all vivax infections relapse; relapse is seen in 20-80% of people following primary infection (46, 47).

Severe malarial anaemia related to vivax infection may be worsened by relapse. There is a significant drop in haemoglobin seen with acute vivax infection (48) but relapses occurring before the erythrocyte levels have had chance to return to normal can lead to severe, and even life-threatening anaemia (49).

1.4.4 Treatment of *P. vivax* malaria

The recommended treatment for uncomplicated vivax malaria is chloroquine, except in areas where chloroquine-resistant vivax has been identified, and in this case an artemisinin-based combination therapy (ACT) should be used. For severe vivax malaria, as for severe falciparum malaria, treatment should be with an injectable artesunate followed by a full course of ACT (21). These drugs are active against the blood-stages of vivax infection but not against the hypnozoite.

Treatment to avoid relapse is limited as primaquine is currently the only licensed drug that is active against the hypnozoite. Primaquine is not 100% effective and causes haemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, so is by no means an ideal drug (46). G6PD deficiency is widespread, with an estimated 350 million people thought to be affected, many of these living in vivax-endemic regions (50). Recently it has also been shown that different genotypes of the cytochrome P450 allele CYP2D6 lead to variations in the ability for individuals to metabolise primaquine, with those heterozygous or homozygous for the null allele demonstrating reduced metabolism and therefore risk of treatment failure and vivax relapse (51). This has obvious implications for the efficacy of primaquine as a drug for radical cure of hypnozoites and therefore as a control/elimination mechanism.

1.5 Naturally-acquired immunity to malaria

Individuals frequently exposed to malaria do develop natural immunity after a period of time. It is for this reason that in endemic settings the mortality rates from malaria are lower after the age of five as, for those who have survived repeated infection in early childhood, a level of immunity is achieved and they are protected from severe disease and high parasitaemia (52). Immunity may also be seen in the form of premunition, where individuals maintain a low-grade parasitaemia, which is usually asymptomatic, but provides protection against new infections (53). The rate of acquisition of immunity is dependent on the level of exposure, i.e. in high intensity transmission settings immunity is acquired at an earlier age than in low or medium transmission settings. The dominant presentation of severe malaria also differs depending on the age of the individual, for example, severe malarial anaemia is more likely in young infants whereas the proportion of severe cases manifest by cerebral malaria increases with age, indicating that aged-related physiological changes as well as the intensity of exposure contribute to the dominant clinical syndromes associated with malaria infection (52). Immunity to malaria is compromised in pregnancy (especially in the first pregnancy) and in individuals whose frequent exposure to infection ceases (for example, by moving to a non-endemic area) (53). This has important implications for interventions which reduce malaria

exposure as there is the possibility of catastrophic rebound if malaria is reintroduced into a population who have lost their naturally-acquired immunity, as was seen in Madagascar in the late 1980s following the malaria control campaigns in the 1950s and 1960s which successfully removed the *Anopheles funestus* vector from the central highlands. The vector was gradually reintroduced and following population movement there were huge outbreaks of malaria leading to an estimated 40,000 deaths in a population which was no longer immune (54).

The mechanisms of natural immunity are not fully understood. Presumed mechanisms of adaptive immunity include antibody responses against the pre-erythrocytic and erythrocytic stages to block hepatocyte invasion by sporozoites, block erythrocyte invasion by merozoites, prevent binding of infected erythrocytes to the vascular endothelium and contribute to inhibition of the development of the sexual-stages. T cells are also thought to play a role in inhibiting development of the parasite during the liver stage ($CD8^+$) and in activating macrophages ($CD4^+$) to phagocytose blood-stage parasites. More recently, the role of the innate immune system has been more closely studied and studies both in malaria naïve individuals exposed to *P. falciparum* malaria in an experimental infection, as well as data from populations exposed to repeated malaria infections indicate that innate immune mechanisms are triggered by malaria infection and serve to limit the maximum parasite density (55). Although multifactorial, it is widely believed that naturally-acquired immunity is primarily directed towards blood-stage parasites, and in *P. vivax* these arise from both primary infection and relapses (56).

Natural immunity to *P. vivax* malaria is acquired more rapidly than to *P. falciparum* but is still seen to the highest degree in adults in holoendemic areas. The peak of *P. vivax* disease occurs in young infants at 1.0 – 1.9 years old, whereas for *P. falciparum* it peaks at 2.0 – 3.9 years of age and the proportion of *P. vivax* infections presenting with severe symptoms rapidly declines with age; so that by 1 year of age there is a significantly lower incidence of severe illness

attributable to *P. vivax* compared with *P. falciparum* in children living in areas endemic for both infections (56).

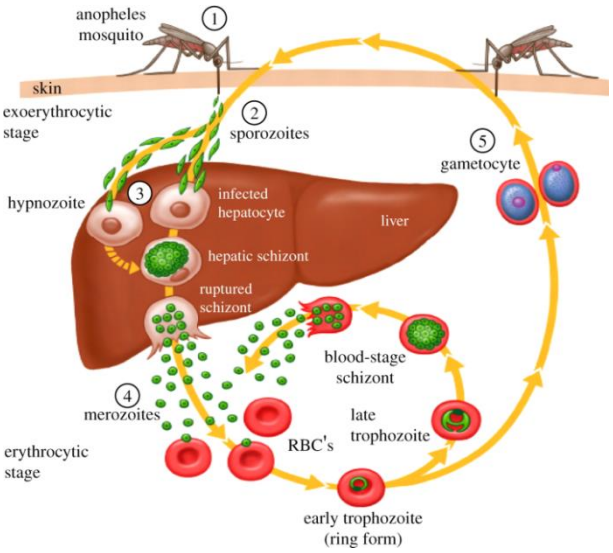
1.6 Vaccine-induced immunity to malaria

There is currently no licensed vaccine for malaria, and attempts at developing a vaccine have been met with many challenges. Only one malaria vaccine candidate, RTS,S, based on the *P. falciparum* circumsporozoite (CS) protein (the major component of the sporozoite coat) has reached Phase III efficacy trials to date (57).

Attempts to develop an effective malaria vaccine have been ongoing for decades. Initial experiments in the 1940s in birds exposed to repeated injections of large numbers of inactivated sporozoites of the avian malaria *P. gallinaceum* demonstrated partial efficacy against infection with the homologous parasite delivered by mosquito bite (58, 59). In the 1960s immunisation studies in mice with X-irradiated sporozoites of the rodent parasite *P. berghei* demonstrated that it was possible to achieve sterile protection (i.e. no patent parasitaemia as detected by blood film) in some immunised mice (60), supporting the theory that a malaria vaccine was possible. Studies by Clyde *et al* in Maryland, USA in the 1970s demonstrated that protective immunity could be induced in humans following exposure to large numbers of X-irradiated sporozoites, both for *P. falciparum* and *P. vivax*. However, the immunity was species-specific and short lived (lasting between 3 – 5 months), and required exposure to hundreds of mosquito bites (61-64).

Since the 1980s malaria vaccine development has focused more on subunit vaccines than whole sporozoites vaccination, although more recently this has been re-examined. The discovery that cellular immunity had an important role in protection against the liver-stage of the parasite led to the development of different vaccination techniques from protein-in-adjuvant preparations to DNA vaccines and later viral vectors, which induce strong T cell responses (65). Most subunit malaria vaccines target a specific stage of the malaria life cycle- either the pre-erythrocytic (sporozoites or liver-stage), erythrocytic or sexual-stages as shown

in Figure 1-3. The whole sporozoite vaccination techniques for *P. falciparum* that have been re-examined are led by two main approaches. The first of these is immunising malaria naïve volunteers by exposing them to the bites of infected mosquitoes whilst preventing the development of clinical disease by simultaneous chloroquine administration, as demonstrated by Sauerwein's group in Nijmegan (66). The immunity induced by this approach has been shown to remain effective over 2 years after immunisation in some volunteers (67). The second approach is the use of cryopreserved sporozoites, as developed by Sanaria®, which allow administration without the need for mosquitoes. These have been administered as aseptic, radiation-attenuated, metabolically active, purified, cryopreserved sporozoites (PfSPZ), which showed protective efficacy when administered intravenously (68). Administration of viable sporozoites with simultaneous chloroquine prophylaxis (PfSPZ-CVac) has been claimed to have high-level protective efficacy with fewer sporozoites required than PfSPZ (69), but this is not the case if the sporozoites are inoculated intradermally (70).



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Life cycle of the malaria parasite illustrating the various stages that are relevant to vaccine design. These are (1) the anopheline mosquito vector, used in experimental protocols to immunize with irradiated sporozoites administered by mosquito bite; (2) the sporozoite, the target of several vaccines, including RTS,S; (3) the liver-stage, usually targeted by vectored vaccines; (4) the blood-stage, usually targeted by protein in adjuvant vaccine candidates. Merozoite antigens have been most often included in blood-stage vaccines; (5) the gametocyte which along with the ookinete, formed after fertilization in the mosquito midgut, is the source of parasite antigens used in sexual-stage transmission-blocking vaccines. Pre-erythrocytic vaccines, which target the sporozoite and the liver-stage parasite are intended to prevent infection as well as disease while blood-stage vaccines are intended to prevent clinical illness and death.

Figure 1-3: Plasmodium life cycle stages relevant to vaccine design

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1.6.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines target sporozoite- and liver-stage parasites. The most widely investigated sporozoite target antigen is the CS protein, which has been developed by GlaxoSmithKline (GSK) as the RTS,S vaccine in which the CS protein is fused to hepatitis B surface antigen (HBsAg) expressed together with unfused HBsAg (71). After expression, the hybrid proteins form particles spontaneously, similar to naturally-occurring HBsAg particles (72). When given with GSK's potent Adjuvant System AS01, RTS,S is able to protect healthy adult volunteers against malaria infection with a vaccine efficacy of around 50% (73). This vaccine has been administered to thousands of children in endemic countries in a Phase III trial, and demonstrated only moderate efficacy (57). The thrombospondin-related adhesion protein (TRAP) is perhaps the most studied liver-stage antigen. This has been administered to

healthy volunteers in the viral vectored heterologous prime-boost vaccination regime using the chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) ME-TRAP (TRAP combined with a multiple epitope string) with a vaccine efficacy of 21% against sporozoite controlled human malaria infection (74). It has also been administered to adults and children in endemic settings with no safety concerns and some early efficacy noted in a trial in Kenyan adults who were monitored for 8 weeks after vaccination (75).

The only vivax pre-erythrocytic antigen to reach clinical trials has been PvCSP, an antigen derived from the *P. vivax* circumsporozoite protein. Differences in the amino acid composition of the circumsporozoite protein divide this *P. vivax* antigen into two subtypes; the dominant VK210 form and the variant VK247 (76). This antigen has been used in several trials, including an efficacy trial in which a chimeric protein containing repeat sequences from both subtypes was given as a protein-in-adjuvant formulation with the AS01 adjuvant (VMP001/AS01B). This vaccine demonstrated no sterile efficacy, but a significant delay in development of patent parasitaemia (thick blood film positivity) in vaccinees compared with controls (77).

1.6.2 Erythrocytic vaccines (blood-stage)

There has been less progress in the blood-stage malaria vaccine field than in pre-erythrocytic vaccines (65). There are many proteins involved in the invasion of erythrocytes by merozoites, and the process is not fully understood. The merozoite is committed to invasion following high affinity binding interactions between erythrocyte membrane proteins and merozoite adhesins such as the the erythrocyte-binding ligand (EBL) and PfRH proteins. In *P. falciparum* infection, invasion is classically divided into two main pathways: sialic acid (SA)-dependent and SA-independent.

The antigens involved in SA-dependent invasion include EBA-175, which binds to the glycophorin A (GPA) receptor; EBL-1, which binds to the glycophorin B receptor; EBA-140, which binds to the glycophorin C receptor as well as EBA-181 and PfRH1, the receptors for which have not yet been identified (78). EBA-175 has been assessed in Phase I trials as a

protein-in-adjuvant formulation alone and with the blood-stage antigen merozoite surface protein 1 (MSP1), but the vaccine has not progressed to Phase II trials to date (79, 80).

SA-independent invasion pathway antigens include PfRH4, which binds to complement receptor 1; PfRH5, which binds to basigin; MTRAP (found on all motile forms of the malaria parasite, including merozoites, sporozoites and ookinetes), which binds to semaphorin 7a; MSP1, which binds to band 3 and GPA (which, together, represent the most abundantly expressed protein within the plasma membrane of the erythrocyte) and, finally, apical membrane antigen 1 (AMA1), which binds to the merozoite rhoptry neck (RON) complex protein RON2 after it has been inserted into the erythrocyte membrane (78). The most widely studied antigens as blood-stage vaccine candidates are MSP1 and AMA1, both of which are considered vital for merozoite attachment to the erythrocyte and subsequent invasion, and both of which are antibody targets following infection with *P. falciparum* (81).

MSP1 has been developed as a vaccine candidate in protein-in-adjuvant formulations as well as in viral vectors (82-84). None of the vaccines have demonstrated efficacy in human trials, but studies with MSP1 vaccines in *Aotus* monkeys have shown that sterile protection can be achieved after vaccination (85, 86), however, very high antibody titres are required and protection is strain-specific (86). This has significant implications for vaccine development as the adjuvants available for human use are not able to induce the same level of antibody response as adjuvants (such as Freunds) used in preclinical studies. Furthermore, the polymorphism of this antigen also has implications, and presumably leads to the strain-specificity of vaccine efficacy. In one study site in Mali there were 14 haplotypes of the MSP1 fragment involved in erythrocyte invasion, $MSP1_{19}$, found in 1363 malaria infections, indicating that even in one geographical site multiple polymorphisms are present (87).

P. falciparum AMA1 is a precursor protein of 83 kDa (88) which is synthesised in the late stages of schizont development, before the N terminus is cleaved to give a 66 kDa form (89, 90). Prior to schizont rupture the 83 kDa AMA1 is located in the apical end of the merozoite in the

micronemes, whereas following schizont rupture AMA1 is localised both apically (in the 83 kDa form) and on the merozoite surface (in the 66 kDa form) (89). This surface protein binds to the rhoptry neck parasite protein, PfRON2, inserted by the parasite into the host red cell membrane, thus forming the tight / moving junction. At the merozoite surface further proteolytic cleavage occurs, producing two soluble fragments: a 44-kDa molecule and a 48-kDa molecule (91). Antibodies to AMA1 have been shown to prevent processing and circum-merozoite redistribution and shedding of the protein. Without AMA1 undergoing these processes red blood cell invasion is inhibited (92). Vaccines against AMA1 have been developed using both protein-in-adjuvant and viral vector approaches. A study in Oxford with the viral vectored vaccines ChAd63/MVA AMA1 administered alone or with ChAd63/MVA MSP1 demonstrated low-level efficacy, but this was likely due to a pre-erythrocytic effect rather than efficacy during the blood-stage of infection (83). The leading recombinant protein vaccine, known as FMP2.1, was developed by the Walter Reed Army Institute of Research (WRAIR) and based upon the 3D7 clone sequence of AMA1 (93). This vaccine was formulated in the Adjuvant System AS01 or AS02 from GlaxoSmithKline (GSK) and had previously been tested in Phase I/IIa trials in the USA (94, 95) and in field trials in Malian adults and children (96, 97). The Phase IIb field trial in 400 Malian children using the FMP2.1/AS02 formulation was reported to show 64.3% efficacy (hazard ratio 0.36, 95% CI 0.08-0.86, *P*=0.03) in a pre-defined secondary analysis against clinical malaria with 3D7-type parasites (defined by eight immunologically important AMA1 polymorphisms in the cluster 1 loop of domain I), although the number of cases meeting this definition was small (97, 98). This allele-specific efficacy, seen in the first malaria season, did not extend into the second season of follow-up (99).

P. falciparum reticulocyte-binding protein homologue 5 is expressed in merozoites and localises to the apical complex. It is not found in *P. vivax* parasites but is expressed in all *P. falciparum* strains tested so far. Two reports have demonstrated that the gene is essential for parasite survival (i.e. cannot be knocked out) (100, 101). PfRH5 binds to its receptor basigin, the Ok blood group antigen, in a complex with two other *P. falciparum* proteins, PfRh5-

interacting protein (PfRipr) and cysteine-rich protective antigen (CyRPA). During invasion, the three proteins co-localise and the junction formed between the merozoite and red blood cell. Binding of the complex to basigin triggers the release of Ca^{2+} and formation of the tight junction (102). This interaction is essential for red blood cell invasion (103). No vaccines against the RH5 protein have previously entered clinical trials, but pre-clinical data have shown very promising results with high-level efficacy induced by PfRH5 vaccination against heterologous strain challenge in an *in vivo Aotus* monkey *P. falciparum* challenge model (104).

No blood-stage *P. vivax* vaccines have previously reached clinical trial. The most promising antigen currently in clinical development is the Duffy-binding protein. As described above, interaction between DARC on the surface of red blood cells and region II of the Duffy-binding protein (PvDBP_RII) on the vivax parasite is essential for merozoite invasion of erythrocytes. Antibodies induced by a vaccine to this protein should therefore inhibit invasion. This is supported by i) the demonstration of naturally-acquired antibodies to this antigen in individuals living in vivax-endemic regions which can partially inhibit merozoite invasion in short term *in vitro* culture (105) and ii) the association of natural responses with protection against blood-stage *P. vivax* infection (31). The serological response to PvDBP_RII increases with age, which suggests a boosting effect through recurrent infections (106). A recombinant PvDBP_RII protein from the Salvador I reference strain has been expressed in *E. coli* and used to immunise *Aotus* monkeys with Freund's adjuvant or Montanide ISA 720. This demonstrated the induction of high-titre specific antibodies and partial protection against challenge with Salvador I strain *P. vivax* blood-stage parasites, with the demonstration of longer pre-patency periods (i.e. before thick blood film positivity) as well as lower parasitaemias in the monkeys immunised with recombinant protein in Freund's adjuvant compared with controls (107).

There are conflicting arguments regarding the issue of polymorphism. The fact that PvDBP_RII is under positive selection has been demonstrated by the fact that most of the polymorphic residues in PvDBP are found in region II (28). This effect of immune pressure is also supported

by a higher frequency of non-synonymous mutations compared to synonymous mutations, suggesting that in populations tested PvDBP is under strong positive selection, and a decline in the frequency and diversity of PvDBP_RII haplotypes in blood samples from individuals living in an endemic area with increasing age. This was assumed to be due to the development of partial immunity to multiple vivax strains in adults, meaning that parasitaemias following infections are lower and more rapidly cleared compared with children who have not developed any natural immunity. However, this finding may also be due to difficulties in detecting variants in blood samples from adults in endemic areas due to the low parasitaemias (108). Polymorphism in PvDBP is likely to be a challenge for vaccine development. A study by Chootong *et al* in Thailand demonstrated that naturally-acquired PvDBP_RII antibodies do inhibit binding between PvDBP_RII and Duffy-positive erythrocytes, but inhibition activity did not correlate with the level of anti-PvDBP_RII responses seen (i.e. some 'low responders' showed high levels of inhibition whilst 'high responders' showed low levels). One explanation for this is a strain-specific response (109). However, it is claimed that conservation of the contact residues that form the DARC-recognition site would mean that vaccine-induced antibodies would provide protection across diverse strains (16). A study using monoclonal antibodies generated against PvBDP_RII examined their reactivity to a panel of seven allelic variants and showed that some monoclonal antibodies were broadly inhibitory when functionally assessed in an erythrocyte binding assay, whereas others were not. This demonstrates that a high titre of antibody alone may not be sufficient for protection, but the optimisation of antibody specificity is vital (28). Recently the structure of the first inhibitory monoclonal antibody (mAb) bound to PvDBP_RII has been identified, along with two other inhibitory mAbs against this antigen. These were found to recognise broadly conserved epitopes in the DBP sequence and will likely be effective against multiple strains. Using these structures, DBP vaccines can be designed to confer strain-transcending protection (110). Interestingly, the use of adjuvants which are Toll-like receptor (TLR) 4 agonists (e.g. AS02) has been shown to increase the breadth of the antibody response to PvDBP_RII and improve

antigen neutralisation when the recombinant protein is formulated with these (111), indicating that correct adjuvant choice for a blood-stage protein vaccine is also very important.

The three blood-stage antigens used in the vaccines described in the trials in this thesis are AMA1, RH5 and PvDBP. Unlike AMA1, which does not have a known host receptor, but instead binds to the RON complex which is deposited in the erythrocyte by the merozoite (102), the receptors for RH5 (basigin) and PvDBP (DARC) are found on the surfaces of many different cells throughout the body. This potentially has implications for these vaccines as the antigen could potentially bind to cells expressing these receptors. Furthermore, other molecules also bind these receptors, for example, IL-8 and melanoma growth stimulating activity (MGSA) chemokines which bind DARC. The binding of these chemokines can block the binding of Duffy positive erythrocytes to *P. vivax* DBL domains, demonstrating that these binding sites overlap with those of the parasite but also that these chemokines bind competitively (16). Basigin also has a large number of molecules which interact with it in its various isoforms in different sites in the body (112). Notably, the binding of RH5 to basigin is a low affinity interaction, and easily blocked with a low concentration of anti-basigin monoclonal antibodies (103), suggesting RH5 antigen would unlikely outcompete natural ligands if RH5 protein is administered as a vaccine. The competitive binding of other molecules to both of these receptors would perhaps also reduce the potential for binding of vaccine antigen. However, the main question arises as to what extent vaccine antigen would bind to these receptors in the context of immunisation. In the case of vaccines described in this thesis, viral vectored vaccines encoding the PvDBP and RH5 antigens are administered intramuscularly and it is therefore unlikely that antigen will be distributed widely beyond the draining lymph nodes; as has been demonstrated in mouse models comparing vaccination routes with MVA vaccines where intramuscular injection was associated with highly localised priming of the immune response (113). Most importantly, the immune responses induced by the vaccinations will be targeting parasite antigens rather than human receptors, so there should not be a risk in safety in inducing these immune responses,

whereas that would obviously not be the case if the responses were directed at widely distributed receptors.

1.6.3 Transmission-blocking vaccines (sexual/mosquito-stages)

The final class of malaria vaccines in development are the transmission-blocking vaccines (TBV). These aim to prevent onward transmission of the disease in endemic communities by blocking development of the parasite within the mosquito vector. Low levels of polymorphisms are expected in antigens expressed in the sexual- or mosquito-stages given they are under low human immune pressure, if any. The aim of a transmission-blocking vaccine is in control and elimination of malaria, particularly in areas of low endemicity or epidemics (23, 114). This type of vaccine potentially has issues regarding acceptability as it is an 'altruistic' vaccine. It does not offer direct protection to the individual, but if a community is vaccinated with a successful TBV the rates of malaria for that community will fall. These vaccines could also be used in combination with a pre-erythrocytic or blood-stage vaccine candidate to afford protection to the vaccinated individual as well as preventing transmission (115).

Two transmission-blocking candidate vaccine antigens have reached Phase I clinical trials: P25 (including the *P. falciparum* antigen Pfs25 and the equivalent *P. vivax* antigen, Pvs25) and Pfs230. A clinical study of a Pfs230 vaccine is currently being conducted in Mali but no results are yet available (NCT02334462). P25 is the most developed candidate in the clinical pipeline and the only TBV for which trial results have been published.

P25 proteins are present on the ookinete surface of all *Plasmodium* species and are important for survival of the parasite within the mosquito midgut. P25 is expressed on the surface of Plasmodium gametes from the initiation of emergence through to ookinete invasion of the mosquito mid-gut (116). The flat, triangular P25 molecule forms a protective interlocking sheet on the surface of the ookinete, along with the Pfs28 protein. Pre-clinical studies have

demonstrated that vaccination with both Pfs25 and Pvs25 can produce transmission-blocking antibodies (117).

Both Pfs25 and Pvs25 vaccines have reached Phase I clinical trials. Both antigens were given with the adjuvant Montanide ISA 51 but the trial had to be stopped early due to unexpected reactogenicity, which was thought to be due to the specific antigen-adjuvant combination (118). Another vivax transmission-blocking vaccine, Pvs25H adsorbed onto the adjuvant Alhydrogel, also reached Phase I clinical trials. Transmission-blocking activity was assessed by the ability of vaccine-induced antibodies to inhibit oocyst development in a mosquito membrane feeding assay using *P. vivax* infected blood from 12 patients. A positive correlation between antibody level (as measured by enzyme-linked immunosorbent assay [ELISA]) and the number of infected mosquitos was seen, with the five sera with the highest antibody levels at day 194 giving a 20-30% reduction in number of infected mosquitos, however transmission-blocking activity was insufficient for a practical malaria transmission-blocking vaccine (114).

A Phase Ia study of the Pfs25 antigen fused to a molecular adjuvant IMX313 (a chimeric version of the oligomerisation domain from chicken complement inhibitor C4bp) expressed in the ChAd63 and MVA viral vectors is currently ongoing in Oxford (NCT02532049). This follows promising pre-clinical data showing that vaccination with the Pfs25-IMX313 fusion led to a 10-fold improvement in antibody immunogenicity as well as significantly better transmission-blocking activity in a membrane feeding assay (119). Whether this improvement in immunogenicity (and efficacy) will be seen following vaccination in humans remains to be seen.

1.7 Immune responses to vaccine adjuvants

Adjuvants have long been used to increase the adaptive immune response to vaccination, particularly for subunit vaccines. Access to effective adjuvants has historically been difficult and therefore, one of the main advantages of the viral vectors used in two of the clinical trials described in this thesis was that, because they are live viruses, an adjuvant is not required.

One of the trials described in this thesis (VAC054; Chapter four) used an adjuvant, namely the Adjuvant System AS01, developed by GSK, with a recombinant protein antigen.

The development of adjuvants has progressed from just aiming to increase the adaptive immune response to vaccination to also guide the type of response, thereby producing the most effective response for a particular pathogen. This has meant developing adjuvants to focus the response produced, e.g. inducing a T helper 1 (Th1) skewed response versus Th2 cells; increasing memory cell generation; inducing a more rapid initial response and altering the breadth, affinity or specificity of the response (120). Developing improved adjuvants, especially for more challenging pathogens such as malaria, has meant recognising that a broader response than merely inducing antibodies is likely to be required, including cell mediated immunity with activated $CD4^+$ and $CD8^+$ T cells. Cytokines secreted by $CD4^+$ T cells are able to activate macrophages as well as assisting the development of high-affinity antibodies and memory B cells by antibody-producing cells (121). Viral vectors are particularly effective at inducing a T cell response, and due to their direct infection of cells and the endogenous MHC class I antigen presentation pathway, are able to induce $CD8^+$ T cell immunity directly. Protein vaccines however are exogenous and therefore require formulation with an adjuvant which facilitates entry into the MHC class I processing pathway, triggers activation of dendritic cells and the production of interferon (120).

Adjuvants interact with the innate immune system initially, and through mimicking some aspects of the natural response to pathogens, can be used to tailor the adaptive response in order to maximise vaccine efficacy (121). The AS01 adjuvant used in VAC054 is liposome-based and contains 3-0-desacyl-4’ monophosphoryl lipid A (MPL), derived from the cell wall lipopolysaccharide of the Gram negative *Salmonella minnesota* R595 strain and QS21, a triterpene glycoside purified from the bark of the South American tree *Quillaja saponaria* (122).

MPL stimulates the activation of the innate immune system via Toll-like receptor 4 (TLR4), directly activating antigen-presenting cells (APCs) which express this receptor. This occurs through activation of NF-κB transcriptional activity and subsequent proinflammatory cytokine expression (e.g. TNF-α and IL-6) (123). The immune response to MPL has been found to be skewed towards a Th1 profile (required for protection against intracellular pathogens), with the promotion of IFN-γ production by Ag-specific CD4+ T cells (123, 124). Use of MPL in vaccines has been shown to typically boost serum antibody titres 10 to 20-fold compared with vaccine alone (125). QS21 stimulates $CD8^+$ T cells and promotes antigen-specific antibody production, although the signalling pathways are not fully understood (121).

Studies in mice have demonstrated that following intramuscular injection of AS01 cytokine levels increase in the muscle within 3 hours, suggesting production by local cells, including stromal cells. The antigen and adjuvant were cleared rapidly from the muscle, excluding a local depot effect that has been demonstrated with other adjuvants. The production of cytokines leads to the recruitment of neutrophils and monocytes to the muscles. Simultaneously, adjuvant and antigen are rapidly transported to the draining lymph nodes. There is an influx of neutrophils, monocytes, dendritic cells and T cell populations into the lymph node (121). The immune responses at the injection site following vaccination with the AS01 adjuvant are demonstrated in Figure 1-4. During migration to draining lymph nodes, dendritic cells recruited at the vaccination site present antigens and mature into efficient APCs. Within the lymph node, AS01 and antigen may activate resident dendritic cells and the activated dendritic cells go on to activate T cells which then differentiate into effector populations and later, into memory cells. $CD4^+$ T cells secrete cytokines which stimulate antigen-specific B cells to divide into antibody-secreting cells and memory B cells. Memory cells and effector cells will ultimately enter the bloodstream from the lymph nodes (121).

The differences between AS01 and other adjuvants include: a lack of depot effect; increased populations of activated APCs and a higher antigen-specific response with a synergistic effect

of MPL and QS21. The response is also directed towards a predominantly IFN-driven pathway, which is thought to contribute towards a stronger cellular immune response and promote antibody isotype switching (121).

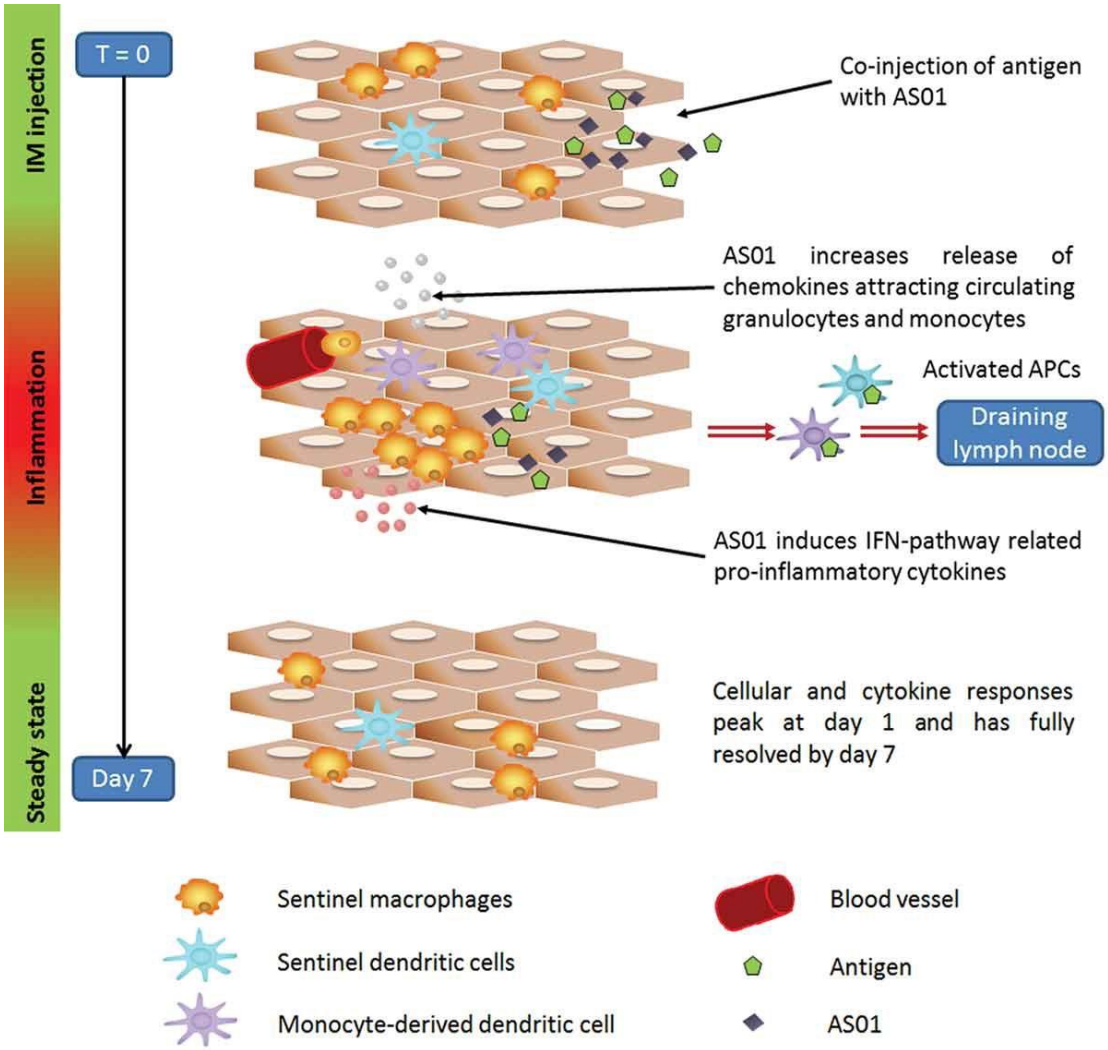

Figure 1-4: Immune responses following intramuscular vaccination with AS01-adjuvanted vaccine at the injection site.

The immune response to an AS01-adjuvanted vaccine injected into a muscle involves multiple cellular interactions. The adjuvant effect requires the spatial and temporal co-localization of AS01 and antigen. The two immune-stimulatory components of AS01, MPL and QS-21, act synergistically together to stimulate the release of immune mediators by innate cells allowing an enhanced neutrophil and monocyte recruitment at the injection site. The AS01-adjuvanted vaccine induces an early and transient activation of the innate immune response. Cellular and cytokine responses peak at day 1 and are fully resolved by day 7 (121).

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1.8 Methods for investigating immune responses to vaccination

Vaccines aim to induce a targeted immune response against the pathogen of interest. For most vaccines this equates to inducing a specific antibody response against the antigen or antigens in the vaccine. Antibodies are a particularly important immune mechanism for extracellular pathogens, but intracellular pathogens are better targeted by activated T cells. The most successful malaria vaccine to date, RTS,S, demonstrated that the main correlate of protection was a high antibody titre against the circumsporozoite protein, although low levels of induced $CD4^+$ T cells may also contribute to vaccine efficacy (126-128).

Measurement of vaccine-induced antibody can be done ELISA, which measures the presence of antigen-specific antibody in serum from a vaccinated or exposed individual. Antibody levels following ChAd63/MVA viral vectored vaccines typically peak around 4 weeks after the MVA boost vaccination (129). Protein-in-adjuvant vaccines are typically given in three doses 4 weeks apart, with the peak response seen 2 – 4 weeks after the final vaccination. This assay allows quantification of the antibody response by determining the antibody level as a μg/mL measure, although this requires conversion either by calibration free concentration analysis (CFCA) of antigen-specific antibody or by affinity purification of antigen-specific IgG (130). ELISAs can also be carried out to evaluate the avidity of the antibodies induced by vaccination, which gives an indication of antibody quality, and to assess the isotypes of the antibodies.

Functional assays can be carried out to evaluate antibodies induced by vaccination using *in vitro* techniques. An example of this type of assay in *P. vivax* is the DARC binding inhibition assay, which measures functional binding of *P. vivax* Duffy-binding protein region II (PvDBP – the vaccine antigen) to the Duffy antigen receptor for chemokines. The presence of functional anti-PvDBP antibodies results in a relative level of percentage binding inhibition (16). In *P. falciparum* malaria, the growth inhibition activity (GIA) assay is used to assess antibody function. This involves adding purified IgG from vaccinated (or naturally immune) volunteers to a culture of parasitised human erythrocytes and measuring subsequent parasite growth after

40 hours of culture using a biochemical assay specific for parasite lactate dehydrogenase (LDH). Results are then compared with results obtained from parasites incubated with a pool of malaria-naïve human serum (control) and with uninfected erythrocytes (131). Antibodies with functional activity against blood-stage malaria will inhibit growth of *P. falciparum* in culture, and this has been found to correlate with the antibody level induced by vaccination (130). This assay cannot be used to assess the functional activity of *P. vivax* blood-stage vaccine candidates as the parasite does not survive long-term culture, but an *ex vivo* invasion inhibition assay has been developed for use in endemic countries (as it requires access to clinical isolates of *P. vivax*) which has demonstrated that a monoclonal antibody against DARC is able to almost completely inhibit invasion and provides a potential method for assessing antibodies induced by vaccination (132).

Cell-mediated vaccine-induced immune responses can be measured by the production of interferon gamma (IFN-γ) by antigen-specific T cells. This is done using the *ex-vivo* IFN-γ enzyme-linked immunospot (ELISPOT). This assay allows enumeration of T cells and detection of individual IFN-γ-secreting cells from stimulated human peripheral blood lymphocytes (133). T cell responses typically peak 2 weeks after vaccination with a simian adenoviral vectored vaccine and a week after MVA boost. Protein-in-adjuvant vaccines typically do not induce a marked T cell response, but the AS01 adjuvant developed by GSK has been designed with a specific aim to improve cell-mediated immunity (122).

1.9 Methods for evaluating malaria vaccine efficacy

Whilst the *in vitro* methods described above give some information about the functional activity of antibodies induced by vaccination, this does not necessarily translate to vaccine efficacy. Animal models have been used for many years to study the mechanisms of *Plasmodium* infection, immunity against the parasite and in the development of vaccines (which first began in the 1940s) (58, 59) and antimalarial drugs. The most widely used model is the murine model in which mice can be infected with one of four rodent malaria species (*P.*

berghei, *P. chabaudi*, *P. vinckei*, or *P. yoelii*) or, more recently, with *P. falciparum* malaria in Severe Combined Immunodeficiency (SCID) mice receiving continuous injection of human erythrocytes (134). The mouse model has been very useful in furthering understanding of the malaria parasite(s), but there are limitations in the comparisons that can be made between murine and human malaria. Non-human primate models have also been used, with various primate malarias providing useful models for studying syndromes seen with human malaria- for example, *P. cynomolgi* which is very similar to *P. vivax* (135). Whilst these models provide key pre-clinical data for vaccine development, an effective vaccine in an animal model often does not translate into an effective human vaccine, necessitating human trials to assess efficacy.

The traditional method for assessing vaccine efficacy is to vaccinate a population at risk of the disease and compare the outcome to unvaccinated controls in Phase II or Phase III efficacy trials. These trials typically require large numbers of participants and there are many unpredictable and confounding factors such as differences in annual rainfall and co-infections with other pathogens. The only malaria vaccine to have reached Phase III trials to date is the RTS,S vaccine developed by GSK. The efficacy of this vaccine was assessed in over 15,000 children at eleven centres in seven sub-Saharan African countries with moderate efficacy which waned during the follow-up period (57). These types of study obviously involve huge cost and logistics, and therefore it is prudent only to take forward vaccines which have shown promise at an earlier phase into these large studies.

For many years the malaria field has used the controlled human malaria infection (CHMI) model to enable small proof-of-concept efficacy studies to take place in non-endemic settings and allow early indications of vaccine efficacy. These studies have several advantages over the traditional field efficacy trials as they require much smaller numbers of participants, can be conducted in non-endemic settings in a controlled environment, allow specific strains of malaria to be assessed and are not subject to the unpredictability of seasonal malaria

outbreaks. The CHMI model has particularly been used for *P. falciparum* studies, but several *P. vivax* CHMI studies have also now been carried out (77, 136, 137). There are several methods for CHMI, including via mosquito bite (11, 83, 95, 138), via injection of cryopreserved sporozoites (139) and by intravenous injection of parasitised red blood cells (11, 140, 141).

CHMI vaccine efficacy studies involve deliberately infecting volunteers vaccinated with the candidate vaccine alongside unvaccinated infectivity controls and monitoring them for development of clinical malaria. Patent parasitaemia is either diagnosed by thick blood film or when a predetermined threshold is reached on parasite level detected by quantitative polymerase chain reaction (qPCR). Treatment is commenced either at the point of diagnosis or at the end of the follow-up period if the volunteer has been protected from malaria. This method of assessing vaccine (or drug) efficacy is used in several centres around the world and has been found to be a safe and useful tool for early vaccine efficacy assessment. The ease with which the infection can be diagnosed, as well as its well-known time course and the availability of completely effective treatment mean that this mechanism for studying malaria has been accepted. These studies are obviously still subject to limitations from both an ethical point of view (using as few volunteers as possible to answer the study question) and a logistical point of view given the close follow-up of volunteers that is required after infection.

1.10 Development of viral vectored malaria vaccines

Development of effective malaria vaccines is difficult for many reasons. Immunity to malaria is complex and not fully understood, and vaccines have struggled to elicit adequate levels of protective antibody or a broad enough response. Protein vaccines have been the most commonly developed vaccines. Proteins have to be given with a potent adjuvant in order for them to achieve adequate immunogenicity. Access to adjuvants is limited and combination with an adjuvant may produce unacceptable side effects. Protein vaccines also have other drawbacks. Producing conformationally correct proteins to Good Manufacturing Practice (GMP) standard can be difficult, as has been seen with production of the PvDBP_RII protein

(Chetan Chitnis, International Centre for Genetic Engineering and Biotechnology, India; personal communication).

For some years viral vectors have been being developed as an alternative method of vaccination to bypass some of these issues. The viruses encode malaria antigens of interest that are then expressed when the viruses are taken up in the body. Viral vectored vaccines have been used in a wide range of pathogens including respiratory syncytial virus (RSV) (142), human immunodeficiency virus (HIV) (143, 144), malaria (84, 145-148), tuberculosis (149), Hepatitis C (150), influenza (151, 152), Ebola (153). and human papilloma virus (HPV) (154) The simian adenovirus ChAd63 and MVA vectors have been used in previous vaccine trials in Oxford encoding the *P. falciparum* malaria antigens ME-TRAP, AMA1, MSP1 and CS (83, 84, 129, 138, 155). The viral vectors are unable to replicate in human cells but are able to produce strong antigen-specific antibody and T cell responses following heterologous prime-boost vaccination (83, 84, 129, 148). Viral vectors had not previously been used in clinical trials of *P. vivax* vaccines but have been shown to be immunogenic in pre-clinical models (156, 157). Viral vectored vaccines have also been developed for the *P. falciparum* antigen RH5, which have been shown to be immunogenic in pre-clinical studies, with efficacy against blood-stage CHMI in *Aotus* monkeys (104, 158).

1.10.1 The ChAd63 Viral Vector

ChAd63 is a simian adenovirus. These adenoviruses exhibit hexon structures homologous to human adenoviruses and are not known to cause pathological illness in humans. Hexons are the major capsid proteins in adenoviruses; they are potently immunogenic and the main target of neutralising antibodies. Simian adenoviruses have been developed as viral vectors following concerns that pre-existing immunity to human adenoviral serotypes could limit future widespread use of these viruses (159). Low titres of neutralising antibodies to chimpanzee adenoviruses are found in less than 5% of humans in the US (160). The essential E1 gene region of the virus has been deleted so the ChAd63 vaccine vector is replication-deficient. The

virus cannot propagate in human cells within the body as it requires cells expressing E1 gene products. The E3 locus is additionally deleted, and the E4orf6 region has been replaced with that from AdHu5. These measures prevent homologous recombination with the E1 gene from the producer cell line and subsequent production of replication competent virus, and improve the yield of the ChAd63 virus during production.

The ChAd63 viral vector encoding various malaria antigens, including ME-TRAP, AMA1, MSP1 and CS has been administered to over 1000 individuals through studies conducted by the Jenner Institute in the UK and Africa. There have been no significant safety concerns relating to these vaccines. ChAd63 vaccine doses are measured in viral particles (vp). Doses of up to 2 x 10^{11} vp have been safely administered to healthy UK adults.

1.10.2 The MVA Viral Vector

MVA is a highly attenuated poxviral vector and as such, is unable to replicate efficiently in human and most other mammalian cells (161). The vector's viral and recombinant gene expression is unimpaired as the replication defect occurs at a late stage of virion assembly (162). The MVA vector was attenuated by continually passaging the chorioallantois vaccinia Ankara (CVA) strain on chicken embryo fibroblast cells over 570 times, resulting in six deletions in the CVA genome and multiple mutations (163, 164). MVA was licensed for use in Germany in 1977 and administered to over 120,000 individuals as a pre-vaccine before the second traditional smallpox vaccination was administered. Pre-vaccination with MVA led to lower reactogenicity following the smallpox vaccine (163).

MVA-based vaccine constructs have now been developed for use in studies of a wide range of infections including HIV (143, 144), malaria (83, 84, 129, 145, 148), Hepatitis C (150), tuberculosis (149) and Ebola (153). These studies have demonstrated that the vector is safe and immunogenic and, despite being highly attenuated, induces strong cellular immune responses (165). Previous vaccine regimes using MVA boost following an adenovirus prime have shown significant boosting of the cellular and humoral immune responses against the

encoded transgene in clinical studies (83, 84, 129). MVA vaccine doses are measured in plaque-forming units (pfu).

1.11 Hypothesis and aim of work

The hypothesis relating to work in this thesis is that blood-stage *Plasmodium falciparum* and *Plasmodium vivax* vaccines can be developed by targeting antigens which the parasites require in order to invade human red blood cells.

The aim of this work is to evaluate three candidate malaria vaccines, one *P. vivax* vaccine and two *P. falciparum* vaccines, in three clinical trials. Two of these trials (VAC051 and VAC057) will be Phase Ia first-in-human studies assessing the safety and immunogenicity of the vaccines in healthy volunteers. The vaccines in both of these studies are based on the ChAd63 and MVA viral vectors encoding a blood-stage malaria antigen.

Chapter three describes a Phase Ia trial (VAC051) of ChAd63 and MVA encoding the PvDBP transgene for the *P. vivax* vaccine. The hypothesis for this vaccination regime is that the vaccine will be safe and antibodies induced by vaccination will have activity against *P. vivax* Duffy-binding protein, as assessed in a functional assay examining binding between DBP and its receptor, DARC.

Chapter four examines a vaccine which has previously demonstrated evidence of some strain-specific efficacy in a field trial but not against CHMI. This study will assess the safety, immunogenicity and efficacy of the *P. falciparum* candidate blood-stage vaccine FMP2.1/AS01. Efficacy will be assessed using blood-stage CHMI with a malaria strain homologous to the vaccine strain. The hypothesis is that the parasite multiplication rate (PMR) in vaccinated volunteers will be lower than in unvaccinated infectivity controls due to the vaccine-induced immune response to the *P. falciparum* antigen AMA1.

Chapter five describes the Phase Ia trial (VAC057) of the ChAd63 and MVA vectors encoding the RH5 transgene for a *P. falciparum* vaccine. The hypothesis for this trial is that the

ChAd63/MVA RH5 vaccination regime will be safe and immunogenic in healthy volunteers, and induce functional antibodies which are able to inhibit parasite growth *in vitro*.

1.12 Authorship statement

The work presented in this Thesis relating to the clinical trials described in Chapters three to five is part of a collaborative effort. Unless otherwise stated, I undertook the work described.

The conduct of the trials was greatly assisted by the Jenner Institute Clinical Trials team, including Alison Lawrie, Rachel Roberts, Ian Poulton, Natalie Lella, Mary Smith, Raquel Lopez Ramon, Oliver Griffiths, Megan Baker, Celia Mitton, Paula Marriott, Charlotte Tyson, Emma Bakpa, Tommy Rampling, Navin Venkatraman and Morven Wilkie. The VAC054 trial (Chapter four) was also conducted at sites in London (Imperial NIHR/ Wellcome Trust Clinical Research Facility [WTCRF]) and Southampton (NIHR WTCRF). VAC057 (Chapter five) was also conducted in Southampton. I am very grateful to all staff involved in the study teams at both sites for their help in recruiting and following-up participants for these studies. All trials were sponsored by the University of Oxford, and Professor Adrian Hill was Chief Investigator.

Sarah Silk, Sean Elias and Kathryn Milne processed and stored samples for immunological analyses (Chapters three to five) and Nick Edwards performed the qPCR (Chapter four). Rebecca Brown and Simon Draper prepared the blood-stage inoculum for blood-stage CHMI (Chapter four). Simon Draper and Alexander Douglas assisted with the parasite modelling (Chapter four).

Blood samples for haematology, biochemistry and serology (for Epstein Barr virus [EBV], cytomegalovirus [CMV], HIV, Hepatitis B and C) were processed at the Oxford University Hospitals' NHS Foundation Trust laboratories, Oxford (Chapters three to five), at the University Hospital Southampton NHS Foundation Trust laboratories, Southampton (Chapters four and five) and at Imperial College Healthcare NHS Trust laboratories, London (Chapter four).

Chapter two:

Materials and Methods

2.1 Materials

2.1.1 Vaccines

2.1.1.1 ChAd63 PvDBP

ChAd63 is a replication-deficient simian adenovirus, as described in Section 1.9.1. ChAd63 PvDBP expresses the *Plasmodium vivax* antigen PvDBP, which is a synthetic gene insert encoding the conserved, cysteine-rich Region II of the *P. vivax* Duffy-binding protein. The sequence in the vaccines is based on the Salvador I (*Sal*I) strain of *P. vivax*, amino acids (αα) 194–521. The PvDBP_RII sequence was fused with the human tissue plasminogen activator (tPA) secretory leader sequence and the transgene is driven by a CMV promoter (157). tPA ensures the PvDBP_RII antigen is secreted from the immunised (virally infected) cell. In the vast majority of cases *P. vivax* requires interaction between the Duffy-binding protein and DARC in order to invade red blood cells.

ChAd63 PvDBP was manufactured under GMP conditions by the Clinical Biomanufacturing Facility (CBF), University of Oxford, and supplied as a liquid in sterile aliquots in 2.0 mL clear glass vials. ChAd63 PvDBP was supplied to the clinical site by the CBF having been labelled for investigational use only. The vaccine was stored in a locked, temperature controlled freezer between -70^{o}C and -90^{o}C, and all movements/ administration of the vaccine were recorded in an accountability log.

2.1.1.2 MVA PvDBP

MVA is a highly attenuated vector that is unable to replicate efficiently in human and most other mammalian cells, as described in Section 1.9.2 (161). MVA PvDBP is a recombinant MVA expressing the *P. vivax* antigen PvDBP. The insert is the same as that used in the ChAd63 PvDBP vaccine.

MVA PvDBP was manufactured under GMP conditions by IDT, Germany and supplied as a liquid formulation in Tris buffer. The virus suspension was supplied as sterile aliquots in 2.0 mL clear glass vials. Final batch certification and associated labelling took place at the CBF. The

vaccine was stored in a locked, temperature controlled freezer between -70°C and -90°C, and all movements/ administration of the vaccine were recorded in an accountability log.

2.1.1.3 FMP2.1/AS01B

The vaccine FMP2.1 is a recombinant protein of the *Plasmodium falciparum* 3D7 clone of AMA1. It was produced under GMP at the WRAIR BioProduction Facility. FMP2.1 is a lyophilised preparation of the majority of the ectodomain of *P. falciparum* AMA1. The gene encoding the FMP2.1 protein was chemically synthesized to contain an *E. coli*-optimized codon usage to encode 478 amino acids representative of amino acids 83 to 531 of the AMA1 protein with two attached His-tags. The amino acid sequence is: MAHHHHHHPGGSGSGTMH-[AMA1 amino acids 83 to 531]-AAALEHHHHHH. 449 of the amino acids are derived from the merozoite protein AMA1 of the 3D7 clone of *P. falciparum*. The protein is produced in and purified from *E. coli* bacteria (93, 95, 97). The vaccine was administered with AS01B, a liposome-based Adjuvant System developed by GlaxoSmithKline (GSK) (122). Mixing of the vaccine and AS01B took place immediately before vaccination.

The vaccine and adjuvant were labelled and released for investigational use only in trial VAC054 by the CBF, and then transferred to the clinical sites. Both the vaccine and adjuvant were stored between +2 and +8°C in a locked fridge.

Further details about the vaccine and adjuvant can be found in Chapter four.

2.1.1.4 ChAd63 RH5

ChAd63 RH5 is a recombinant replication-defective chimpanzee adenovirus expressing the *Plasmodium falciparum* antigen RH5; a synthetic gene insert encoding the reticulocyte-binding protein homologue 5 (PfRH5) which is one of the reticulocyte binding-like (RBL or *P. falciparum* RBP homologue (PfRH)) proteins which are involved in parasite invasion of red blood cells. PfRH5 is expressed in all *P. falciparum* strains tested so far, and is essential for parasite survival given two reports that the gene cannot be knocked out (100, 101). PfRH5 binds to its receptor basigin, the Ok blood group antigen, and this interaction mediates an essential interaction

required for red blood cell invasion by all tested strains of *P. falciparum* (103). A full-length PfRH5 transgene based on the 3D7 clone of *P. falciparum* was used in the ChAd63 RH5 vaccine (104). Similar to the ChAd63 PvDBP vaccine described above, the antigen was fused with the tPA secretory leader sequence and the transgene is driven by a CMV promoter.

ChAd63 RH5 was manufactured under Good Manufacturing Practice (GMP) conditions by ADVENT S.r.l. (GSK) and supplied as a liquid in sterile aliquots in 2.0 mL clear glass vials. Final batch certification and associated labelling took place at the CBF before transfer to the clinical sites. The vaccine was stored in a locked, temperature controlled freezer between -70^{o}C and -90^{o}C, and all movements/ administration of the vaccine were recorded in an accountability log.

2.1.1.5 MVA RH5

MVA RH5 is a recombinant MVA expressing the *P. falciparum* antigen PfRH5. MVA RH5 used the same vector described above for MVA PvDBP and the same antigen insert as ChAd63 RH5.

MVA RH5 was manufactured under GMP conditions by IDT, Germany and supplied as a liquid formulation in Tris buffer. The virus suspension is supplied as sterile aliquots in 2.0 mL clear glass vials. Final batch certification and associated labelling took place at the CBF before transfer to the clinical sites. The vaccine was stored in a locked, temperature controlled freezer between -70^{o}C and -90^{o}C, and all movements/ administration of the vaccine were recorded in an accountability log.

2.1.2 *Plasmodium falciparum* Controlled Human Malaria Infection Inoculum

The infectious inoculum for the CHMI study (VAC054; Chapter four) was produced by Drs Gregor Lawrence, Allan Saul and colleagues at the Queensland Institute of Medical Research (QIMR) in Brisbane, Australia in 1994 (166). The protocol for the study was reviewed and approved by the QIMR Ethics Committee and the Healthy Volunteer Studies Research Ethics Subcommittee, Lothian Health Board (Edinburgh). Procedures were designed to minimise the risk of other infectious agents in the cryopreserved samples.

Laboratory-reared *Anopheles stephensi* mosquitoes were infected with the *P. falciparum* clone 3D7 (a chloroquine-sensitive strain) by membrane feeding on a blood meal containing gametocytes. Ten and fourteen days later, the mosquitoes were fed on two volunteers. Parasitaemia in the volunteers was followed by daily microscopy from day 4 after infection. Blood was taken from the volunteers 6 hours after they developed fever, when both were microscopically parasite positive. The volunteers were treated with chloroquine soon after blood was drawn with complete recovery.

Initial development of the blood inoculum used in VAC054 is described by Cheng *et al.* 1997 (166). The inoculum used for all volunteers comes from one of the donors described above. This is because the second donor (whose blood has not been used) had a much lower parasitaemia (140).Blood was collected at the Australian Red Cross Blood Bank in an aseptic manner using standard blood bank equipment. The leukocytes were removed with a leukocytic filter. The thawing and washing of the cells reduced the amount of serum transferred with the red cells by a factor of 1000, compared to injecting the same volume of blood. The volume of inoculum to be given to each volunteer contains a very small volume of red blood cells, equivalent to only 1.5 to 4 microlitres of blood.

The red cells were cryopreserved using a protocol from the American Association of Blood Banks Technical Manual that is normally employed for freezing blood from patients and donors with rare blood groups. Blood from both volunteers was group O and Rhesus negative (166).

The *P. falciparum* CHMI inoculum is stored cryopreserved in vapour phase liquid nitrogen at -178^{o}C (-160^{o}C to -196^{o}C) at Fisher BioSciences in Herefordshire, UK, in compliance with the requirements of ISO 9001:2015. The inoculum has been temperature monitored throughout the long term storage at this facility.

2.1.3 Buffers and solutions

Lymphoprep™: ready-made, sterile and endotoxin tested solution for isolation of pure lymphocyte suspensions (Axis Shield 1114545).

R0: Cell culture media consisting of 500mL RPMI (Sigma R0883) with 5 mL Pen/strep (0.1 mg/mL) (Gibco BRL/Invitrogen 15140-122) and 5mL L-glutamine (4mM) (Gibco 25030-24).

R10: Cell culture media consisting of 500mL RPMI (Sigma R0883) with 5mL Pen/strep (0.1 mg/mL) (Gibco BRL/Invitrogen 15140-122), 5mL L-glutamine (4mM) (Gibco 25030-24) and 50mL heat-inactivated, filtered standard foetal calf serum (FCS) (Biosera S1810).

Red blood cell (RBC) lysis solution: (Qiagen 158902/4).

Phosphate buffered saline (**PBS**): 0.01 M (Sigma P3813): NaCl 0.138 M; KCl - 0.0027 M, pH 7.4. Sachets reconstituted in distilled or deionised water.

PBS/Tween: PBS (Gibco 21600-069) 0.01M Tween 0.05%: made by dissolving sachets in dH_2O.

Dimethyl sulfoxide (**DMSO**): sterile filtered and endotoxin tested (Sigma D2650).

Staphylococcal enterotoxin B (**SEB**): Superantigen consisting of a single polypeptide chain containing 239 amino acids which induces apoptosis in T cells (Sigma S4881).

StartingBlock™ T20 Solution: T20 in PBS blocking buffer (Fisher 10270404).

Development Buffer (**DB**): 4-Nitrophenylphosphate tablets (Sigma N2765) dissolved in diethanolamine buffer (Fisher 34064) to give final concentration of 1mg/ml.

Casein: blocking buffer (ThermoFisher 37528).

DPBS: Dulbecco's phosphate buffered saline (Sigma D8537).

NaSCN: Sodium thiocyanate (Sigma 251410).

Extravidin-AP: Extravidin-alkaline phosphatase buffered aqueous solution in 0.05 M Tris-HCl buffer, pH 8.0, containing 1 mM $MgCl_2$, 1% bovine serum albumin and 15 mM sodium azide (Sigma E2636).

BCIP/NBT: 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium plus solution – ELISPOT developing buffer (Europa bioproducts Ltd M0711A-1000).

Diphtheria toxoid: diluted in PBS to 10 μg/mL (NIBSC 02/176)

SAC: Staphylococcus aureus Cowans Strain (Calbiochem 507858).

CpG-2006: 0.2mg/mL (TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT) (Invivogen)

PWM: Pokeweed mitogen made up to 1 mg/mL stock (Sigma L-9379)

2.1.4 Recombinant proteins

Recombinant PvDBP_RII protein was produced at the Jenner Institute in the *Drosophila* S2 expression system (Expres^{2}ion). Recombinant DARC (His-tagged) was produced at the Jenner Institute. Recombinant PfAMA1 was produced at the Jenner Institute in HEK293 cells (3D7 clone) (167) and provided by an external collaborator, Dr Mike Blackman (NIMR, London, UK) (FVO clone). Recombinant PfMSP1$_{19}$-GST fusion proteins were produced in *E. coli* (168) and purified by affinity chromatography according to Jenner Protocol J136. Recombinant PfRH5 was produced at the Jenner Institute in *Drosophila* S2 cells (169). These recombinant proteins were used for secondary assays described below.

2.2 Methods: Study preparation, participant enrolment and safety analysis

For each trial I prepared the study documents which were then reviewed and approved by the Sponsor (Clinical Trials Research Governance department, CTRG, University of Oxford). Following Sponsor approval, each trial was submitted for ethical approval to the National Research Ethics Service (NRES) Oxford A ethics committee and to the Medicines and Healthcare products Regulatory Agency (MHRA). For VAC054 (Chapter four) and VAC057 (Chapter five) approval from the Research and Development departments at the NHS collaborating sites was applied for and gained prior to starting the trials at these sites. The VAC054 trial also underwent ethical review by the Western Institutional Review Board (WIRB)

in the USA as a condition of funding (the trial was funded by USAID). The VAC051 (Chapter three) and VAC057 trials also received approval by the Genetic Modification Safety Committee, Oxford University Hospitals NHS Trust to use the viral vectored vaccines as a Class I activity under the Genetically Modified (Contained Use) Regulations 2000.

The Chief Investigator for all trials was Professor Adrian Hill, but the conduct of the trials was delegated to me as lead clinician. All trials were monitored to ensure the rights and wellbeing of human subjects were protected and that the conduct of the study was in compliance with the protocol, regulatory and ICH GCP requirements. All monitoring for these studies was carried out by CTRG.

Study-specific methods, such as study design, approvals and inclusion/exclusion criteria are described in the Chapters relating to each trial (see Chapters three to five).

2.2.1 Recruitment of volunteers

Healthy volunteers were recruited to the studies by various means. Advertising was carried out with posters approved by the Oxford ethics committee in public places and in newspapers/advertising leaflets. There are healthy volunteer databases at the sites used in Oxford, Southampton and London through which some volunteers were contacted. The department has a website through which information about the trials and contact details for arranging a screening visit can be found. Social networking sites (Facebook and Twitter) were also used to inform potential participants about the trials. Recruitment of volunteers was coordinated in Oxford and London by the Recruitment Coordinator (Sam French and Natalie Lella) and in Southampton by their local study team. Once interest in a trial had been registered the volunteer was sent the Participant Information Sheet (Appendix 1) by email and invited to attend a screening visit. For all studies there was a minimum of 24 hours between the information sheet being sent out and the volunteer attending a screening visit.

2.2.2 Screening Visit and Informed Consent

The screening visits took place up to 90 days before participants were enrolled in the study (participants were considered to be enrolled once they had received a vaccination). At this visit the information sheet is explained to the volunteer and they were given the opportunity to ask any questions. This was carried out by me for volunteers recruited in Oxford and London, and by the local study team in Southampton. The details of the trial, the potential risks and the responsibilities of the participant once enrolled were explained before volunteers signed a consent form (Appendix 2). For the VAC054 trial (Chapter four) volunteers were also required to complete an informed consent questionnaire (Appendix 3) to ensure they had understood the information and risk relating to the trial, in particular to malaria infection. Participants had to answer all questions correctly but were allowed to re-take the same questionnaire following further discussion if they failed to do this on their first attempt. The following general principles were emphasised:

- Participation in the study is entirely voluntary.
- Refusal to participate involves no penalty or loss of medical benefits.
- The volunteer may withdraw from the study at any time.
- The volunteer is free to ask questions at any time to allow him or her to understand the purpose of the study and the procedures involved.
- The study involves research of an investigational vaccine.
- There is no direct benefit from participating.
- The volunteer's GP will be contacted to corroborate their medical history.
- The volunteer will be registered on the TOPS database (The Over-volunteering Prevention System; www.tops.org.uk).
- The volunteer's blood samples taken as part of the study will be stored indefinitely and samples may be sent outside of the UK and Europe to laboratories in collaboration with the University of Oxford. These will be anonymised.

If the volunteer consented to participate in the study, they were then asked details of their medical history and underwent a physical examination, as well as baseline blood tests (full blood count, urea & electrolytes, liver function tests and serology for Hepatitis B, Hepatitis C and HIV). Their GP was contacted by letter, giving information about the trial, and asked to respond to try to ensure any medical or psychiatric reasons for exclusion from the trials (which had not been disclosed by the volunteer at screening) were picked up. For VAC054 an ECG was carried out, and cholesterol and magnesium levels were also checked with the other biochemistry tests.

The inclusion and exclusion criteria for each trial are detailed in each trial Chapter.

2.2.3 Study design and procedures

The study design for each trial is detailed in the Chapter relating to that trial (i.e. Chapter three for VAC051, Chapter four for VAC054 and Chapter five for VAC057). The schedules of attendance for volunteers taking part in each study can be found in Appendix 4. Data at clinic visits were recorded into the case report form (CRF) by study staff. This was a paper CRF in the VAC051 trial, with anonymised data subsequently entered into an OpenClinica database, with double data entry carried out by two members of the study team for each visit entry. In the VAC054 and VAC057 trials, data were entered directly into an electronic CRF (eCRF) in clinic so this functioned as both source data and database. Blood tests carried out at the NHS laboratories were received as paper reports which were signed off by me in Oxford, or one of the clinicians at other trial sites. These results were then entered into the OpenClinica database by study staff. For all interim and final analyses data were exported from the OpenClinica database and processed by the data manager (Jack Quaddy) before I analysed the data.

2.2.4 Assessment of Safety

The safety of the vaccines in the three clinical trials discussed in this thesis was assessed using actively and passively collected data on any adverse events (AEs) as described in the 'Methods'

section of each trial Chapter. Participants were asked to record both solicited and unsolicited AEs on diary cards (paper or electronic) given to them on the day of vaccination. Examples of the diary cards used are included in Appendix 5. Data from paper diary cards were entered into the OpenClinica database by study staff. The electronic diaries (eDiaries) were analysed directly as AE data were exported already processed into an Excel spreadsheet directly from the diaries. Safety was assessed by the frequency, incidence and nature of AEs and serious adverse vents (SAEs) arising during the study.

For each vaccine a Development safety update report (DSUR) was submitted to the regulatory authority annually whilst the trials were ongoing. This contained safety information relating to the vaccine over the preceding year. In addition, the Investigator Brochures were also updated annually with new safety information.

2.2.4.1 Definitions

Adverse Event (AE)

An AE is any untoward medical occurrence in a volunteer, which may occur during or after administration of an Investigational Medicinal Product (IMP) and does not necessarily have a causal relationship with the intervention. An AE can therefore be any unfavourable and 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.

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 the reporting medical Investigator 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.

Unexpected Adverse Reaction

An adverse reaction, the nature or severity of which is not consistent with the applicable product information (*e.g.* the Investigator's Brochure [IB] for an unapproved IMP) is considered as an unexpected adverse drug reaction (ADR).

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
- Life-threatening event (i.e., the volunteer 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 severe 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, regardless of length of stay, even if it is a precautionary measure for continued observation. Hospitalisation (including inpatient or outpatient hospitalisation 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 hospitalisation) that may, based upon appropriate medical judgment, jeopardise the volunteer and/or require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic reaction requiring intensive treatment in an emergency room or clinic, blood dyscrasias, or convulsions that do not result in inpatient hospitalisation.
- Congenital anomaly or birth defect.

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.

Suspected Unexpected Serious Adverse Reaction (SUSAR)

A serious adverse reaction, the nature and severity of which is not consistent with the information about the medicinal product in question set out in the IB or Summary of Product Characteristics (SmPC).

Solicited AEs

Solicited AEs are those commonly seen after vaccination, and are divided into local and systemic AEs. Local AEs include redness, swelling, itching, warmth and pain at the injection site. Systemic AEs include fever/ feverishness, headache, nausea, malaise, myalgia and arthralgia. Solicited AEs are expected to occur, and generally resolve, within the first seven days after vaccination. Any adverse events not listed as a solicited AE or occurring after the defined time (usually seven days after vaccination) are termed unsolicited AEs. Local AEs are graded by severity according to the criteria shown in Table 2.1. Criteria for assessing abnormalities in physical observations (vital signs) are shown in Table 2.2. These criteria were not specified for the VAC051 trial, and were developed through VAC054 and VAC057. The table shows the criteria for VAC057 and VAC054 where they differed. Changes were made to reflect the UK National Institute for Health and Care Excellence (NICE) clinical guidelines on stages of hypertension (170). Systemic AEs other than fever (solicited and unsolicited) are graded according to the criteria shown in Table 2.3

The maximum reported severity of an AE is reported in the results section of each trial Chapter, i.e. if a volunteer had an AE which lasted 3 days and was mild for 2 days but severe for one this has been reported as ‘severe’.

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 - ≤50 mm
	2	>50 - ≤100 mm
	3	>100 mm
Swelling at injection site	1	>0 - ≤20 mm
	2	>20 - ≤50 mm
	3	>50 mm

Table 2.1: Severity grading criteria for fever, injection site pain, erythema and swelling.

**erythema ≤3mm is an expected consequence of skin puncture and will therefore not be considered an adverse event.*

Physical observations		Grade 1 (mild)	Grade 2 (moderate)	Grade 3 (severe)
Fever (oral) °C		37.6°C - 38.0°C	38.1°C – 39.0°C	>39.0°C
Tachycardia (beats per minute [bpm])		101 - 115	116 – 130	>130
Bradycardia (bpm)*		50 – 54	40 – 49	<40
Systolic hypertension (mmHg)	VAC057	141 - 159	160 – 179	≥180
	VAC054	141 - 150	151 - 155	>155
Diastolic hypertension (mmHg)	VAC057	91 - 99	100 – 109	≥110
	VAC054	91 - 95	96 - 100	>100
Systolic hypotension (mmHg)**		85 - 89	80 – 84	<80

Table 2.2: Criteria for grading severity of clinically significant abnormal physical observations.

Observations should have been taken at rest (after ≥10 minutes at rest defined for VAC057)

**Applicable only when resting heart rate is between 60 – 100 beats per minute. Use clinical judgement when characterising bradycardia among some healthy subject populations, for example, conditioned athletes. This criterion was not defined for VAC054.*

***Only if symptomatic (e.g. dizzy/ light-headed)*

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; medical intervention/therapy required, hospitalisation possible

Table 2.3: Severity grading criteria for systemic AEs.

2.2.4.2 Causality Assessment

In each trial, for each unsolicited AE, an assessment of the relationship of the AE to the study intervention(s) was undertaken. Solicited AEs occurring within the first seven days after vaccination were considered to be at least possibly related to vaccination. I categorised the relationship of the AE as unrelated, unlikely to be related, possibly related, probably related or definitely related (Table 2.4). Alternative causes of the AE, such as the natural history of pre-existing medical conditions, concomitant therapy, other risk factors and the temporal relationship of the event to vaccination were considered.

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

Table 2.4: Guidelines for assessing the relationship of vaccine administration to an AE.

2.2.4.3 Laboratory AEs

Blood samples were taken during each trial to monitor for any AEs relating to haematological or biochemical changes following vaccination of volunteers. These blood samples were analysed either at the Oxford NHS laboratory or at the local NHS laboratories in trials involving other sites. For VAC051, laboratory abnormalities were assessed according to Table 2.5. After this trial I set up a more comprehensive laboratory AE severity grading table to be used across trials conducted at the Jenner Institute. It was also recognised that each laboratory has different normal reference ranges for some blood tests, so site-specific grading tables were produced by me, with support from other clinical research fellows based at the Jenner Institute and Southampton. These can be found in Appendix 6.

Laboratory Test	Grade 1	Grade 2	Grade 3
Hgb (female) – decrease from testing laboratory LLN in gm/dl	>1.0 - <1.5	≥1.5 & <2.0	≥2.0
Hgb (male) – decrease from testing laboratory LLN in gm/dl	≥1.5 & <2.0	≥2.0 & <2.5	≥2.5
Absolute neutrophil count (ANC, cells/mm^3)	1000-1499	500-<1000	<500
Leukopenia (WBC, cells/mm^3)	<3500 - ≥2500	<2500 - ≥1500	<1500
Lymphocytes Decrease (cell/mm^3)	750 – 1,000	500 – <750	250 – <500
Platelets (cells/mm^3)	125,000 – 135,000	100,000 – <125,000	20,000-<100,000
Bilirubin – when accompanied by any increase in Liver Function Test increase by factor	1.1–1.25 x ULN	>1.25–1.5 x ULN	>1.5–1.75 x ULN
ALT	1.25–2.5 x ULN	>2.5–5.0 x ULN	>5.0 x ULN
Creatinine	1.1–1.5 x ULN	>1.6–3.0 x ULN	>3.0 x ULN

Table 2.5: Severity grading criteria for clinically significant abnormalities for VAC051 trial (171).

Hgb = Haemoglobin, LLN = lower limit of normal, ANC = absolute neutrophil count, WBC = white blood cells, ALT = alanine transferase, ULN = upper limit of normal.

2.2.5 Compensation for trial participants

Compensation for volunteers taking part in the trials described in this thesis was calculated on a pro rata basis. Participants were compensated according to the following:

- Travel expenses:
    - £10 per visit. Where travel expenses were greater than £10 per visit because the volunteer lived outside the city of the trial site, the volunteer was given further reimbursement to meet the cost of travel necessary for study visits.
- Inconvenience of blood tests:
    - £10 per blood donation
- Time required for visit:
    - £20 per hour

Volunteers taking part in the VAC054 CHMI trial (Chapter four) were also compensated £20/hour illness compensation for 24 hours following CHMI.

2.3 Methods: Immunology

2.3.1 Blood separation

In all trials detailed in this thesis, blood samples for immunology were collected before vaccination (as baseline) and at several time-points following vaccination(s), as per the study designs. Blood was collected in serum and lithium heparinised vacutainer tubes (BD bioscience) and transported to the Jenner Institute laboratories for processing. This was carried out by Kathryn Milne, Sarah Silk and Sean Elias.

Separation of peripheral blood mononuclear cells (PBMC) and serum were carried out as per Jenner Standard Operating Procedure (SOP) *ML002: Malaria PBMC Separation and Freezing* (Appendix 8). Briefly:

- Serum tubes were spun at 706 *xg* for five minutes; serum was collected with a transfer pipette and stored in aliquots. These were then stored at -80^{o}C.
- Heparinised blood was poured into Leucosep tubes (Greiner 227209) which had been pre-prepared with 15 mL Lymphoprep^{TM}, spun so that it was below the porous filter disc. Leucosep tubes containing heparinised blood were spun at 1000 *xg* for 13 minutes at room temperature (brakes off). The plasma fraction was aliquoted and

stored at -80°C before the excess plasma containing PBMC was poured into a Falcon tube. This was then topped up with R0 and spun at 706 *xg* at room temperature for five minutes. The supernatant was poured off, cells were re-suspended in R0 and the spin/wash process was repeated. RBC lysis solution was used if there was significant contamination of the pellet with RBC. The cells were then re-suspended in 10 mL of R10 for counting. Cells were counted using a CasyCounter. Following counting, cells were re-suspended and used for the *ex-vivo* IFN-γ ELISPOT assay or stored in 1mL aliquots of 5 - 10 million cells/cryovial in filtered, heat-treated FCS and 20% DMSO. These were initially stored at -80°C before transfer to liquid nitrogen storage 1 – 3 days later.

2.3.2 Thawing of PBMC

ASC and mBC ELISPOTS were carried out using frozen PBMC for most assays. PBMC had been stored in liquid nitrogen following the separation process described above. Vials were removed from liquid nitrogen on the day of the assays and thawed in a water bath at 37°C before being added to warmed (37°C) R10 media in Falcon tubes. Cells were spun at 706 *xg* for 5 minutes at room temperature, supernatant was discarded and cells were then re-suspended in R10. Benzonase was added to each Falcon tube (1 µL per 1 x 10^6 PBMC in the thawed cryovial) and suspended cells were incubated for at least 1 hour at 37°C. Tubes were spun again at 706 *xg* for 5 minutes, supernatant was discarded and 10 mL of R10 was added to resuspend cells prior to counting using a Casycounter. After counting, cells were suspended in R10 at 5 x 10^6 cells/mL for ASC ELISPOTs and 2 x 10^6 cells/mL for mBC ELISPOTs.

2.3.3 *Ex-vivo* IFN-γ ELISPOTs

Ex-vivo IFN-γ ELISPOTs were carried out on fresh PBMC for all participants in each trial to assess the kinetics and magnitude of the vaccine-induced T cell responses over time. Samples were processed and analysed according to the Jenner Institute SOP *ML006: Ex Vivo ELISPOT* (Appendix 8). Assays were carried out within 4 hours of blood being collected from the participant. These assays were carried out by Kathryn Milne, Sarah Silk and Sean Elias.

ELISPOT plates (Millipore, MAIPS4510) were prepared the preceding day with 50 µL of coating solution (10 µL of catcher antibody [1-D1K] per 1 mL of ELISPOT coating buffer) added to each well and then left overnight at 4^{o}C. Peptide pools (5 µg/mL) were pre-aliquoted into 96 well plates at the start of the trial and stored at -80^{o}C until needed.

On the day of sample processing the plates were blocked with R10 for 1 – 8 hours after being washed three times with sterile PBS. The PBMC isolated from whole blood (as described in Section 1.3.1) were plated at 250,000 cells per well for an 18-20 hour re-stimulation with overlapping peptides spanning the antigen of interest: *P. vivax* DBP_RII (Salvador I allele) for VAC051; *P. falciparum* AMA1 (3D7) for VAC054; and *P. falciparum* RH5 (3D7) for VAC057. The positive control for the assay was 10 µg/mL PHA plus 0.02 mg/mL SEB and negative control was R10 media + DMSO. All samples were tested in triplicate for each peptide pool. After overnight incubation, cells were discarded, plates were washed and 50 µL of a second, biotinylated monoclonal antibody against human IFN-γ (7-B6-1-Biotin, concentration 1 µg/mL) (Mabtech, 3420-2A) was added and the plate incubated for 2 - 4 hours (room temperature). After another wash step, streptavidin alkaline phosphate (Mabtech, 3420-2A) (diluted 1:1000 in PBS) was added before a further incubation at room temperature for 1-2 hours. Plates were washed and the alkaline phosphate substrate (developer) was added (50 µL/well). The plate was left to develop for approximately 3 – 15 minutes until spots were clearly visible. Spots were counted using an automated plate counter and results obtained by subtracting any background response (from negative control wells) and then taking the average of the triplicate wells. Results are expressed as IFN-γ spot-forming-units (SFU) / million PBMC.

2.3.4 ELISAs

2.3.4.1 Antigen-specific IgG ELISAs

The kinetics and magnitude of the serum IgG antibody responses against the antigen of interest for each trial (PvDBP_RII for VAC051, AMA1 for VAC054 and RH5 for VAC057) were assessed over time by ELISA. Following CHMI in VAC054 an anti-$MSP1_{19}$ ELISA was done looking for evidence of seroconversion in infectivity control volunteers and in control volunteers from six previous CHMI studies with samples from before CHMI (dC-1) and 4 weeks after blood stream infection (74, 83, 138, 172, 173). These assays were carried out by me and Sarah Silk.

Anti-PvDBP_RII IgG ELISA (VAC051; Chapter three)

This ELISA was carried out as per Jenner Institute SOP *ML026: DBP ELISA* (Appendix 8). Briefly, 96 well Nunc-Immuno Maxisorp plates were coated with recombinant PvDBP_RII (Salvador I strain) at a concentration of 2µg/ml in PBS and left overnight. Plates were washed with PBS/Tween six times and blocked for 1 hour with StartingBlock™ T20 buffer (Fisher, UK). A standard curve and internal control samples were prepared from the reference serum (VAC051 volunteer 028 day 84 serum sample) and added to the plate with the serum samples from trial volunteers. All samples were diluted in StartingBlock™ T20 solution. The serum samples were added in triplicate to the ELISA plate (Figure 2-1). After a two hour incubation the plates were washed and alkaline phosphatase conjugated goat anti-human IgG (γ-chain) (Sigma) diluted 1:1000 in StartingBlock™ T20 solution was added. After a further one hour incubation, followed by a wash step, 4-nitrophenyl phosphate substrate (Sigma) diluted in diethanolamine buffer (Fisher Scientific, UK) (DB) was added to detect antibodies. An ELx800 microplate reader (BioTek, UK) was used to read optical density at 405nm (OD_{405}). The reciprocal of the dilution giving an OD_{405} of 1.0 in the standardised assay was used to assign an ELISA unit value of the standard. The standard curve and Gen5 ELISA software v2.07 (BioTek, UK) was used to convert the OD_{405} of individual test samples into ELISA units.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
G	1	2	3	4	5	6	7	8	9	10	Blank	
H	1	2	3	4	5	6	7	8	9	10		

Figure 2-1: Antigen-specific IgG ELISA plate layout

Blue S1 – S22 = test sera (added in triplicate); Pink 1 – 10 = standard curve dilutions (in duplicate); Internal control (as per ELISA protocol); Blank = blocking solution (as per ELISA protocol, e.g. StartingBlock™ T20)

Anti-AMA1 and anti-MSP1 IgG ELISAs (VAC054; Chapter four)

These ELISAs were carried out as per Jenner Institute SOP *ML023: P. falciparum MSP1 and AMA1 ELISA* (Appendix 8). Briefly, 96 well Nunc-Immuno Maxisorp plates were coated with recombinant AMA1 or recombinant $MSP1_{19}$ at a concentration of 2µg/ml in PBS and left overnight. Plates were washed six times with PBS/Tween and then blocked for 1 hour with Casein blocking buffer. A standard curve and internal control samples were prepared from the reference serum (human hyperimmune serum from Kilifi, Kenya diluted 1:100) and added to the plate with the serum samples from trial volunteers. The serum samples were diluted and added in triplicate to the ELISA plate (see Figure 2-1). After a two hour incubation the plates were washed and the alkaline phosphatase-conjugated secondary polyclonal goat anti-human IgG (γ-chain) antibody was added. After a further one hour incubation, followed by a wash step, developer (DB) was added. The developer was left for 10 - 25 mins (antigen dependent) and the absorbance at 405nm (OD_{405}) was read using a plate reader. The result was obtained by taking an average of the triplicate wells for each test sample, and using the standard curve to assign MSP1 or AMA1 ELISA arbitrary units (AU). Samples with an OD <0.15 were considered negative.

Calibration-free concentration analysis (VAC054; Chapter four)

CFCA was used to convert AMA1 OD-based ELISA units to antigen-specific µg/mL using a Biocore T200 instrument, a Biotin CAP chip and T200 control and evaluation software (all from GE Lifesciences, UK) with methodology previously published (167). This work was performed by Sandy Douglas using serum samples from three individuals with a range of ELISA-measured anti-AMA1 IgG responses. The CFCA-measured antigen-specific antibody concentrations for each individual against 3D7 and FVO sequence AMA1 were combined with the known total IgG ELISA AU measurements for the same samples to derive an AU-to-µg/mL conversion factor. For each AMA1 allele, the mean of the conversion factors measured for the three subjects was calculated and applied to the AU ELISA measurements for other participants so that all ELISA results could be expressed in µg/mL units.

Anti-RH5 IgG ELISA (VAC057; Chapter five)

Anti-RH5 IgG ELISAs were carried out as per Jenner Institute SOP *ML011: RH5 ELISA* (Appendix 8). Briefly, 96 well Nunc-Immuno Maxisorp plates were coated with recombinant PfRH5 at a concentration of 2µg/ml in PBS and left overnight. Plates were washed six times with PBS/Tween and then blocked for 1 hour with Casein blocking buffer. A standard curve and internal control samples were prepared from the reference serum (VAC057 Volunteer 1020 G2B d84 serum sample) and added to the plate with the serum samples from trial volunteers. The serum samples were diluted and added in triplicate to the ELISA plate (see Figure 2-1). Secondary antibody, development and ELISA result readout steps were carried out as described above for PvDBP, AMA1 and MSP1 ELISAs.

2.3.4.2 Avidity ELISAs

The avidity ELISAs for all trials were carried out as per Jenner Laboratory Protocol *J177: Human Whole IgG Avidity ELISA*. Briefly, 96 well Nunc-Immuno Maxisorp plates were coated with the recombinant protein of interest (e.g. AMA1) diluted in DPBS to a concentration of 2 µg/mL and left overnight. Following a wash step with PBS/Tween, plates were blocked with Casein for 1 hour and then a further wash step was carried out. Serum samples were diluted using

standardised ELISA results so that each individual serum would reach an OD of 1 when read (e.g. if sample has an ELISA result of 3000 AU, dilute serum 1:3000) and added in duplicate down the plate. After a 2 hour incubation, plates were washed and an increasing concentration of NaSCN diluted in PBS was added down the plate from 0M to 7M (Figure 2-2). Plates were left for 15 minutes before a further wash step. Secondary goat anti-human γ-chain whole IgG alkaline phosphatase conjugate antibody (diluted 1:1000 in Casein) was added and plates were left for 1 hour. After a final wash step, DB was added and plates were left to develop until the OD of the wells in row A (see Figure 2-2) reached 1. The OD was the read at 405 nm on a Biotek ELx800 microplate reader with Gen5 software.

		1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
B	**1M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
C	**2M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
D	**3M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
E	**4M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
F	**5M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
G	**6M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**
H	**7M**	**S1**	**S1**	**S2**	**S2**	**S3**	**S3**	**S4**	**S4**	**S5**	**S5**	**S6**	**S6**

Figure 2-2: Avidity ELISA plate layout

S1 – S6: Diluted serum samples are added in duplicate down the plate. NaSCN is added in increasing concentration (0 - 7M down the plate).

2.3.4.3 Isotype ELISAs

The isotype ELISAs for all trials were carried out as per Jenner Laboratory Protocol *J255: Human Isotype ELISA*. Briefly, six 96 well Nunc-Immuno Maxisorp plates were coated with the recombinant protein of interest (e.g. AMA1 at a concentration of 2 µg/mL), native human IgG1-4, IgA and positive control serum diluted 1:1000 as per Figure 2-3. One plate was coated for each isotype measured (IgG1-4, IgA and IgM). Plates were labelled IgG1, IgG2, IgG3, IgG4, IgA and IgM. All antigens and isotype controls were diluted in DPBS. Coated plates were left overnight.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
B	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
C	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
D	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
E	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
F	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
G	1	2	3	4	A	K	CA	CA	CA	CA	CA	CA
H	1	2	3	4	A	K	CA	CA	CA	CA	CA	CA

Figure 2-3: Plate coating layout for isotype ELISA

1-4 – Native human IgG1-4.
A – Native human IgA.
K – Kilifi serum, 5H at 1:1000 dilution.
CA- Coating antigen (e.g. AMA1)

Following a wash step (PBS/Tween x 6) plates were blocked for 1 hour with Casein. Serum samples were diluted at 1:100 and added to plates in duplicate after a wash step. Following a 2 hour incubation, plates were washed again with PBS/Tween before the corresponding secondary antibody (1:1000) was added (e.g. anti-human IgG1 added to IgG1 plate). Plates were left for 1 hour before a further wash step, then Extravidin-AP (1:5000) was added to all plates except the IgA plate (Extravidin-AP not required for this plate as anti-IgA antibody is already conjugated to AP) and plates were left for 30 minutes. Following a final wash step, DB was added and OD read at 405 nm on a Biotek ELx800 Microplate Reader with Gen5 software.

2.3.5 ASC ELISPOTs

ASC ELISPOTs were carried out as per Jenner Protocol *J205: Blood Stage Malaria Human ex-vivo ASC ELISPOT*. These assays were carried out by me, Sarah Silk and Sean Elias and were done in a Class II microbiological safety cabinet. Briefly, plates were coated with the antigen of interest (e.g. AMA1) (to detect antigen-specific IgG-secreting cells), PBS (negative control wells), polyvalent goat anti-human IgG (for detection of total IgG secreting cells) and diphtheria toxoid (positive control) and left overnight. Plates were washed three times with sterile PBS and then blocked with R10 for at least 1 hour. PBMC were retrieved from liquid nitrogen storage, thawed and counted using a Casycounter (see Section 2.3.2). Cells were then

re-suspended in R10 at a concentration of 5 x 10^6 cells/mL. The cell suspension was added to the plate with around 250,000 cells per well in the control wells (negative and positive) and some of the IgG and antigen wells. Dilutions of 125,000 cells and 50,000 cells were also added for the antigen wells, and a dilution of 10,000 cells per well was also added for two of the IgG wells. The plates were incubated at 37^{o}C overnight. The following day, plates were washed with PBS/Tween (six times) and secondary goat-anti-human IgG (γ chain specific) antibody was added (1:5000). Plates were left for 4 hours before a further wash step. BCIP/NBT development buffer was then added until spots had developed (3 - 5 minutes). Plates were washed and left to dry before spots were counted using an AID ELISPOT reader. The automated counts were checked and corrected by eye to ensure only spots consistent with IgG secreting ASCs were counted.

2.3.6 mBC ELISPOTs

mBC ELISPOT assays were carried out as per Jenner Protocol *J204: Blood-Stage Human Memory B cell ELISPOT*. These assays were carried out in a Class II microbiological safety cabinet by me, Sarah Silk and Sean Elias. PBMC were removed from liquid nitrogen storage and thawed as described in Section 2.3.2, before being re-suspended in R10 at a concentration of 2 x 10^6 cells/mL. A minimum of 6 million cells/volunteer were prepared. For each volunteer, 500 µL of the cell suspension were added to 6 wells of a 24 well plate, yielding 1 x 10^6 cells/well. A stimulation mix containing SAC (diluted 1:2400), PWM (diluted 1:6000) and CpG (diluted 1:200) was added to 5 of the 6 wells per volunteer, with R10 added to the final 'unstimulated' well. The 24 well plates were transferred to an incubator at 37^{o}C and 5% CO_2 for 6 days.

MAIP ELISPOT plates were prepared on day five by coating with the antigen of interest (e.g. AMA1) at the concentration specified in the protocol (typically 5 µg/mL) of the antigen solution for peak time-points for the stimulated cells. The antigen was also added to wells for the unstimulated cells for each volunteer. Positive and negative control wells were coated with Diphtheria toxoid and PBS respectively. Wells were also coated with polyvalent goat anti-

human IgG at 50 µg/mL, and at serial dilutions of 1:100 and 1:1000. The plate layout is shown in Figure 2-4. Once coated, plates were left overnight at 4°C.

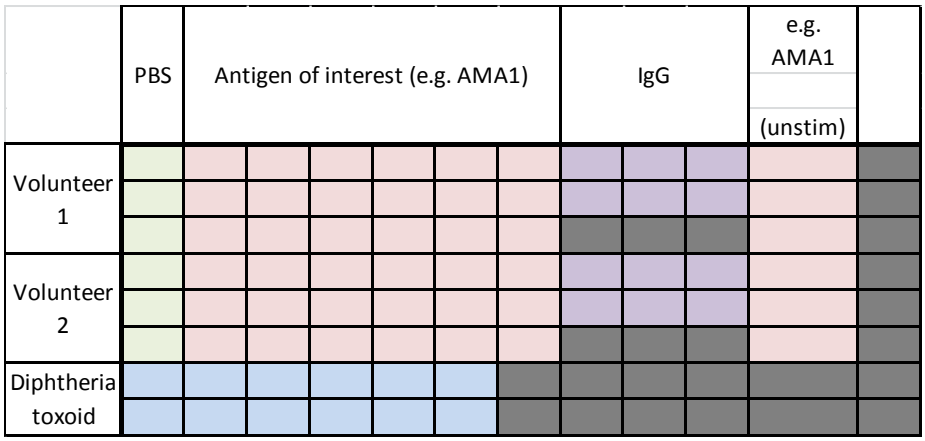


Figure 2-4: Plate layout for mBC ELISPOTs at peak time-points.

ELISPOT plates were coated with PBS (green) for negative control wells, the antigen of interest (e.g. AMA1) (pink) for stimulated cells and unstimulated cells (undiluted), polyvalent goat anti-human IgG (purple) and diphtheria toxoid (blue) for positive control wells. Remaining wells (blanks) were coated with PBS (grey).

On day six, plates were washed with sterile PBS and then blocked with R10 for at least 1 hour at 37°C. The cultured cells from the 24 well plates were harvested by gentle resuspension in R10. All stimulated cells from a single volunteer were re-suspended in the same 50 mL Falcon tube. Unstimulated cells were re-suspended in a 15 mL Falcon tube and all tubes were spun at 706 *xg* for 5 minutes. Supernatant was discarded and cells were re-suspended in R10 for a further wash step. Following this, cells were again re-suspended in R10 before being counted using the Casycounter. Cells were then re-suspended in R10 to a concentration of 2×10^6 cells/mL for stimulated cells and 1×10^6 cells/mL for unstimulated cells. Stimulated cells were added to the ELISPOT plate at 100 µL/well for control wells and for six of the antigen wells as well as two of the IgG wells for each volunteer. Dilutions of 1:2 and 1:5 of the cell suspension were added to six wells for each volunteer, and dilutions of 1:100 and 1:1000 were added to two wells for each volunteer. 100 µL of the unstimulated cell suspension was added to 3 wells for each volunteer. Plates were transferred to an incubator and left at 37°C and 5% CO_2 overnight.

On day seven, plates were developed using the same method described above for ASC ELISPOTs, and after being left to dry were counted on the ELISPOT reader as described for ASC ELISPOTs.

2.3.7 Preparation of *P. falciparum* inoculum for blood-stage CHMI (Chapter four)

The inoculum used for CHMI was produced by Drs Gregor Lawrence, Allan Saul and colleagues at QIMR in Brisbane, Australia in 1994 and consists of aliquots of *P. falciparum* (clone 3D7) infected erythrocytes taken from a single donor (140, 141, 166). Aliquots have been cryopreserved in vapour phase liquid nitrogen at -178^{o}C in the UK, and one of these was used for the VAC054 trial to infect all participants with *P. falciparum* malaria. Preparation was carried out as per Jenner Institute SOP *ML021: Preparation of malaria-infected blood for injection* (Appendix 8). This was carried out by Rebecca Brown and Simon Draper. Briefly, a single vial was thawed in a derogated containment level III laboratory area using solutions licensed for clinical use and single-use disposable consumables. A class II microbiological safety cabinet (MSC) was used to prepare the inocula. The MSC was fumigated with hydrogen peroxide and decontamination validated prior to use. 0.2 volume 12 % saline was added dropwise to 1.6 mL of thawed infected blood, left for 5 min, and an additional 10 volumes of 1.6% saline added dropwise. This was centrifuged for 4 min at 830 x*g*, the supernatant was removed, and 10 mL of 0.9% saline was added dropwise. The cell pellet was washed twice in 0.9% saline and re-suspended in 0.9% saline in a sterile syringe for injection. The injection volume per volunteer was 5 mL containing an estimated 1000 parasitised erythrocytes based on microscopic estimates of the donor's parasite density prior to freezing. The clinical inoculum was also added to aerobic and anaerobic culture bottles (BACTEC^{TM}) following preparation and shown to be negative for bacterial contamination.

Parasite viability was assayed by limiting dilution assay with a 10 day culture period as per Jenner Institute SOP *ML022: Blood Stage Challenge Viability Assay* (Appendix 8). The methods

were similar to those described previously for blood-stage CHMI trials at the Jenner Institute using the same inoculum (174, 175). Wells were scored positive or negative for replicating parasites using qPCR. A plate of identical dilutions of the inoculum that had been frozen without incubation was used as a negative control because the qPCR assay (see Section 2.3.9) can also detect dead parasites. There was no detectable amplification from negative control wells, and unincubated wells which had received a 100-fold greater parasite inoculum gave results of 323-445 arbitrary amplification units. Cultured wells plated at an estimated 1.5 parasites/well gave a clear bimodal distribution, with 20/40 wells giving results of <54 units (suggesting they contained no viable parasite), while 20/40 wells gave results of >24,000 units (suggesting they had contained at least one viable parasite at the start of the culture period). The number of viable parasites/mL of inoculum could then be calculated with reference to the Poisson distribution, and viability expressed as a percentage of the pre-freezing microscopy-estimated parasitaemia calculated using the RBC count/mL of inoculum. This was calculated to be 69%, i.e. approximately 690 viable parasites injected per volunteer.

2.3.8 Thick blood film preparation and interpretation (Chapter four)

Thick blood films were prepared and interpreted according to Jenner Institute SOP *ML009: Collection, preparation and slide reading: Malaria Challenge Studies* (Appendix 8). This work was carried out by experienced microscopists from Kemri, Kenya (Joseph Muita, Pauline Titus and Kebba Konteh), who were resident in Oxford for the duration of the CHMI period in VAC054. Briefly, a small drop of blood, collected from the volunteer in an EDTA vacutainer tube, was prepared on a microscope slide by spreading the drop thinly and allowing it to dry. The slide was then dipped in Field's stain A for 3 seconds, before being rinsed in clean water for 3 seconds and then dipped in Field's stain B for a further 3 seconds. The slide was allowed to dry and was then examined under oil immersion at high power (1000x) with 200 fields read by an experienced microscopist. Any parasites identified by the microscopist were reviewed by

either me or another clinical research fellow for visual confirmation. Thick blood films were not quantified for parasite density.

2.3.9 Parasite qPCR (VAC054; Chapter four)

Quantitative PCR was carried out on EDTA blood samples collected from participants after CHMI in the VAC054 trial and processed as per Jenner Institute SOP *ML008: Malaria qPCR* (Appendix 8). This work was led by Nick Edwards, with support from Jenner laboratory staff. Briefly, blood was filtered to reduce white cell content and DNA was extracted from filtered blood using the Qiagen Blood Mini Kit. 10% of each extraction (total eluate volume = 50 µL, with 5 µL used per assay) was run in triplicate for qPCR – equivalent to 150 µL blood directly assessed. The qPCR assay used Forward and Reverse primers 80 µMol scale (Custom; Applied Biosystems, 4304971), Taqman FAM-NFQ-MGB Probe 20 µMol scale (Custom; Applied Biosystems, 4316033) and Taqman Universal PCR Master Mix (with Amperase UNG; Applied Biosystems, 4304437). The Taqman probe-based PCR amplifies a 133 base pair (bp) product from the multicopy (three per parasite) 18S (small subunit) ribosomal RNA genes of *P. falciparum*. The probe then binds to the PCR product and is hydrolysed on each PCR cycle, releasing the fluorophore which is detected in a quantitative manner (176).

Parasites per mL (p/mL) equivalent mean values were generated by a standard Taqman absolute quantitation, against a defined plasmid standard curve with an ABI StepOne Plus machine and v2.3 software. Default Universal qPCR and quality control (QC) settings were used apart from the use of 45 cycles and 25 µL reaction volume. Based upon results obtained using dilution series of microscopically-counted cultured parasites, this method has a lower limit of quantification (LLQ, defined as % coefficient of variation [CV] <20%) of around 20 p/mL blood (177). Counted parasite dilution series results suggest that the lower limit of probable detection (LLD, i.e. a probability of >50% of ≥1 positive result among three replicate qPCR reactions) is in the region of 5 p/mL, while samples at 1 p/mL are consistently negative (24/24 qPCR reactions). Positive results in this assay are therefore essentially 100% specific for

genuine parasitaemia, with positive results beneath the LLQ likely to signify parasitaemia in the range 2-20 p/mL.

For quality control purposes, qPCR samples were re-tested if;

- Replicates included a mixture of positive and negative (in terms of amplification) results with one or more positive results >100 p/mL.
- The % CV of any results were high outliers.

Following the QC steps above, qPCR data, including any 0 values, were used to generate the mean result for each time-point.

2.3.10 Parasite multiplication rate (PMR) modelling (Chapter four)

Modelling of qPCR-derived PMR was carried out by Sandy Douglas and Simon Draper. This had been pre-specified in the VAC054 trial protocol as the primary study endpoint, and the comparison of the endpoint between the two groups constituted the pre-specified primary analysis for vaccine efficacy.

The arithmetic mean of the three replicate qPCR results obtained for each individual at each time-point was used for model-fitting. Negative individual replicates were assigned a value of 0 p/mL for the purposes of calculating the arithmetic mean of triplicates (where at least one of the three readings was positive). As previously reported, qPCR data points which, based upon the mean of the three replicates, are negative or below the LLQ (< 20 p/mL) up until the first quantifiable result for that subject (i.e. ≥20 p/mL) were treated as missing, while negative or sub-quantifiable data points after that subject's first quantifiably positive result were replaced with values of half the LLQ (i.e. 10 p/mL) (177). PMR was then calculated using a linear model fitted to $\log_{10}$-transformed qPCR data (177).

On the day of CHMI, all volunteers were inoculated by 11:30am, within 2 hours and 13 minutes of the inoculum being thawed. For modelling purposes, the follow-up visits, where blood samples were collected for thick blood film and qPCR were taken to occur at 9:00am in the morning (i.e. dC+1 was 0.9 d post-infection), and 6:00pm in the evening (i.e. 0.37 d later). As

previously, fitted lines were constrained to pass through the known starting parasitaemia, calculated from the results of the limiting-dilution-based assay of the number of viable parasites in the inoculum, and a weight-based estimate of each volunteer's blood volume (70 mL/kg) (83). PMR was modelled for all volunteers that underwent blood-stage CHMI, given they all had ≥5 data points above the LLQ (the criterion for proceeding to model the PMR) (83).

2.3.11 Functional assays of immunity

2.3.11.1 PvDBP – DARC Binding inhibition assays (Chapter three)

The functional activity of IgG induced by vaccination with ChAd63/MVA PvDBP in VAC051 was assessed by a binding inhibition assay. These assays were carried out at the Jenner Institute by David Llewellyn and Tom Rawlinson, according to Jenner Laboratory Protocol *J363: DARC-Duffy binding protein binding inhibition assay with human and mouse serum*. Briefly, 96-well ELISA plates were coated with recombinant DARC (His-tagged) and incubated at 4^{o}C overnight. Plates were washed with PBS/Tween (x 6) the following day and blocked with 2% milk (diluted in PBS) for 2 hours at 37^{o}C. Recombinant PvDBP_RII was diluted to 0.1 µg/mL in 0.25% milk (in PBS). Serum was also diluted in 0.25% milk. Recombinant PvDBP and sera were combined 30 minutes before the end of the blocking incubation and left at room temperature. Following the blocking incubation, the ELISA plates were washed in PBS/Tween (x 6) and the PvDBP_RII-serum mix was added to each well, excluding negative control wells (PvDBP_RII incubated with day 0 serum or no serum). The plates were then incubated at 37^{o}C for 1 hour. After a further wash step, diluted serum from rabbits immunised with PvDBP_RII (157) (1:1000 in 0.25% milk) was added to each well. Plates were left for 1 hour then washed again before anti-rabbit IgG AP 1:1000 antibody was added to each well. The plates were left for a further hour before a final wash step. DB was then added and the OD read at 405 nm on Bio-tek ELx800 Microplate Reader with Gen5 software. Plates were developed to a point determined by the OD of the development controls wells (i.e. when the negative control wells, which did not contain any potential blocking agents, reached an OD = 1.0).

Serum was also sent to the International Centre for Genetic Engineering and Biotechnology (ICGEB), India were DARC-PvDBP binding inhibition was assessed by Rushdi Shakri (178). At ICGEB, sera were analysed for binding inhibition against four variants of PvDBP_RII: Sal I (homologous to the vaccine), PvP, PvAH and PvO. Two kinds of binding assays were used. The first of these was a Bioplex-based assay in which recombinant DARC-Fc was coated on magnetic, fluorescent beads and recombinant PvDBP_RII with C-terminal 6-His tag was allowed to bind in the presence or absence of sera from volunteers in VAC051. Binding was detected with anti-6-His monoclonal antibodies. The second of these assays was an ELISA-based assay (178). Briefly, recombinant DARC-Fc was coated on an ELISA plate and recombinant PvDBP_RII was allowed to bind in the presence or absence of sera from VAC051 volunteers. Binding was detected with antibodies against the 6-His tag. Standard curves based on known amounts of PvDBP_RII were developed for both the Bioplex and ELISA based binding assays and used to determine the percent inhibition.

2.3.11.2 In vitro assay of GIA (Chapters four & five)

Serum samples for vaccinated volunteers in VAC054 and VAC057 were sent to the NIH Reference Center in the USA to assess GIA. This work was led by Kazutoyo Miura. The methods used in this assay have been published previously (130, 131). Briefly, IgG was purified from serum and concentrated to 10 mg/mL. The purified IgGs were preadsorbed with uninfected human O^+ red blood cells for 1 hour in order to remove any anti-human erythrocyte antibodies and were sterilised and heat inactivated before being used in the assay. Each test IgG was incubated with human erythrocytes which contained late trophozoite and schizont stages of *P. falciparum* parasites prepared by Percoll gradient and/or 5% sorbitol treatment. GIA was assessed over a single growth cycle, measuring parasite LDH after 40 hours of culture and using this to calculate relative parasitaemia levels. Results obtained using the test IgGs were compared with those obtained with parasites incubated with a pool of malaria-naïve human serum and uninfected erythrocytes as controls (131). The IgG samples were all initially tested

at 10 mg/mL, followed by a dilution series for positive samples to determine the concentration of purified IgG that gave 50% GIA (EC_{50}).

2.4 Statistical analyses

The immunology data for all trials were analysed using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., California, USA). A value of $P < 0.05$ was considered significant. Safety data were analysed in Microsoft Excel 2010; studies were not powered to analyse statistically significant differences in safety data so these were assessed descriptively.

The Phase Ia trials (VAC051 and VAC057) were powered to detect a mean two-fold improvement in either T cell or antibody immunogenicity between the two boosted groups (Group 2B and Group 2C) at a significance level of $P = 0.05$ with 76% power.

The VAC054 study was powered to detect a 33% decrease in mean PMR in vaccinees compared with controls with ≥80% power. The power calculations for this study were performed by Nicola Williams at the Centre for Statistics in Medicine at the University of Oxford using data from previous blood-stage CHMI trials in Oxford and at the Radboud University Nijmegen Medical Centre in the Netherlands (179). These historical data suggested the CV in the controls may range from 22% (Nijmegen where the mean PMR was 10) to 33% (Oxford where the mean PMR was 12). Given the logistical challenges of a blood-stage CHMI study, the decision was made not to include any more than thirty volunteers. A study design with 15 controls versus 15 vaccinees consistently provided the best power to observe a 33% reduction in mean PMR when allowing for this CV in the controls, and an increased CV in the vaccinees.

Chapter three:

A Phase Ia clinical trial to assess the safety and immunogenicity of new *Plasmodium vivax* malaria vaccine candidates ChAd63/MVA PvDBP (VAC051)

3.1 Authorship statement

I set up the VAC051 clinical trial with the assistance of Alison Lawrie, Rachel Roberts, Ian Poulton, Susanne Hodgson, Adrian Hill and Simon Draper. This involved preparation of the study documents with submission for ethical and regulatory approval. The Chief Investigator (CI) for this trial was Adrian Hill.

I screened and enrolled volunteers for the trial. Vaccinations and follow-up visits were conducted by me and also by the nursing team: Ian Poulton, Mary Smith and Adrienne Cook.

Laboratory assays in Oxford were carried out by me and by Kathryn Milne, Sarah Silk, Sean Elias and Tom Rawlinson. Assays were also carried out at the ICGEB by Rushdi Shakri. Details about who carried out each assay can be found in Section 1.3.3.

3.2 Introduction

3.2.1 Development of a blood-stage *P. vivax* vaccine

A viral vectored *Plasmodium vivax* vaccine has been developed at the University of Oxford in collaboration with Chetan Chitnis at the ICGEB, India. Similar to vaccine candidates for *P. falciparum* previously developed at the Jenner Institute in Oxford, this uses the simian adenovirus ChAd63 and MVA in a heterologous prime-boost regime. The antigen in these vaccines is the Duffy-binding protein region II (PvDBP) of the *P. vivax* malaria parasite Salvador I strain, and these vaccines are therefore referred to as ChAd63 PvDBP and MVA PvDBP (157). The VAC051 Phase Ia clinical trial was the first trial to use the ChAd63/MVA PvDBP vaccines in humans.

3.2.2 PvDBP as an antigen

The micronemal parasite ligands (Duffy-binding proteins, DBP or erythrocyte binding antigens, EBAs) are a family of antigens that are functionally conserved across *Plasmodium* species and are thought to be involved in the tight attachment step between the parasite and new host cell, but only one gene copy exists in *P. vivax*. Knockout studies of the orthologous *P. knowlesi*

DBPα gene prevent invasion of Duffy-positive erythrocytes by this highly related parasite *in vitro* (180).

Unlike *P. falciparum* which utilises multiple redundant invasion pathways for human erythrocyte invasion (2, 17), *P. vivax* requires interaction with DARC in the vast majority of cases. Interaction with DARC is mediated by PvDBP, in particular with the receptor-binding domain which maps to a conserved cysteine-rich region, referred to as region II. The Duffy-binding protein belongs to the EBL family of proteins found in all *Plasmodium* species. The other family of *Plasmodium* proteins known to be involved in red blood cell invasion are the RBL family. In *Plasmodium vivax* this family of proteins are referred to as reticulocyte binding proteins (PvRBPs), whereas in *P. falciparum* they are referred to as reticulocyte binding protein homologues (PfRHs) (26). *P. vivax* was initially thought to express two reticulocyte binding proteins, PvRBP1 and PvRBP2 which bind specifically to reticulocytes and not normocytes, presumably explaining the host-cell preference of *P. vivax* (2). However, since the *P. vivax* genome sequence became available it has been demonstrated that there are in fact many more than two members in the *Pvrbp* gene family, with 8 genes predicted to be protein-coding (2 of these correspond to the genes encoding the originally discovered PvRBP1 and PvRBP2 proteins, *Pvrbp1a* and *Pvrbp2c*) (26). There are some significant similarities between some of the PvRBP proteins and some of the PfRH proteins, which are known to be involved in invasion of red blood cells in *P. falciparum* infection. This suggests that these proteins may be involved in reticulocyte sensing, and even in erythrocyte invasion (26, 181). In particular, the PvRBP2a protein shows significant similarity with PfRH5 which is essential for the invasion erythrocytes by *P. falciparum* (this protein is discussed in more detail in Chapter five). The crystal structures for both of these proteins have been determined (181, 182) and show similar general architecture, although also some striking differences have also been identified, particularly in terms of the surface properties. PvRBP2a has been shown to bind erythrocytes (not only reticulocytes) but the erythrocyte surface receptor has not yet been identified (181).

Individuals who lack the Duffy blood group antigen are resistant to blood-stage *P. vivax* infection, providing evidence for the non-redundant nature of this invasion pathway. Duffy blood group negativity has essentially led to the disappearance of *P. vivax* from much of sub-Saharan Africa and has arisen independently in Papua New Guinea (16). However, it is likely there are other invasion pathways possible, in which the RBPs may be implicated, as *P. vivax* infections have been reported to occur in Duffy-negative individuals (183-187), although infections are still less likely than in Duffy-positive individuals (187).

The induction of binding inhibitory antibodies against the receptor binding residues within PvDBP region II does not commonly occur following natural exposure (188, 189). However, these antibodies (when they occur) were associated with strain-transcending protection against *P. vivax* re-infection in a prospective cohort study in Papua New Guinea (31). Importantly, the presence of naturally-acquired high-titre binding inhibitory antibodies against PvDBP_RII were associated with reduced risk of *P. vivax* infection, as well as lower *P. vivax* parasite densities, following infection in the prospective study. This observation suggests that antibodies produced following vaccination with PvDBP_RII should be able to block diverse strains of *P. vivax* (16). Similar observations show that antibodies raised against recombinant SalI strain PvDBP_RII in rabbits can inhibit binding of heterologous polymorphic PvDBP_RII domains derived from diverse field isolates (105).

Immunisation with recombinant PvDBP_RII can elicit high-titre antibodies in mice and monkeys that block binding of this antigen to recombinant DARC or Duffy-positive erythrocytes by *in vitro* assay (157, 190, 191). Following intravenous challenge of New World *Aotus* monkeys with blood-stage *P. vivax*, longer pre-patent periods and lower parasitaemias were observed in immunised animals in comparison to controls. Protection was conferred following immunisation with protein vaccine in Freund's adjuvant but not Montanide ISA 720 (although antibody levels induced by ISA 720 were surprisingly low in this study) (107).

In this Chapter I will describe the first clinical trial to assess a vaccine against the blood stage of *P. vivax*, using the viral vectored vaccines previously developed in Oxford, ChAd63 PvDBP and MVA PvDBP. As this was a first-in-human trial it was primarily conducted to assess safety in healthy malaria-naïve adult volunteers, through collection and analysis of adverse event data. The immunogenicity of the vaccines was also assessed and the results of these assays are also discussed in this Chapter, along with the safety data.

3.3 VAC051 Methods

Detailed methods of the recruitment and enrolment of volunteers, as well as the assays used in this trial can be found in Chapter two: Materials and Methods.

3.3.1 VAC051 Study Design

This study was a Phase Ia open-label, dose escalation, first-in-human, non-randomised trial of the viral vectored vaccines ChAd63 PvDBP and MVA PvDBP given in a prime-boost regime with an eight week interval (Figure 3-1). The study was conducted at the Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, UK. Healthy, malaria-naïve males and non-pregnant females aged 18-50 were invited to participate in the study. Allocation to study groups occurred at screening based on sequential recruitment of groups and volunteer preference.

Groups 1 and 2A received ChAd63 PvDBP alone on Day 0. Groups 2B and 2C received the ChAd63 PvDBP vaccine at day 0 and the MVA PvDBP vaccine at day 56 (nominal study days are reported throughout; a window of 7 days either side of day 56 was permitted in the study protocol). The sample size for this study was chosen to allow determination of the magnitude of the outcome measures, especially of serious and severe adverse events, rather than aiming to obtain statistical significance. Four volunteers were enrolled into Groups 1 and 2A as initial safety cohorts. The decision to enrol eight volunteers into Groups 2B and 2C was based on a power calculation to have 76% power (1-β) to detect a mean two-fold improvement (two-

tailed) at a significance level (α) of $P = 0.05$ in immunogenicity (IgG and T cell response) between Groups 2B and 2C following MVA PvDBP boost vaccination.

Group Number		Number of volunteers	Dose ChAd63 PvDBP	Dose MVA PvDBP
1		4	5 x 10^9 vp IM	--
2	**A**	4	5 x 10^{10} vp IM	**--**
	B	8	5 x 10^{10} vp IM	1 x 10^8 pfu IM
	C	8	5 x 10^{10} vp IM	2 x 10^8 pfu IM

Figure 3-1: VAC051 study groups

3.3.2 VAC051 Ethics

The study received ethical approval from the Oxfordshire Research Ethics Committee A in the UK (REC reference 13/SC/0001). The study was also reviewed and approved by the MHRA (reference 21584/0312/001-0001). Volunteers signed written consent forms and consent was verified before each vaccination. The trial was registered on Clinicaltrials.gov (NCT 01816113).

3.3.3 VAC051 Study Objectives and Endpoints

Primary Objective

To assess the safety of ChAd63 PvDBP when administered alone, and in heterologous prime-boost with MVA PvDBP in healthy volunteers.

Primary Outcome Measures

The specific endpoints for safety and reactogenicity were actively and passively collected data on adverse events. These data were collected using diary cards which were completed by volunteers for 14 days following ChAd63 PvDBP and 7 days following MVA PvDBP, as well as

recording any adverse event reported or detected (e.g. laboratory abnormalities) at clinic visits throughout the trial.

Secondary Objectives

To assess the cellular and humoral immunogenicity of ChAd63 PvDBP when administered alone, and in a heterologous prime-boost regime with MVA PvDBP in healthy volunteers.

Secondary Outcome Measures

Immunogenicity outcome measures following ChAd63/MVA PvDBP included:

- Induction of antigen-specific T cells (assessed by *ex-vivo* IFN-γ ELISPOT)
- Induction of antigen-specific IgG (assessed by anti-PvDBP ELISA)
- Measurement of antigen-specific ASCs and mBCs following vaccination.
- Functional activity of antigen-specific IgG (assessed using a PvDBP – DARC binding inhibition assay).

T cell ELISPOT assays, PvDBP ELISA assays, ASC ELISPOT assays and mBC ELISPOT assays were carried out in the Jenner Institute laboratories in Oxford. Kathryn Milne and Sarah Silk carried out the T cell ELISPOT s, Sarah Silk and I carried out the ELISA assays and I carried out the ASC and mBC ELISPOT assays, with support from Sean Elias. The PvDBP – DARC binding inhibition assays were carried out both Oxford by Tom Rawlinson and in a collaborating laboratory outside of the UK by Rushdi Shakri- at the ICGEB. Volunteers were consented for samples to be shipped to collaborating laboratories.

3.3.4 VAC051 Participants

All volunteers were recruited and vaccinated at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), part of the Oxford Vaccine Centre (OVC) at the University of Oxford. Twenty four volunteers were enrolled in total. All volunteers signed written consent forms, and consent was checked to ensure volunteers were willing to proceed before each vaccination. The inclusion and exclusion criteria for participation are described below.

3.3.4.1 VAC051 Inclusion Criteria

The volunteer had to satisfy all the following criteria to be eligible for the study:

- Healthy adult aged 18 to 50 years.
- Able and willing (in the Investigator's opinion) to comply with all study requirements.
- Willing to allow discussion of their medical history with their GP.
- For females only, willingness to practice continuous effective contraception during the study and a negative pregnancy test on the days of screening and vaccination.
- Agreement to refrain from blood donation during the course of the study.
- Provision of written informed consent.

3.3.4.2 VAC051 Exclusion Criteria

The volunteer could not enter the study if any of the following applied:

- Participation in another research study involving receipt of an investigational product in the 30 days preceding enrolment or during the study period.
- Prior receipt of an investigational malaria vaccine or any other investigational vaccine likely to impact on interpretation of the trial data.
- Administration of immunoglobulins and/or any blood products within the three months preceding vaccination.
- 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 were allowed).
- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, e.g. egg products, Kathon.
- History of clinically significant contact dermatitis.
- Any history of anaphylaxis in reaction to vaccination.
- Pregnancy, lactation or willingness/intention to become pregnant during the study.
- History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ).
- History of a serious psychiatric condition.

- Any other serious chronic illness requiring hospital specialist supervision.
- Suspected or known alcohol abuse as defined by an alcohol intake of greater than 42 units every week.
- Suspected or known injecting drug abuse in the 5 years preceding enrolment.
- Seropositive for hepatitis B surface antigen (HBsAg).
- Seropositive for hepatitis C virus (antibodies to HCV) with positive PCR for hepatitis C at screening.
- History of clinical malaria (any species).
- Travel to a malaria endemic region during the study period or within the previous six months.
- Any clinically significant abnormal finding on screening biochemistry or haematology blood tests or urinalysis.
- 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.
- Inability of the study team to contact the volunteer's GP to confirm medical history and safety to participate.

3.3.5 ChAd63 PvDBP and MVA PvDBP Vaccines

The ChAd63 PvDBP and MVA PvDBP vaccines are described in Chapter two, Section 2.1.1. Final certification of these products and associated labelling took place at the CBF in Oxford by a qualified person (QP).

An eight week interval between adenovirus prime and heterologous MVA boost has been shown to optimise IgG induction in animal models as well as previous clinical trials (192). This was therefore chosen as the regime for this study.

3.3.6 VAC051 Interventions

Vaccination of volunteers was carried out at the CCVTM in Oxford. Vaccines were stored at -80°C in a locked freezer. Administration of vaccines was carried out by me and a research nurse after I had checked consent and ongoing eligibility (i.e. no change in medical status or

medications that would exclude the volunteer from the trial). One of us was required to give the vaccine and the other to check the volume drawn and countersign the procedure. Vaccines were all administered intramuscularly (IM) into the deltoid muscle. If more than one vaccine was given (i.e. volunteers in Groups 2B and 2C) these were given into opposite arms. The Investigator administering the vaccine wore an apron, gloves and eye protection. The vaccines are genetically modified organisms (GMOs) and therefore all waste from a vaccination procedure was autoclaved to minimise dissemination of the recombinant vectored vaccine virus into the environment. This is in accordance with UK Genetically Modified Organisms (Contained Use) Regulations (2000).

Volunteers were required to remain at the CCVTM for an hour after vaccination (2 hours for the first recipient of a new vaccine dose). Their vital signs and the vaccination site were checked at 30 minutes (at which time the vaccination site dressing was removed and discarded as GMO waste) and 60 minutes to check for evidence of any immediate reactions to the vaccine.

Four volunteers were vaccinated with 5×10^9 vp ChAd63 PvDBP (diluted in 0.9% NaCl and administered in 310 µL) in Group 1. Following a safety review, twelve volunteers (Groups 2A and 2B) were vaccinated with 5×10^{10} vp ChAd63 PvDBP. Volunteers in Group 2B were boosted with MVA PvDBP 1×10^8 pfu in the opposite arm 8 weeks later. A further safety review was carried out before the final eight volunteers received full dose (2×10^8 pfu) MVA PvDBP eight weeks after vaccination with ChAd63 PvDBP 5×10^{10} vp (Group 2C). Volunteers in Group 1 were followed up for approximately 3 months, and volunteers in Group 2 were followed up for approximately 5 months. Volunteers attended follow-up visits on days 2, 14, 28, 56 and 84 in Group 1; days 0, 2 ,14, 28, 56, 63, 84 and 140 in Group 2A; and days 0, 2, 14, 28, 56, 58, 63, 84 and 140 in Groups 2B and 2C. Safety data were collected throughout the study, as detailed in the next Section. Baseline antibody and T cell responses were checked on day 0.

3.3.7 Assessment of Safety of ChAd63/MVA PvDBP

Safety data were assessed by actively and passively collected data on adverse events occurring throughout the VAC051 trial. Volunteers were asked to complete a paper diary card for 14 days after vaccination with ChAd63 PvDBP and a second diary for7 days after vaccination with MVA PvDBP, with details of any adverse events experienced during these periods, and any medication taken. Severity grading of adverse events was as described in Chapter two. A longer diary card period was chosen following the initial vaccination because, at the time, there had been less experience of the ChAd63 viral vector compared with MVA. Adverse event data were also collected at all follow-up visits by study staff who asked the participant to recall any adverse events since the last visit, which were then recorded in the participant's CRF. Blood tests for safety (full blood count [FBC], liver function tests [LFTs], urea and electrolytes [U&Es]) were carried out at all visits after vaccination except days 2 and 58.

Prior to each dose escalation (i.e. between Groups 1 and 2 for ChAd63 PvDBP, and between Groups 2B and 2C for MVA PvDBP) safety of the vaccines was reviewed by the Local Safety Monitor (LSM), who chairs the Local Safety Committee (LSC). The LSC consists of at least two other appropriately qualified committee members. The LSM reviewed a report on the adverse event profiles of the vaccines (solicited and unsolicited adverse events) and advised on whether the study should proceed. The LSM for this study was Dr Brian Angus, a Clinical Tutor in Medicine, Honorary Consultant Physician and Director, Centre for Tropical Medicine at the University of Oxford.

3.4 VAC051 Results

3.4.1 VAC051 Participant Flow

Thirty volunteers were screened in total, of which twenty four were enrolled. Recruitment took place between May 2013 and February 2014. Four volunteers were recruited to Groups 1 and 2A, and eight volunteers to Groups 2B and 2C. In total, fifteen females and nine males were enrolled. The mean age of volunteers was 25 years 9 months (range 18 – 40 years). Four

volunteers were enrolled into Group 1 and received 5 x 10^9 vp of the ChAd63 PvDBP vaccine. There was a 3 week interval between vaccination of the final Group 1 volunteer and the first Group 2 volunteer to allow a safety review prior to dose escalation of ChAd63 PvDBP to 5 x10^{10} vp. Four volunteers in Group 2A received ChAd63 PvDBP alone. Eight volunteers were enrolled into Groups 2B and 2C. These volunteers received ChAd63 PvDBP followed 8 weeks later with a 'boost' vaccination of MVA PvDBP at a dose of 1 x 10^8 pfu (Group 2B) or 2 x 10^8 pfu (Group 2C). There was a 2 week interval between the final vaccination in Group 2B with MVA PvDBP at the lower dose of 1 x 10^8 pfu and the first vaccination with MVA PvDBP 2 x 10^8 pfu in Group 2C, with a safety review prior to dose escalation. One volunteer withdrew from Group 2B prior to the MVA PvDBP vaccination due to personal commitments. Following a discussion with the safety monitor, the decision was made not to replace her but continue the study with a total of 7 volunteers in Group 2B. The trial flow diagram is shown in Figure 3-2.

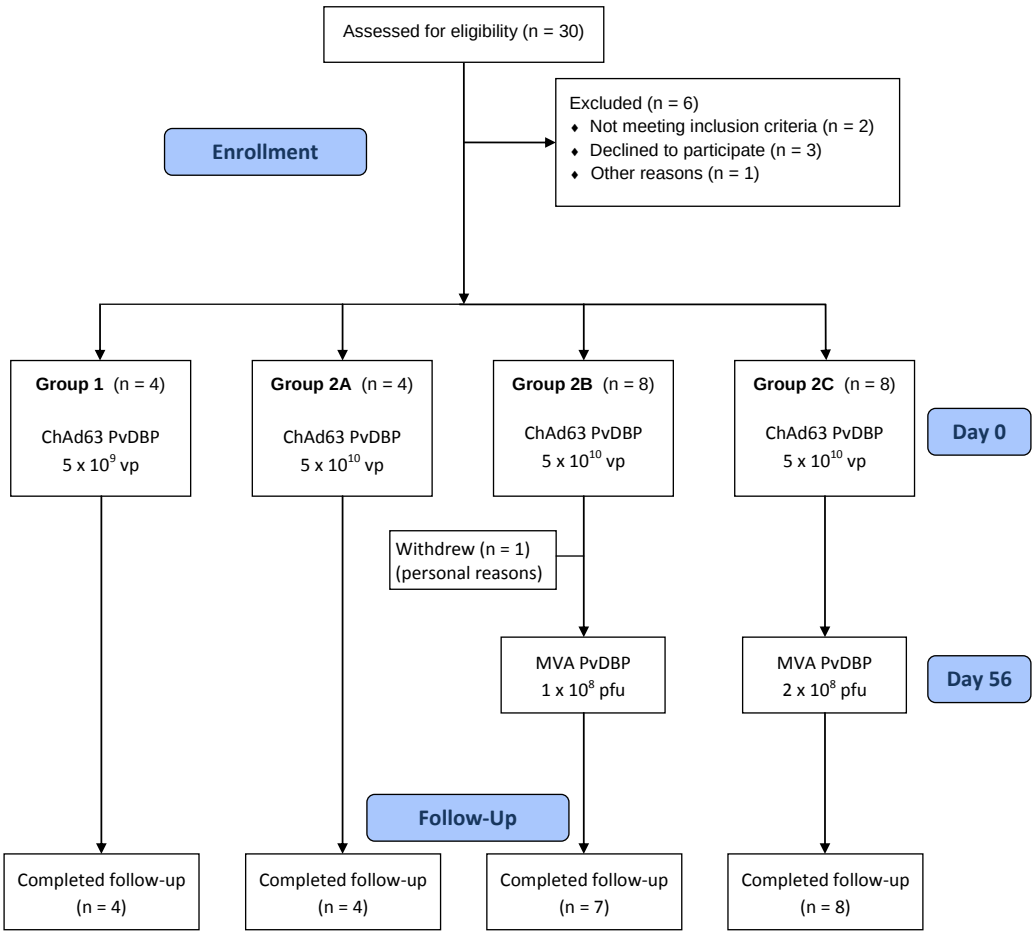


Figure 3-2: VAC051 flow chart of study design and volunteer recruitment.

3.4.2 VAC051 Vaccine safety and reactogenicity

There were no SAEs or unexpected reactions during the course of the trial and no volunteers withdrew due to vaccine-related AEs. ChAd63 PvDBP and MVA PvDBP demonstrated favourable safety profiles, similar to those seen in previous clinical trials with the same viral vectors (with different malaria antigens) at similar doses in healthy adults (84, 155).

The maximum severity of solicited AEs reported by volunteers following ChAd63 PvDBP is shown in Figure 3-3. All AEs following ChAd63 PvDBP 5 x 10^9 vp were mild, as were the vast majority following the higher dose in Group 2, although some volunteers did report moderate or severe adverse events following the higher dose. The majority of solicited adverse events following ChAd63 PvDBP occurred within 48 hours of vaccination (Figure 3-5A, B).

Unsolicited adverse events occurring after ChAd63 PvDBP and deemed possibly, probably or definitely related to vaccination were all mild in nature. These are shown in Table 3.1.

There was only one laboratory adverse event following ChAd63 PvDBP that was considered possibly, probably or definitely related to vaccination. This was mild lymphopenia in one volunteer vaccinated with ChAd63 PvDBP 5 x 10^{10} vp which resolved spontaneously.

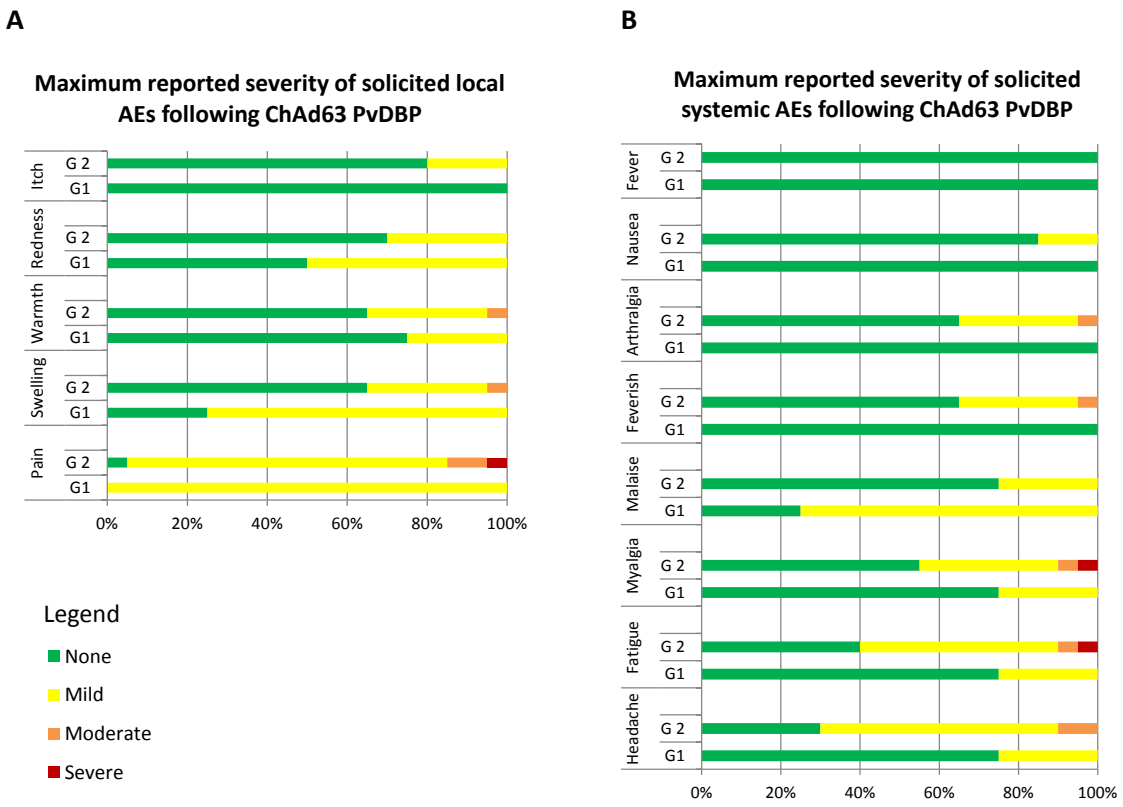


Figure 3-3: Solicited local and systemic AEs following ChAd63 PvDBP.

Only the highest intensity of each AE per subject is listed. Data are combined for all AEs for all volunteers receiving the same vaccine at the stated dose. ***(A)*** *Local AEs post ChAd63 PvDBP at 5 x 10^9 vp (G1; 4 volunteers) and 5 x 10^{10} vp (G2; 20 volunteers).* ***(B)*** *Systemic AEs post ChAd63 PvDBP at 5 x 10^9 vp (G1) and 5 x 10^{10} vp (G2). Data were exported from the OpenClinica database into Excel and the percentages of volunteers experiencing each AE following vaccinations at different doses were calculated.*

Unsolicited AEs post ChAd63 PvDBP 5 x 10^9 vp	**AE start day**	**Severity**
Bad dreams overnight	1	Mild
General malaise following lack of sleep and drinking alcohol	2	Mild
Unsolicited AEs post ChAd63 PvDBP 5 x 10^{10} vp	**AE start day**	**Severity**
Arthralgia in hip area	0	Mild
Blocked nose	0	Mild
Bruising at site of vaccination	1	Mild
Coryzal symptoms	1	Mild
Unsolicited AEs post MVA PvDBP 1 x 10^8 pfu	**AE start day**	**Severity**
Bruise at vaccine site	0	Mild
Unsolicited AEs post MVA PvDBP 2 x 10^8 pfu	**AE start day**	**Severity**
Sore throat	0	Mild
Dry cough	1	Mild
5mm diameter red mark 2 inches from vaccination site, felt warm	1	Mild
Slight tightness of the chest	5	Mild
Raised lymph nodes in neck	3	Mild

Table 3.1: Unsolicited adverse events considered possibly, probably or definitely related to vaccination with ChAd63 PvDBP or MVA PvDBP.

AE data were extracted from the Openclinica database and grouped by vaccination dose. Causality was assessed as per the criteria in Table 2.4

Solicited adverse events following MVA PvDBP at 1 – 2 x 10^8 pfu are shown in Figure 3-4A (local) and Figure 3-4B (systemic). The higher dose of MVA PvDBP was more reactogenic with half of the volunteers reporting at least 1 severe AE, although no systemic AE was reported as severe for more than 24 hours. Reactogenicity following MVA PvDBP peaked in the first two days after vaccination (Figure 3-5C, D).

Unsolicited AEs following MVA PvDBP that were considered possibly, probably or definitely related to vaccination were mild in nature and are shown in Table 3.2.

There was only one laboratory AE following MVA PvDBP that was considered possibly, probably or definitely related to vaccination. This was moderate eosinophilia in one volunteer vaccinated with MVA PvDBP 1 x 10^8 pfu which peaked more the 4 weeks after vaccination and resolved spontaneously.

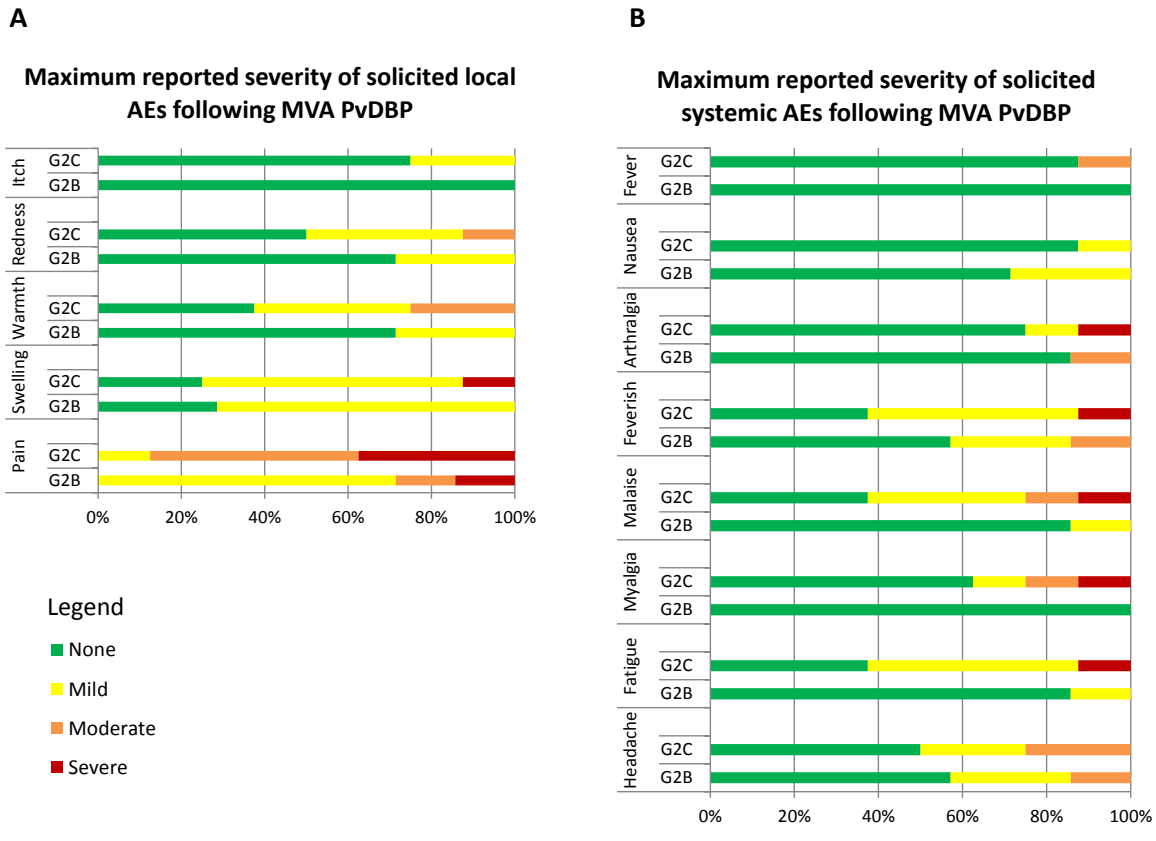


Figure 3-4: Solicited local and systemic AEs following MVA PvDBP.

Only the highest intensity of each AE per subject is listed. Data are combined for all AEs for all volunteers receiving the same vaccine at the stated dose. There were no immunisation related serious AEs. ***(A)*** *Local AEs post MVA PvDBP 1 x 10^8 pfu (G2B; 7 volunteers) and MVA PvDBP 2 x 10^8 pfu (G2C; 8 volunteers).* ***(B)*** *Systemic AEs post MVA PvDBP (G2B and G2C). Data were exported from the OpenClinica database into Excel and the percentage of volunteers experiencing each AE following vaccinations at different doses were calculated.*

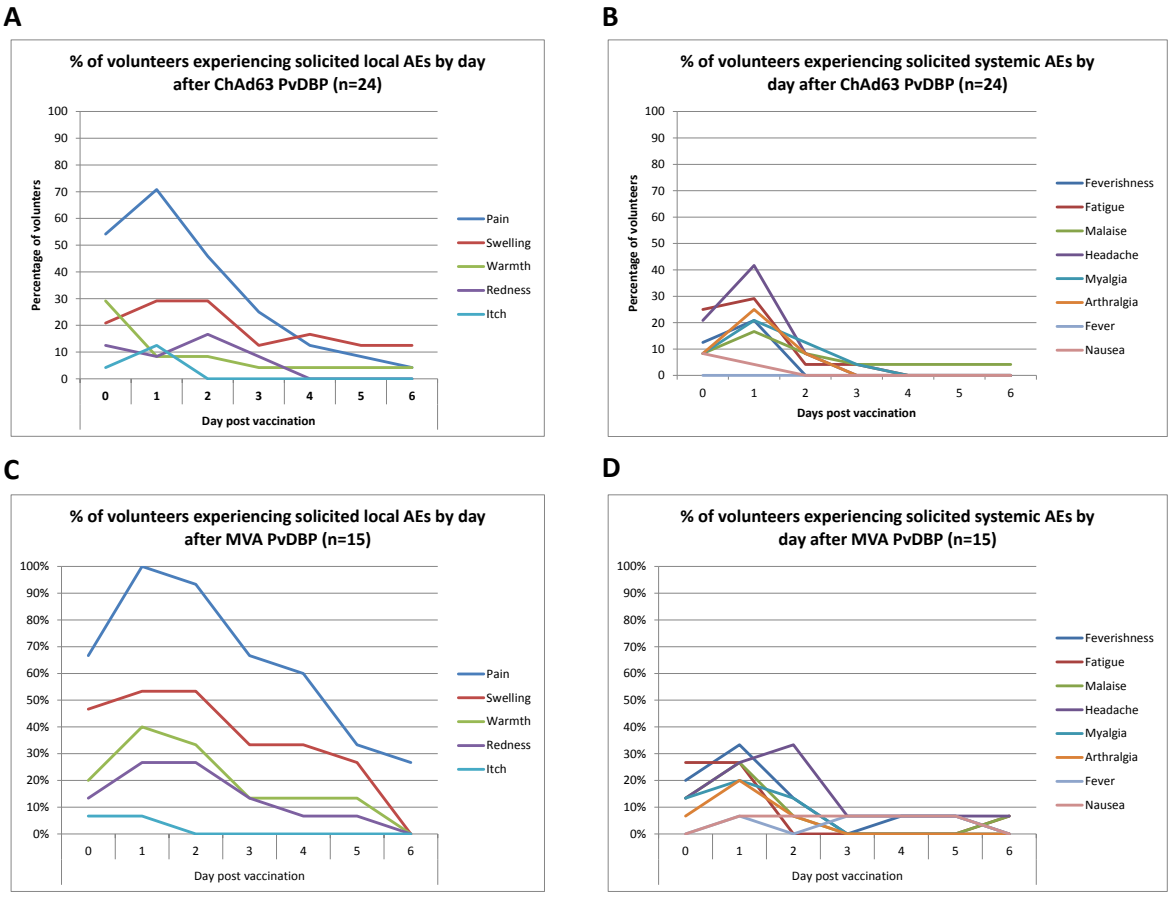


Figure 3-5: VAC051 Percentage of volunteers reporting solicited adverse events by day.

Data shown for all volunteers post ChAd63 PvDBP (A, B) and MVA PvDBP (C, D), regardless of dose received. Data were exported from the Openclinica database into Excel and the percentages of volunteers reporting each solicited AE by day post vaccination were calculated.

3.4.3 ChAd63/MVA PvDBP T cell immunogenicity assessed by *ex-vivo* IFN-γ ELISPOT

The kinetics and magnitude of the T cell response to PvDBP were assessed over time by *ex-vivo* IFN-γ ELISPOT following re-stimulation of PBMC with overlapping peptides spanning the entire PvDBP_RII insert present in the viral vectored vaccines.

Vaccination with ChAd63 PvDBP and MVA PvDBP induced antigen-specific T cell responses in all volunteers as measured by *ex-vivo* IFN-γ ELISPOT, with median group responses shown in Figure 3-6A Following the ChAd63 PvDBP prime at doses of 5 x 10^9 vp (Group 1 [G1]) and 5 x 10^{10} vp (Group 2 [G2]) there was no significant difference between the groups two weeks after vaccination, Figure 3-6B. However, one week after the MVA PvDBP boost (Figure 3-6C) there

were significantly stronger median responses in G2B and G2C compared to G2A who did not receive the boost (median of 368, 2061 and 2459 SFU/million PBMC in G2A, G2B and G2C respectively). There was a trend towards better maintained responses at day 140 in G2C compared with G2B (median 1871 vs 385.3 SFU/million PBMC).

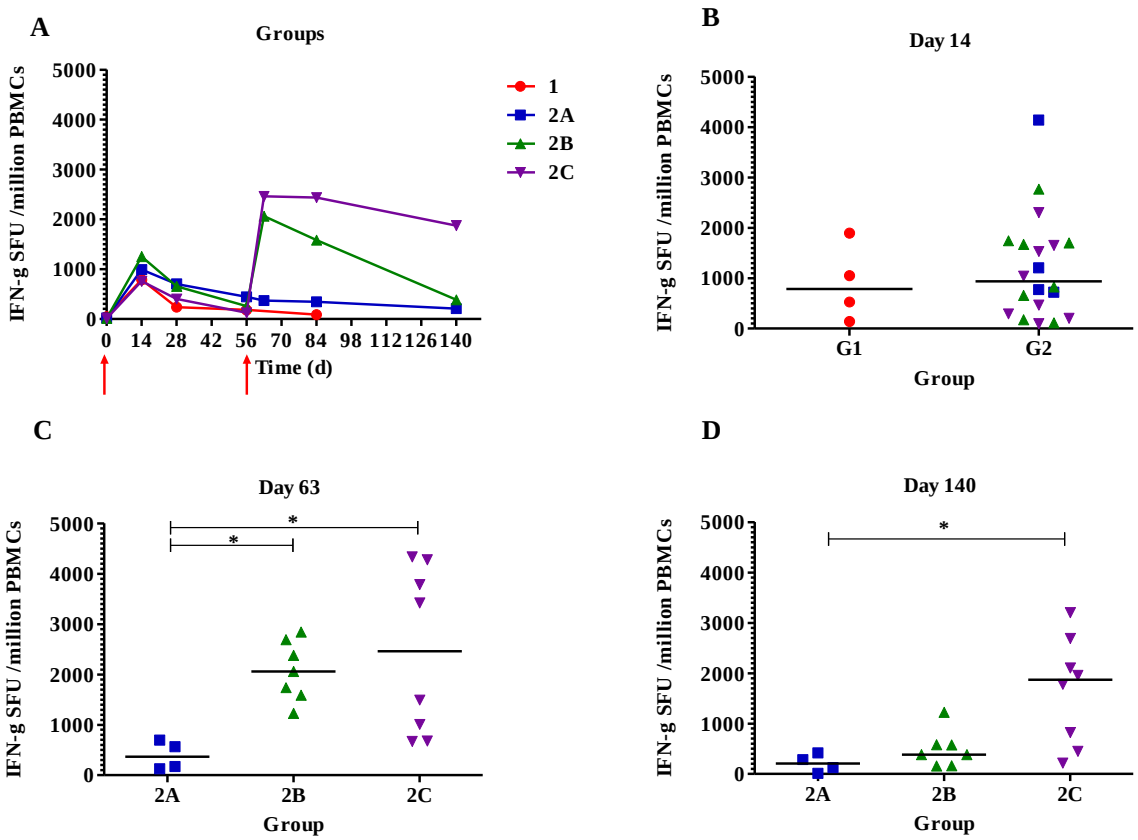


Figure 3-6: T cell responses following ChAd63/MVA PvDBP vaccination.

*T cell responses were measured by ex vivo IFNγ ELISPOT using fresh PBMC following ChAd63 PvDBP prime vaccination (d0) for G1 ($5x10^9$ vp; n=4) and G2 ($5x10^{10}$ vp; n=20) and heterologous boost with MVA PvDBP (d56) to G2B ($1x10^8$ pfu; n=7) and G2C ($2x10^8$ pfu; n=8). All vaccinations were given IM. (A) Group median responses. (B) Two weeks post ChAd63 PvDBP vaccination (d14) low dose G1 vs full dose G2. (C) One week post MVA PvDBP boost vaccination (d63) in G2B and G2C compared to G2A (ChAd63 PvDBP prime only). (D) 12 weeks post boost of G2B and G2C compared to G2A prime only. Spots were counted using an automated plate counter and exported into an Excel worksheet where results were obtained by subtracting any background response (from negative control wells) and then taking the average of triplicate wells. Data were then imported into GraphPad Prism for statistical analyses. *P<0.05, Kruskal-Wallis test with Dunn's correction for multiple comparisons.*

3.4.4 ChAd63/MVA PvDBP antibody response assessed by ELISA

The kinetics and magnitude of the serum IgG antibody responses were assessed over time by an ELISA to recombinant PvDBP_RII protein (Figure 3-7). Vaccination with the full dose of

ChAd63 PvDBP ($5x10^{10}$vp) induced antigen-specific antibody responses in all volunteers, with responses boosted following the MVA PvDBP vaccination in Groups 2B and 2C. Median responses are shown in Figure 3-7A. Following the ChAd63 PvDBP prime doses of 5×10^9 vp and 5×10^{10} vp there was no significant difference between the two groups four weeks post vaccination, but only volunteers in G2 seroconverted to above the threshold of 20 AU. However, four weeks post MVA DBP boost (Figure 3-7C), there were significantly stronger median responses in G2C compared with G2A who received ChAd63 PvDBP alone (median 3899 vs 95.5 anti-PvDBP_RII IgG AU), and responses were still significantly higher at day 140 in G2C compared to G2A (median 1378 vs 71.70 anti-PvDBP_RII IgG AU).

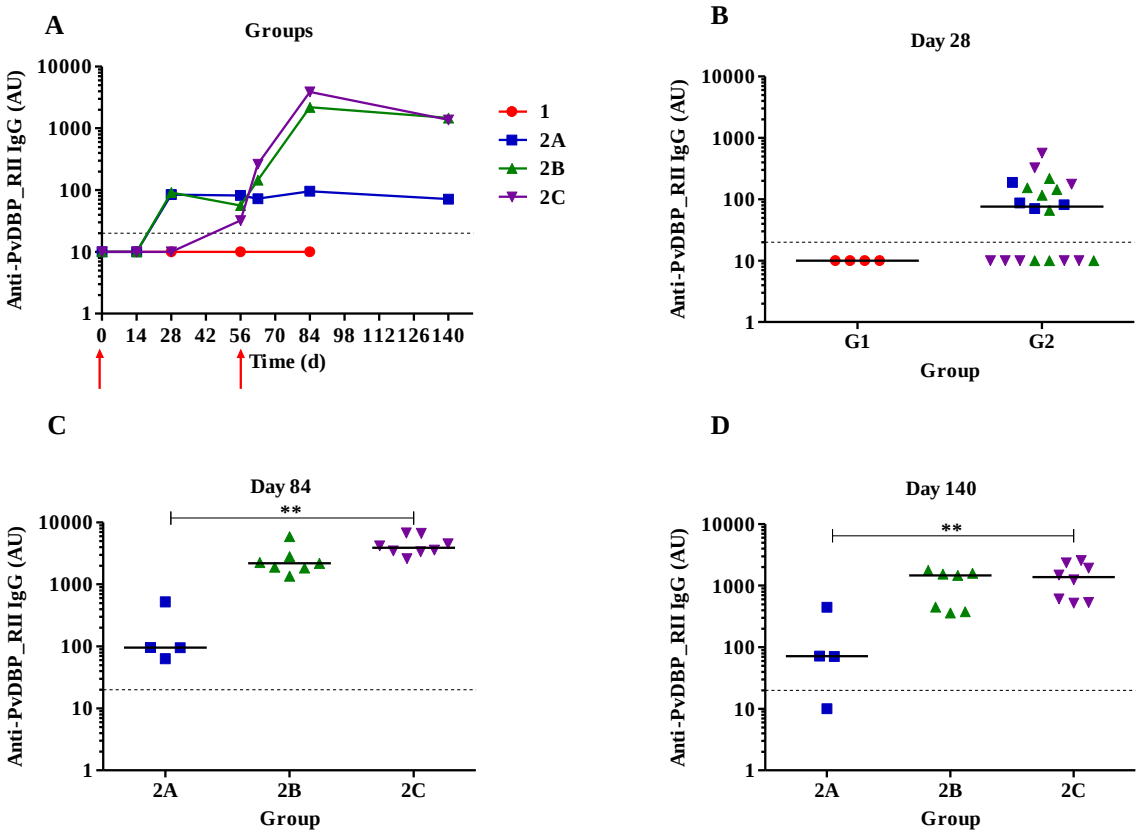


Figure 3-7: Serum IgG antibody responses following ChAd63/MVA PvDBP measured by ELISA.

PvDBP_RII-specific IgG responses are shown following ChAd63 PvDBP (d0) in G1 ($5x10^9$ vp; n=4) and G2 ($5x10^{10}$ vp; n=20) and MVA PvDBP boost (d56) in G2B ($1x10^8$ pfu; n=7) and G2C ($2x10^8$ pfu; n=8). (A) Group median responses. (B) Four weeks post ChAd63 PvDBP (d28) low dose G1 vs full dose G2. (C) Four weeks post MVA PvDBP boost (d84) in G2B and G2C compared G2A prime only. (D) 12 weeks post boost of G2B and G2C compared to G2A prime only. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and values of internal controls and samples in triplicate were assessed for any aberrant readings. The results were obtained by taking an average of triplicate wells, and using the standard curve to assign ELISA arbitrary units (AU). The limit of the assay is 20 AU (marked by a dotted line), below which is classed as negative.

*Data were then imported into GraphPad Prism for statistical analyses. **P<0.01, Kruskal-Wallis test with Dunn's correction for multiple comparisons.*

3.4.5 Anti-PvDBP IgG avidity and antibody isotype response profiles

An avidity ELISA was carried out on Group 2 samples four weeks post MVA PvDBP boost (d84; the peak timepoint for IgG response). This assay used displacement of sodium thiocyanate at different concentrations to ascertain the avidity of the IgG antibodies for each volunteer. The avidity of the anti-PvDBP IgG was similar for all responders in G2 (Figure 3-8) with the IC_{50} ranging from 2.5-3.3 M, similar to antibodies produced in boosted PfMSP1 vaccinees (193) and higher than PfAMA1 vaccine induced IgG (167).

An isotype ELISA was also carried out in order to determine the antibody subclass response profile following ChAd63/MVA PvDBP vaccination. The response was predominantly composed of IgG1, and IgG3 (Figure 3-9), as has been seen previously with viral vectored vaccines (193).

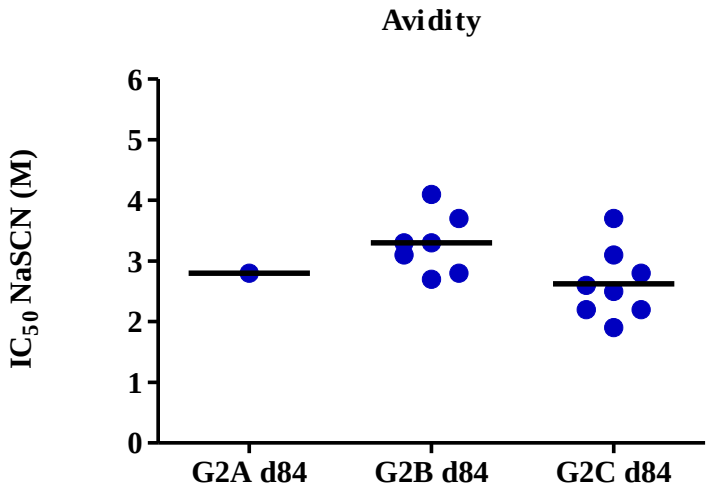


Figure 3-8: IgG Avidity assessment following ChAd63/MVA PvDBP vaccinations.

Anti-PvDBP total IgG avidity was assessed by NaSCN-displacement ELISA against Sal1 PvDBP_RII protein and is reported as the molar concentration of NaSCN required to reduce the OD_{405} to 50% of that without NaSCN (IC_{50}). Sera were diluted to give an OD_{405} = 1.0 and exposed to a dilution curve of NaSCN (0-7M). The IC_{50} is shown for individual responses in each group at the peak time-point (d84) (G2A n=1, G2B n=7, G2C n=8). Avidity for samples negative for Total IgG ELISA could not be measured. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and the average value of duplicate readings calculated. Data were then imported into GraphPad Prism for statistical analyses. There were no significant differences between groups using the Kruskal-Wallis test with Dunn's correction for multiple comparisons.

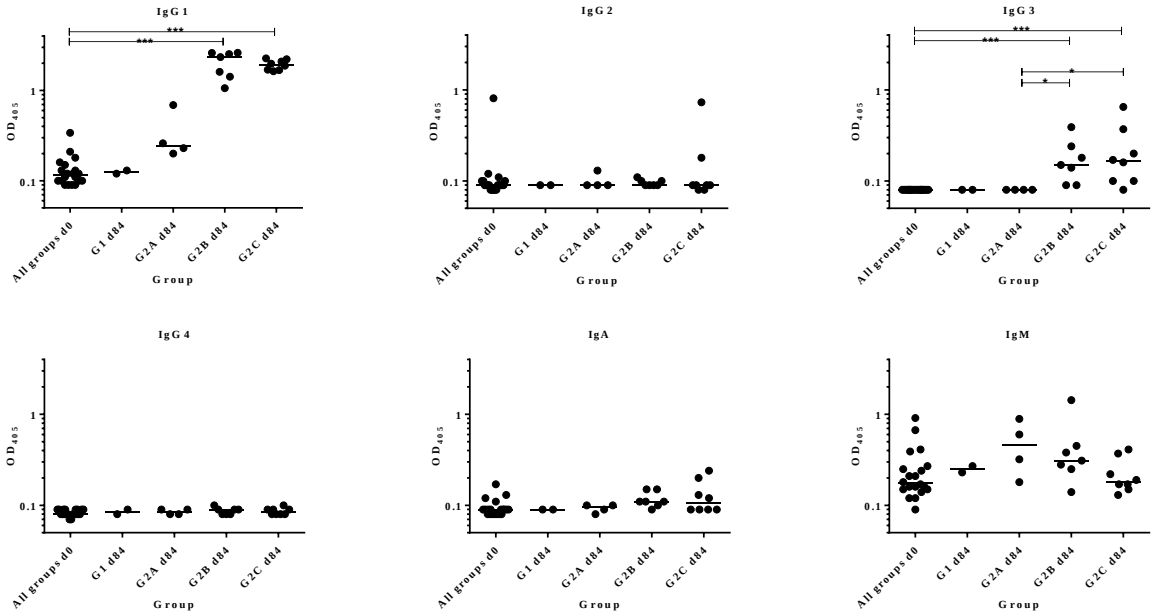


Figure 3-9: Antibody isotype profile following ChAd63/MVA PvDBP vaccination.

*Antibody isotype was assessed by ELISA. Baseline (d0) response for all Groups (n=20), 12 weeks post ChAd63-PvDBP prime for G1 (n=2) and G2A (n=4) and 4 weeks post MVA-PvDBP boost for G2B (n=7) and G2C (n=8) (d84) are shown. Individual and median responses are shown for every isotype. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and the average OD values of duplicate samples calculated. Data were then imported into GraphPad Prism for statistical analyses *P<0.05, ***P<0.001, Kruskal-Wallis test with Dunn's correction for multiple comparisons.*

3.4.6 Detection of anti-PvDBP ASCs following ChAd63/MVA PvDBP

Previous studies have demonstrated that ASC responses are detectable in peripheral blood around 7 days after boost vaccination using a ChAd63/MVA viral vectored heterologous prime-boost regime (194). Samples from boosted volunteers (G2B and G2C) were assessed by *ex-vivo* ASC ELISPOT at the d63 timepoint using frozen PBMC. There was no significant difference in the responses between the two groups (Figure 3-10).

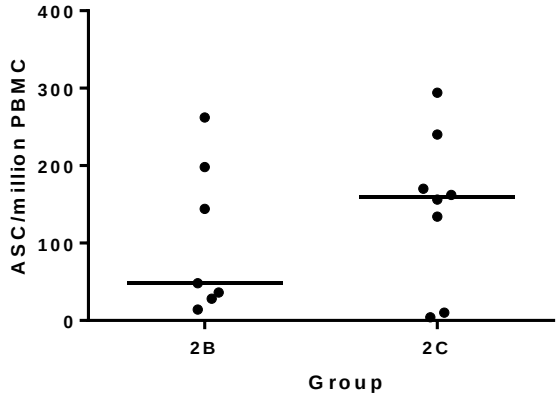


Figure 3-10: ASC responses following ChAd63/MVA PvDBP vaccination.

PvDBP-specific peripheral blood ASCs measured from frozen PBMC isolated 7 days following MVA PvDBP vaccination (d63) using ex-vivo ELISPOT from volunteers in G2B (n=7) and G2C (n=8). Spots were counted using an AID ELISPOT reader and automated counts were corrected by eye to ensure only spots consistent with IgG secreting ASCs were counted. Data were exported into an Excel worksheet and then imported into GraphPad Prism for statistical analyses. There was no significant difference between the two groups using a Mann Whitney t test.

3.4.7 Anti-PvDBP peripheral mBC responses following ChAd63/MVA PvDBP

Peripheral mBC responses were assessed by identifying PvDBP-specific mBC-derived plasma cells by *ex-vivo* ELISPOT following a 6-day polyclonal culture of PBMC (Figure 3-11). The responses were measured at the peak IgG response (d84) and demonstrated a significant difference between the median response in G2B and G2C, both as a measure of mBC-derived ASC per million PBMC (Figure 3-11A), and when comparing antigen-specific cells as a percentage of total IgG^+ (Figure 3-11B). As has been seen previously (194), the mBC derived ASC response correlated with the peak (d84) antibody response measured by ELISA (Figure 3-11C, D).

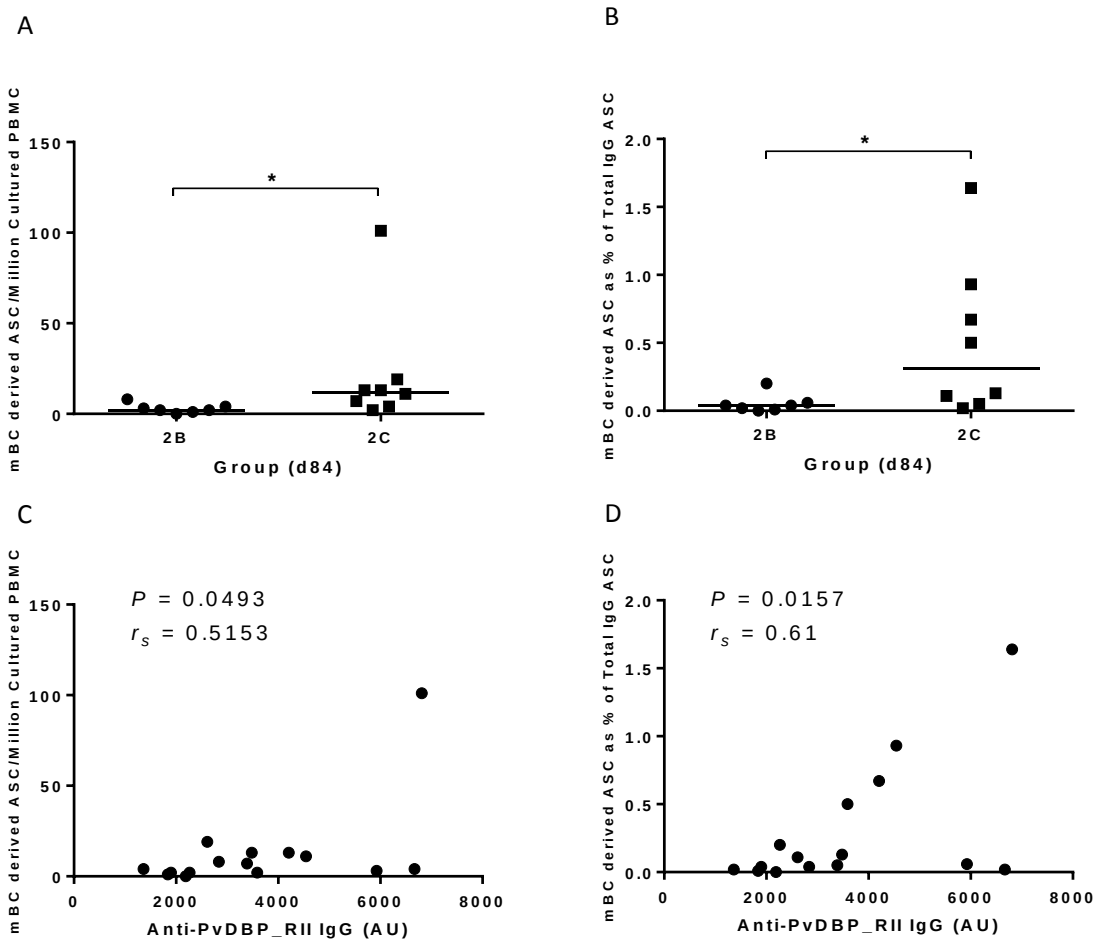


Figure 3-11: mBC responses following ChAd63/MVA PvDBP vaccination.

*mBC derived ASC measured at the peak of the response at d84 using ex-vivo ELISPOT. (A) Comparison between vaccine Groups 2B (n=7) and 2C (n=8) (mBC derived ASC per million cultured PBMC). (B) Comparison between vaccines Groups 2B and 2C (mBC derived ASC as a % of total IgG ASC). Individual and median responses are shown. (C-D) Correlations between mBC derived ASC and peak (d84) antibody response as measured by anti-DBP ELISA mBC ELISPOT spots were counted using an AID ELISPOT reader and automated counts were corrected by eye .Data were exported into an Excel worksheet and then imported into GraphPad Prism for statistical analyses. *P<0.05, Mann-Whitney test for comparing groups, Spearman rank for correlation analysis.*

3.4.8 PvDBP_RII – DARC Binding Inhibition Assay

The functional activity of the antibodies induced by vaccination with ChAd63/MVA PvDBP was assessed in a binding inhibition assay using recombinant PvDBP_RII and its receptor, DARC (Figures 3-12 and 3-13). These assays were carried out both in Oxford and in a collaborating laboratory at ICGEB in India. Both assays demonstrated inhibition of binding in all volunteers who received the prime-boost regime and one volunteer who received full-dose ChAd63 PvDBP alone. The assay carried out in Oxford demonstrated that as serum was diluted the

binding inhibition decreased (Figure 3-12). The assay carried out at ICGEB (Figure 13-3) demonstrated that the antibodies induced by vaccination were able to block the binding of not just the homologous PvDBP allele (Sal I) but three other variants as well. This is important as an effective *P. vivax* vaccine based on PvDBP would need to have strain-transcending activity, particularly given the concerns about polymorphism in this antigen.

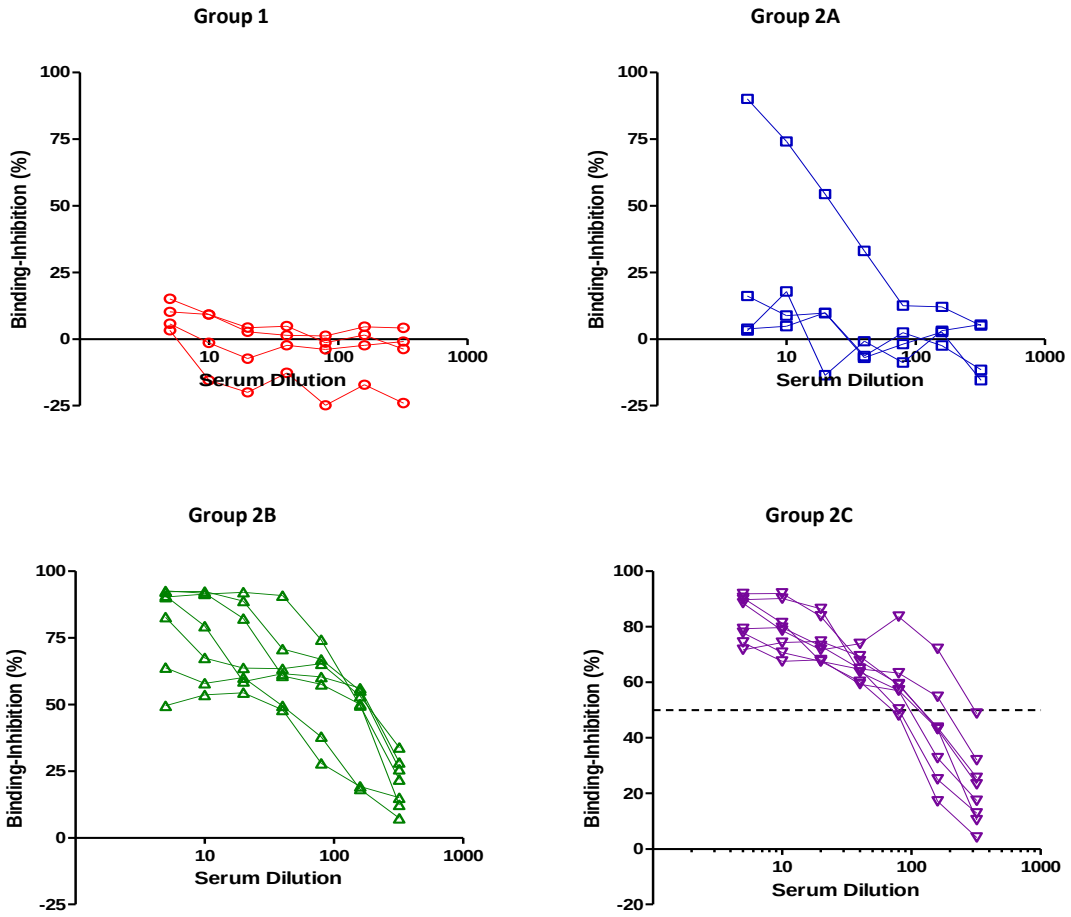


Figure 3-12: PvDBP_RII – DARC Binding Inhibition Assay (Oxford).

Sera were tested for ability to block binding of PvDBP_RII protein to its receptor DARC in an ELISA-based assay carried out in Oxford for the Sal I allele using a serum dilution series. Percentage binding inhibition is shown for Groups 1 (n=4), 2A (n=4), 2B (n=7) and 2C (n=8). The OD of the ELISA plates was read at 405 nm on Bio-tek ELx800 Microplate Reader with Gen5 software. Plates were developed to a point determined by the OD of the development controls wells (i.e. when the negative control wells reached an OD = 1.0). Data were exported into and Excel worksheet and then imported into GraphPad Prism for graphical presentation and analyses.

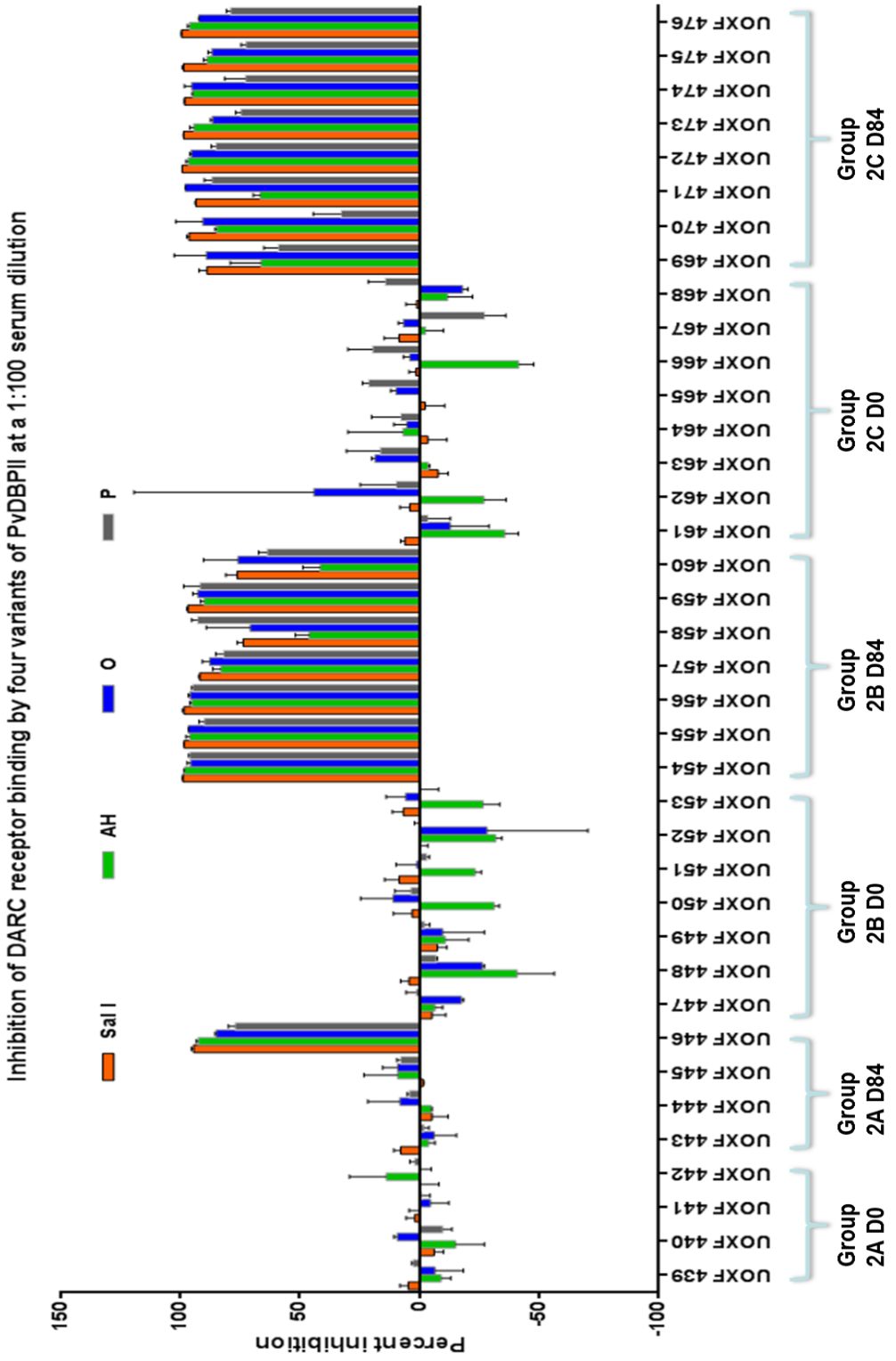


Figure 3-13: PvDBP_RII – DARC Binding Inhibition Assay (ICGEB).

Sera from G2A (n=4), G2B (n=7) and G2C (n=8) were independently tested in an ELISA-based assay at ICGEB in India using four different alleles (Sal I, AH, O and P) and serum diluted in series (1:10, 1:50, 1:100, 1:500 and 1:1,000). Each serum dilution was pre-incubated with the relevant test variant of PvDBP_RII (0.025 µg/mL) before being added to DARC-coated plates. A standard curve was generated from a series of concentrations (0 – 0.025 µg/mL) of the relevant test PvDBP_RII protein variant. The OD was measured at 492 nm using SoftMax Pro software, that interpreted the OD values as concentrations of bound PvDBP_RII based on the standard curve on each plate. Percent inhibition at each dilution was determined as (100 % – % binding).

3.5 Discussion

There are an estimated 2.5 billion people living at risk of *P. vivax* malaria globally (195). The revised 2030 Malaria Vaccine Technology Roadmap now recognises the urgent need for a vaccine to tackle *P. vivax* if the goal of malaria eradication is to ever be achieved (7). This is particularly important given the development of resistance to standard *P. vivax* treatment, with associated mortality (43). In 2015, the World Health Assembly endorsed the target of eliminating malaria from 35 countries and reducing case incidence and mortality rates by 90% globally by 2030 (196). This is an ambitious goal, for which *P. vivax* presents particular challenges. In countries with few cases of malaria each year, where eradication would appear more feasible, *P. vivax* is the predominant form of malaria accounting for over 70% of cases (197). The difficulty of detecting asymptomatic infection, the ability of *P. vivax* to relapse, and the appearance of gametocytes prior to the onset of disease all contribute to difficulties in eradication of this infection. A blood-stage *P. vivax* vaccine, even if only partially effective, would allow natural immunity to develop with reduced disease severity (198). A blood-stage vaccine would also, importantly, protect against relapses due to the hypnozoite (as long as relapses occurred within the duration of protective blood-stage immunity provided by vaccination), which would not be the case for pre-erythrocytic or transmission-blocking vaccine candidates.

There are many hurdles to be overcome in development of an effective vaccine against *P. vivax*. Polymorphism of the parasite has been well described, particularly in blood-stage antigens (199, 200) with limited polymorphism described in transmission-blocking candidates – presumably due to reduced human immune pressure as these antigen targets occur within the mosquito (201). This is likely to cause significant problems in vaccine development unless a conserved antigen can be found or multiple antigens are included in a vaccine. Cost-effective formulation and ease of delivery are also likely to be challenges, particularly if vaccines are used as a control method where vaccine coverage will have to be extremely high and migrant populations will pose some difficulty. A vaccine will have to be acceptable to the populations

targeted with the intervention, and a vaccine active against disease may be more readily accepted than a transmission-blocking vaccine.

For many years the *P. vivax* Duffy binding protein has been considered a leading vaccine candidate but has never previously reached clinical trial. The VAC051 Phase Ia clinical trial was the first study to evaluate this antigen (and, in fact any blood-stage *P. vivax* antigen) as a vaccine candidate. Having long been considered an attractive antigen for vaccine development, production of the protein to GMP standards has proved difficult (202). The use of the viral vectors meant that a vaccine regime suitable for human use was successfully produced (157). This trial demonstrated that the vaccines were well tolerated, with no safety concerns, and an acceptable reactogenicity profile. As has been seen in previous vaccine trials with the same viral vectors (84, 155), the higher doses of both vaccines were associated with increased frequency and severity of AEs.

The ChAd63/MVA PvDBP heterologous prime-boost regime was immunogenic, producing both antigen-specific T cells and B cells. There was no significant difference between either of the boosted groups (2B or 2C) in the levels of antigen-specific T cells or IgG at peak time-points, although there was a trend towards better maintenance of T cell levels at the final time-point following the higher MVA PvDBP dose.

The T cell response seen in the VAC051 trial was similar in magnitude to other malaria vaccines using the same viral vectors (83, 84). The ChAd63/MVA heterologous prime-boost regimen has previously been shown to induce increases in both $CD4^+$ and $CD8^+$ T cells, with broader and more potent responses than those seen using prime-boost regimens of DNA and poxvirus vectors. (148). This broader response is anticipated to not only act by helping to facilitate an antibody response through $CD4^+$ helper cells but also to contribute to direct clearance of pathogens through cell-mediated effector mechanisms. The role of cell-mediated immunity in a blood-stage malaria vaccine is not fully understood. However, both $CD4^+$ and $CD8^+$ T cells have been shown to be activated in acute blood-stage malaria infection, and probably

maintained after infection, in a mouse model (using the murine parasite *P. yoelli*), with cells phenotypically similar to those seen after other bacterial and viral infections (203). This supports the view that an optimised blood-stage malaria vaccine should be tailored to induce both an antibody and T-cell immune response.

The ChAd63/MVA PvDBP vaccines were able to induce an antigen-specific IgG response, with a predominance of IgG1 and IgG3 isotypes, as has been seen previously with viral vectored vaccines (193). I also demonstrated induction of antigen-specific memory B cells in boosted volunteers, and the appearance of antigen-specific antibody-secreting cells in peripheral blood around 7 days post boost vaccination with MVA PvDBP. This has been described previously following ChAd63/MVA viral vectored vaccines and these cells are thought to arise from mBC re-stimulation in the lymphoid system (following the boost vaccination). They are seen transiently in the blood stream and this is likely to be as they travel to the bone marrow where they remain as plasma cells (194). The antibodies induced by vaccination were able to block the binding of the Duffy-binding protein to its receptor, DARC, in an *in vitro* assay carried out in Oxford, with similar results seen in both Groups 2B and 2C. This functional activity was also demonstrated in an independent experiment carried out in a collaborating laboratory in the ICGEB. Encouragingly, the antibodies were able to block not only the reference strain (Salvador I) but several other *P. vivax* strains as well. This is encouraging as one of the potential problems of using viral vectored vaccines is that they cause protein expression to occur *in situ* following vaccination, meaning that the immunogen produced cannot be fully characterised and may not have folded correctly or been transported through secretory pathways successfully (204). The ability of the antibodies produced by vaccination to bind to recombinant PvDBP and block binding of the protein to DARC implies that expression of a correctly folded protein occurred following ChAd63/MVA PvDBP vaccination.

The functional activity seen in the VAC051 trial may, however, not translate into vaccine efficacy. Assays to determine invasion inhibition of antibodies against *P. vivax* blood-stage

antigens are more complicated to carry out than in *P. falciparum* studies due to the lack of ability for long term culture of vivax parasites *in vitro*. This generally limits these assays to being carried out in countries where *P. vivax* is endemic, and necessitates collection of blood from vivax-infected individuals for use in the assays (132). Translation of *in vitro* blocking activity to efficacy in an *in vivo* infection setting has not previously been assessed.

The use of a standardised, reproducible CHMI model for early proof-of-concept efficacy testing has been a useful tool in the development of *P. falciparum* antimalarial drugs (205, 206) and vaccines (11). The ability to induce infection consistently and closely observe infected individuals means that much smaller numbers are needed for these Phase IIa clinical trials than would be needed to test efficacy in a Phase IIb field trial. This has substantial implications for cost and time, with only candidates that show efficacy being taken forward to larger, more expensive trials. CHMI for *P. vivax* has been far less utilised than for *P. falciparum*, with only a small handful of studies reported in the last few years, with only one published study assessing the efficacy of a vaccine against vivax malaria, the pre-erythrocytic vaccine candidate VMP001 (77). Deliberate infection with *P. vivax* was practiced for the treatment of neurosyphilis patients almost a century ago by the Austrian psychiatrist Julius Wagner-Jauregg. This initially involved injecting neurosyphilis patients with blood taken from soldiers hospitalised with malaria, and later passaging malaria through patients (207). The practice was widely adopted as it was the only effective treatment for neurosyphilis available at the time, with up to 50% efficacy in curing or improving the disease, but was not without risk – mortality rates of 5-15% were reported in treated patients, although these patients had significant comorbidities (22). CHMI studies conducted in the USA in the 1940s to 1970s explored the biology of *P. vivax* and the potential for inducing immunity through exposure to irradiated sporozoites delivered by mosquito bite which conferred short term (3 – 5 months) protection from subsequent exposure to infection (61, 64). CHMI studies with *P. vivax* were discontinued, and only relatively recently have been reinstated as a potential method for assessing vaccine and drug efficacy.

Several CHMI trials by mosquito bite have been assessed by a group in Cali, Columbia using mosquitoes infected with *P. vivax* from patients presenting for treatment (136, 137, 208). Additionally, the VMP001 candidate vaccine was assessed at WRAIR in the USA using mosquitoes infected from a donor in Thailand and then transported to the USA (77). These studies have demonstrated that the mosquito-bite CHMI model is possible for vivax malaria, but there are several limitations with this method. Fresh gametocytes from an infected patient are required because long-term *P. vivax* culture is not currently possible. This poses significant logistical challenges as it requires at least part of the trial to be undertaken in an endemic setting with appropriate entomological facilities established to produce an infected mosquito lot after screening and enrolling a donor patient. Subsequently, the mosquito lot can be used in trials in the same location, or transported to non-endemic areas but the timing for this in the setting of vaccine efficacy testing will be crucial. Furthermore, a different isolate of *P. vivax* will inevitably be used for every trial, meaning CHMI assessment of vaccines is almost certainto be with a heterologous strain to that used in the vaccine; that the parasites may have different susceptibility to antimalarial treatment between strains; and these differences will be unknown at the time of CHMI. As seen in the trials carried out in Cali, different strains are likely to have different pre-patent periods which can limit comparability between trials (137). The use of sporozoites for CHMI also necessitates a liver-stage of infection, with a high risk of hypnozoite formation and potential relapse. This requires participants to be screened for G6PD deficiency in order to avoid haemolysis with primaquine, and now also requires assessment of the volunteers' ability to metabolize primaquine (requiring analysis of cytochrome P450 2D6 phenotype) to maximise safety. Volunteers with poor or intermediate metaboliser CYP2D6 phenotypes should not be enrolled for such studies (51).

An alternative approach to mosquito-bite CHMI is to use blood-stage CHMI, as has been developed for *P. falciparum* (Chapter four, (209)). There have been four *P. vivax* blood-stage CHMI studies to-date successfully carried out at QIMR Berghofer in Brisbane, Australia using two different inocula (James McCarthy, personal communication). The first pilot study, carried

out in two healthy volunteers, demonstrated that infection with *P. vivax* from a frozen inoculum was possible and there were no safety concerns (210). Blood-stage CHMI is obviously limited to studies of vaccines or drugs targeting the blood-stage of infection so cannot be used for pre-erythrocytic vaccine candidates. It does however have several advantages in situations where it can be used. Practical advantages include the ability to carry out CHMI studies more easily in a non-endemic setting; having access to the *P. vivax* strain genetic data before CHMI; being able to carry out multiple studies with the same strain (for which a safety database can be established); and being able to use the same inoculum size for each volunteer. There are also advantages for participants with this method – the use of blood-stage parasites means there is no liver-stage of infection, and therefore no risk of hypnozoite formation or relapse. This means participants do not require primaquine treatment, and therefore do not require G6PD deficiency or cytochrome P450 2D6 phenotype screening. The sensitivity of the parasite to antimalarial treatment can also be known in this scenario prior to CHMI, removing the risk of using a drug-resistant strain which is a potential possibility with the mosquito-bite CHMI model.

In this Chapter I have described the results of the first Phase Ia clinical trial of a blood-stage *P. vivax* vaccine candidate, ChAd63/MVA PvDBP, demonstrating that in healthy malaria-naïve volunteers the vaccine was well tolerated and immunogenic, inducing functional antibodies against the PvDBP antigen. Future work will aim at developing the blood-stage CHMI model for *P. vivax* to assess the efficacy of this and other blood-stage *P. vivax* vaccines. This model has proved very useful for *P. falciparum*, as discussed in Chapter four, for the blood-stage *P. falciparum* vaccine candidate, FMP2.1/AS01B. I conducted a Phase I/IIa study to assess the safety, immunogenicity and efficacy of this vaccine using a homologous blood-stage CHMI model (209).

The WHO Malaria Vaccine Technology Roadmap calls for a vaccine with an efficacy against clinical disease of 75% for both *P. falciparum* and *P. vivax* by 2030 (7). The focus on vaccine

development must therefore not be based solely on one parasite or the other but work to target both. This is unlikely to be achieved with a single antigen, and will probably require a multi-component malaria vaccine. Chapters four and five discuss the development and assessment of candidate vaccines against *P. falciparum* blood-stage antigens conducted alongside this *P. vivax* study with this eventual goal in mind.

Chapter Four:

A Phase I/IIa clinical trial to assess the safety, immunogenicity and efficacy of FMP2.1/AS01B, an asexual blood-stage vaccine for *Plasmodium falciparum* Malaria (VAC054)

4.1 Authorship statement

I set up the VAC054 clinical trial with the assistance of Alison Lawrie, Rachel Roberts, Ian Poulton, Adrian Hill (CI) and Simon Draper. This involved preparation of the study documents with submission for ethical, regulatory approval for all sites, and Research and Development (R&D) department approval for NHS sites (in Southampton and London).

I screened and enrolled volunteers from Oxford and London (NIHR WTCRF, Imperial College Healthcare NHS Trust) for the trial. Study staff at the NIHR WTCRF in Southampton carried out screening and enrolment of volunteers at this site. Vaccinations and follow-up visits were conducted by local site study teams, including myself. The Principal Investigator (PI) in Southampton was Saul Faust and the PI in London was Graham Cooke.

The blood-stage CHMI inoculum was prepared in the Jenner Institute laboratories by Rebecca Brown and Simon Draper. It was administered to volunteers at the CCVTM in Oxford by me, assisted by Morven Wilkie, Navin Venkatraman, Ian Poulton, Mary Smith, Paula Marriott and Raquel Lopez-Ramon. Thick blood films following CHMI were read by qualified microscopists Joseph Muita, Pauline Titus and Kebba Konteh from Kemri, Kenya. qPCR was carried out in Oxford by Nick Edwards and a team of laboratory staff.

Laboratory assays assessing vaccine and CHMI immune responses were carried out in Oxford by Kathryn Milne, Sarah Silk, Sean Elias and me. The GIA assays were carried out at the NIH reference center laboratory in the USA by Kazutoyo Miura.

The clinical trial described in this Chapter has been published in the Journal of Infectious Diseases (209).

4.2 Introduction

4.2.1 Blood stage *P. falciparum* vaccines

There has been considerably more research into *P. falciparum* vaccines than *P. vivax* vaccines in the past. The antigens that have been most widely investigated as blood-stage vaccine candidates are AMA1 and MSP1, both of which are proteins found on the merozoite surface. Field studies have shown that merozoite surface antigens including AMA1 and MSP1 are targets of naturally occurring protective blood-stage immunity (211, 212). Both of these antigens have been tested as protein-in-adjuvant formulations (95) and in viral vectored vaccines (83), with limited success.

The ability of a blood-stage vaccine to significantly reduce parasite replication in the blood is deemed essential, in order for a blood-stage vaccine to effectively prevent illness when pre-erythrocytic control measures have failed (25). Numerous factors have hindered development of vaccines against the merozoite including: substantial levels of polymorphism in candidate antigens; redundant erythrocyte invasion pathways; and the apparent need for very high antibody concentrations to prevent rapid erythrocyte invasion (25, 213). Furthermore, the best approach to assessment of vaccine efficacy, including 'proof of concept' (POC) studies, against the blood-stage parasite in humans has been widely debated (214).

This trial looked at the safety, immunogenicity and efficacy of a recombinant protein vaccine based on the 3D7 clone sequence of *P. falciparum* AMA1, known as FMP2.1 (93) and formulated in the Adjuvant System AS01 from GSK. The vaccine has previously been developed and tested in a series of Phase Ia/b safety and immunogenicity trials using the AS01 and AS02 Adjuvant Systems (94-96, 215). A subsequent Phase IIb field trial in 400 Malian children using the FMP2.1/AS02 formulation reported strain-specific efficacy against parasites with 3D7 AMA1-like sequence in a secondary efficacy endpoint analysis (97). The trial I led, as described in this Chapter, aimed to assess POC blood-stage efficacy by inoculating volunteers with blood-

stage malaria parasites after vaccination and comparing the results in PMR between vaccinated volunteers and unvaccinated infectivity controls.

4.2.2 AMA1 as an antigen

As described in Chapter one, *P. falciparum* AMA1 is a precursor protein of 83 kDa (93) located in the apical end of the merozoite in the micronemes prior to schizont rupture. The N terminus is cleaved to give a 66 kDa form which is found on the merozoite surface following schizont rupture, whereas the 83 kDa form is localised apically (89, 90). AMA1 binds to another parasite protein, PfRON2, which is inserted by the parasite into the host red cell membrane, forming the tight / moving junction and allowing invasion to take place. Antibodies to AMA1 have been shown to prevent processing and circum-merozoite redistribution and shedding of the protein. Without AMA1 undergoing these processes red blood cell invasion is inhibited (92).

FMP2.1 is a lyophilised preparation of the majority of the ectodomain of *P. falciparum* AMA1. The gene encoding the FMP2.1 protein was chemically synthesised to contain an *E. coli*-optimised codon usage to encode 478 amino acids representative of amino acids 83 to 531 of the AMA1 protein with two attached His-tags. The amino acid sequence is: MAHHHHHHPGGSGSGTMH-[AMA1 amino acids 83 to 531]-AAALEHHHHHH. 449 of the amino acids are derived from the merozoite protein AMA1 of the 3D7 clone of *P. falciparum*. The protein was produced in and purified from *E. coli* bacteria at the WRAIR BioProduction Facility under GMP (93, 95, 97).

4.2.3 The AS01 Adjuvant System

Adjuvants have been known to increase the immune response against a given antigen for over 80 years. GSK Biologicals have developed 'Adjuvant Systems' which are formulations of classical adjuvants (e.g. aluminium salts, liposomes) mixed with immunomodulatory molecules (e.g. Toll-like receptor [TLR] agonists), with an aim of impacting the innate and/or adaptive immune responses. AS01 is a liposome-based Adjuvant system with a specific aim to improve cell-mediated immunity (122). It is also one of the best adjuvants available for inducing

antibody production following vaccination (216). The Adjuvant System contains 3-0-desacyl-4' monophosphoryl lipid A (MPL), a TLR4 ligand derived from the cell wall lipopolysaccharide (LPS) of the Gram negative *Salmonella minnesota* R595 strain. The LPS is detoxified by hydrolytic treatment and purification to provide a powerful adjuvant without the toxic effects of the parent molecule. The AS01 adjuvant system also contains QS21, a triterpene glycoside purified from the bark of the South American tree *Quillaja saponaria.* QS-21 has been shown to impact antigen presentation to antigen presenting cells (APCs) and favours the induction of cytotoxic T lymphocytes (122). AS01 is produced in an adult formulation, AS01B and a paediatric formulation, AS01E, which contains half the amount of MPL and QS21 as the adult version (217). The AS01B adjuvant system used in this trial contains 50 micrograms of MPL and 50 micrograms of Stimulon QS21 in a liposome-based formulation in a 0.5 mL dose (95).

AS01 is closely related to another Adjuvant System, AS02, which contains the same immunostimulants MPL and QS21. AS02 is an oil-in-water, rather than liposomal, formulation so has smaller particle size than AS01 (218). The adult formulation is termed AS02A whilst the paediatric formulation is termed AS02D. The clinical evaluation of GSK's leading malaria vaccine candidate RTS,S started with AS02 but a Phase IIa sporozoite CHMI study demonstrated that the safety and reactogenicity of RTS,S/AS01 was comparable to that of RTS,S/AS02, with a trend towards improved vaccine efficacy (VE) against infection (50.5% [95% CI: 32.9, 67.1] vs 31.8% [95% CI: 17.6, 47.6]) (73). This was confirmed in a subsequent study in Ghent, Belgium (219). The RTS,S vaccine has been given to thousands of individuals, including children in phase I – III trials with no significant safety concerns and moderate efficacy (57). AS01 has been taken forward as the preferential Adjuvant System for malaria vaccines given the improvement in immunogenicity seen with compared with AS02. The VAC054 trial therefore used this rather than AS02.

4.2.4 Previous trials of FMP2.1

4.2.4.1 FMP2.1 with AS02A

Phase Ia

FMP2.1 was evaluated with AS02A in a Phase Ia dose escalation study in 23 healthy, malaria-naïve adult volunteers at WRAIR in 2007 by Polhemus *et al*. The final dose used was 43 µg of lyophilised protein which was mixed with AS02A just prior to immunisation, so that approximately 8, 20 or 40 µg of FMP2.1 was delivered in a final volume of 0.5mL of AS02A. There were 8 volunteers enrolled into each of the lower dose groups and 7 volunteers in the group receiving 40 µg. Three vaccinations were scheduled for each volunteer at 0, 1 and 2 months. Nineteen volunteers completed the study, receiving all vaccines. There were no SAEs related to vaccination and the most common local AE was injection site pain. Swelling at the injection site was also noted, particularly in the high dose group, but this was generally mild. The most common systemic AEs noted were headache (18 incidents over 63 vaccinations) and myalgia (14 incidents over 63 vaccinations). The vast majority of AEs resolved within 72 hours of vaccination. The vaccine showed favourable safety data and was immunogenic, with the induction of humoral and Th1-biased cellular immune responses (94).

Phase Ib (Adults)

A Phase Ib study was carried out in Mali in 60 healthy adults exposed to seasonal malaria. The doses used were FMP2.1 25 µg/AS02A 0.25 mL (half dose) or FMP2.1 50 µg/AS02A 0.5 mL (full dose). 20 volunteers were recruited into each of the groups to receive FMP2.1/AS02A and the remaining 20 received rabies vaccine as controls. The dosing schedule was as for the Phase Ia study – 0, 1 and 2 months. Local solicited AEs were higher in the FMP2.1/AS02A vaccine groups compared with controls. The most common local AEs were pain and swelling at the injection site. Grade 3 swelling was much more common in the full dose FMP2.1/AS02A group. The swelling was generally not associated with significant functional impairment, and all local AEs resolved within the 8 day follow-up period following vaccination. Headache, myalgia and

malaise were the most common systemic AEs, and were more common in the groups receiving the full dose FMP2.1 vaccine. There were no severe systemic AEs, and all solicited systemic AEs resolved within the 8 day follow up period. There were no SAEs noted in this study. The vaccine showed favourable safety data and was immunogenic, with a trend towards higher antibody responses in the full dose FMP2.1/AS02A group compared to the half dose group. There was also significantly greater *in vitro* GIA in the post-vaccination sera from the full dose group than sera from the control group, but not in sera from the half dose group, against both homologous (3D7) and heterologous (FVO) parasites (96).

Phase Ib (Children)

A Phase Ib double-blind randomised controlled dose escalation trial was conducted in healthy Malian children exposed to seasonal *P. falciparum* malaria. 100 children aged 1-6 years were enrolled, and placed into 3 cohorts (1 group of 20 and 2 groups of 40). Within each cohort, participants were randomised in a 3:1 fashion to receive approximately 10, 25 or 50 µg of FMP2.1 with a proportionate volume of the AS02A adjuvant system, or rabies vaccine as a control. In the first cohort (*n*=20) 14 of the 15 children vaccinated with 10 µg FMP2.1 with 0.1mL AS02A received all 3 vaccinations and all 5 controls received 3 vaccinations. The whole cohort completed 1 year of follow up. In the second cohort (*n*=40) 27 of the 30 children randomised to receive 25 µg FMP2.1 received all three vaccinations and 8 of the 10 controls in this arm received three doses of rabies vaccine. 27 of the 30 children in the vaccine arm and all 10 controls completed 1 year of follow up. In the third cohort (*n*=40) 27 children received all three vaccinations of 50 µg FMP2.1 with 0.5 mL AS02A and all 10 controls received 3 doses of rabies vaccine. 29 children in the vaccine arm and all 10 controls completed 1 year of follow up. The most common local AEs were injection site swelling and pain, which tended to diminish with subsequent vaccinations (particularly in the lowest dose group). Grade 3 swelling was seen in all vaccine groups, but more so in the 50 µg malaria vaccine group. All local AEs resolved within the 7 day follow-up period after vaccination. The most common systemic AE

was fever and this was more frequent in malaria vaccine recipients than controls. There were no severe solicited systemic AEs and all resolved within the 7 day follow-up period. Four SAEs were reported in the study. One of these was a raised white blood cell count, which was thought to be secondary to malaria infection. The other three SAEs were related to raised liver transaminases, one of which was found to be due to acute hepatitis A infection and the other two related to hepatitis B, although an additional increase in ALT related to vaccination could not be ruled out. All four SAEs resolved within 3 to 4 weeks of follow up with no sequelae. FMP2.1/AS02A showed favourable safety data in children, with acceptable tolerability. All three dose levels of the FMP2.1/AS02A vaccine elicited high levels of antibodies recognising AMA1 after a single vaccination, peaking a month after the third vaccination. Based on this study the dose of 50 µg was selected to go forward for further evaluation, although there was no significant difference in anti-AMA1 antibody titres between the different dose groups (215).

Phase IIb (Children)

A Phase IIb double-blind, randomised efficacy study was carried out in which 400 healthy Malian children aged 1 to 6 years were vaccinated with either FMP2.1/AS02A or a control (rabies) vaccine and followed up for 6 months. The dose of FMP2.1 used was 50 µg suspended in 0.5 mL of AS02A. Children were randomised in a 1:1 ratio to either receive the malaria vaccine or control vaccine at 0, 1 and 2 months. The primary endpoint was a clinical episode of malaria (defined as fever with an asexual *P. falciparum* parasite density of >2500 parasites/mm^3). Secondary endpoints included one or more episodes of clinical malaria with AMA1 genotypes identical to the 3D7 vaccine strain with respect to eight designated immunologically important AMA1 polymorphisms in the cluster 1 loop of domain I and multiple episodes of clinical malaria. Children received the vaccines at 0, 1 and 2 months and were then followed up for 6 months with blood tests. Blood smears were only read at the time of collection if the child was symptomatic. DNA was extracted from dried-blood spots collected during clinical malaria episodes, and the gene encoding *P. falciparum* AMA1 was sequenced.

Clinical episodes were classified into those that matched the vaccine strain (in terms of AMA1 genotype) and those that did not. The unadjusted efficacy of the vaccine was 17.4% (hazard ratio for the primary endpoint 0.83; 95% confidence interval [CI], 0.63 to 1.09; *P*=0.18), however, efficacy against clinical malaria with the vaccine strain AMA1 was 64.3% (hazard ratio of vaccine vs. control 0.36; CI, 0.08 to 0.86; *P*=0.03). There were no safety concerns relating to the vaccine and no vaccine-related SAEs reported. Swelling was the most common local AE, and was more common in the malaria vaccine group than controls. Fever was the most common systemic AE and was also significantly more common in the malaria vaccine group (97). The allele-specific efficacy against homologous parasites seen in the first malaria season did not extend into the second season of follow-up (99).

4.2.4.2 FMP2.1 with AS01B and AS02A

Phase I/IIa (Adults)

AS01B has been used with FMP2.1 in 20 healthy volunteers in a trial by Spring *et al.* at WRAIR (95). This trial compared FMP2.1 given with the AS02A and AS01B adjuvant systems. Five volunteers received low dose FMP 2.1/AS01B with 3 doses of 10 µg FMP2.1 in 0.5 mL of AS01B. 14/15 volunteers received 3 doses of full dose FMP2.1/AS01B (50 µg FMP2.1 in 0.5 mL of AS01B). A further 14/15 volunteers received 3 doses of full dose FMP2.1/AS02A (50 µg FMP2.1 in 0.5 mL of AS02A). There was no significant difference in the antibody titres across the groups, except at day 42 (two weeks after the second vaccination) when full dose FMP2.1/AS01B induced higher concentrations than low dose FMP2.1/AS01B and full dose FMP2.1/AS02A. The geometric mean concentration (with 95% CI) for anti-AMA1 IgG measured by ELISA two weeks after the third vaccination were 196 µg/mL (103–371 µg/mL) in the low dose FMP2.1/AS01B group, 279 µg/mL (210–369 µg/mL) in the full dose FMP2.1/AS01B and 216 µg/mL (169–276 µg/mL) in the full dose FMP2.1/AS02A group. Some boosting of IgG was seen after the third vaccination but the levels were not significantly higher than those reached after the second vaccination in any vaccine group.

There were no safety concerns with the FMP2.1/AS01B vaccine and no SAEs were noted. Local AEs following vaccination were common, and tended to increase with subsequent vaccinations. The most common local AEs were injection site pain, erythema and swelling. No severe pain was noted and the erythema and swelling were not associated with significant functional impairment. Headache, malaise and fatigue were the most common systemic effects noted. Systemic AEs also tended to increase with subsequent vaccinations, and were most severe in the full dose AS01B group, with 5 volunteers reporting a grade 3 systemic AE in this group. The majority of AEs occurred and resolved within 72 hours of vaccination. Vaccine efficacy of both vaccines was tested following CHMI via five infectious mosquito bites (3D7 clone parasites). Six of the volunteers vaccinated with full dose FMP2.1/AS01B and ten of the volunteers vaccinated with full dose FMP2.1/AS02A underwent CHMI, along with six unvaccinated controls. All vaccinees became parasitaemic with no delay to parasitaemia as determined by thick blood film compared with controls. Level of parasitaemia was examined by qPCR post-CHMI from day 7 to day 12 (inclusive). There was a statistically significant difference among both vaccination groups and the infectivity control group in the longitudinal measurement of peripheral parasitaemia on days 7-9 when parasitaemia was detectable by qPCR but antimalarial treatment had not yet been initiated (P=0.0002). Post-test analysis using Tukey's Test showed a significantly lower parasitaemia in the volunteers who received full dose FMP2.1/AS02A (P=<0.0001) and a trend towards lower parasite burden in the volunteers who received full dose FMP2.1/AS01B P=0.084). There was a trend towards a decreased hepatic parasite burden with both vaccines, but this was not statistically significant.

4.2.5 Blood-stage controlled human malaria infection (CHMI)

As discussed in Chapter one, an alternative to infecting volunteers with malaria via mosquito bite is the intravenous administration of infected erythrocytes. This enables the blood-stage of infection to be examined in isolation, unlike the more widely used sporozoite CHMI models which have historically been used to assess vaccine efficacy of pre-erythrocytic vaccine

candidates in small proof-of-concept Phase IIa clinical trials (11, 220), although a few mosquito-bite CHMI trials have also been carried out for blood-stage vaccine candidates (83, 95). More typically, the efficacy testing of blood-stage vaccines has relied on larger-scale, expensive Phase IIb field trials in endemic populations. Reasons for this include the assumption that blood-stage vaccine efficacy could not be assessed in the short time interval between parasite emergence from the liver (around days 6 or 7 post-sporozoite CHMI) and diagnosis of blood-stage infection by thick-film microscopy (typically 4-6 days later).

The work in this Chapter aimed to further develop the blood-stage *P. falciparum* CHMI model to enable more accurate and rapid efficacy assessment of blood-stage vaccine candidates prior to field trial assessment. The underlying hypothesis for this study was that an effective blood-stage vaccine should demonstrate a measurable effect on the PMR in malaria-naïve individuals, most likely to be seen against homologous challenge. PMR can be modelled for each individual from quantitative real-time PCR (qPCR) data of blood-stage parasitaemia, prior to patency and diagnosis by thick-film microscopy (177). This CHMI model should allow for a longer period of qPCR monitoring, homologous challenge and, in comparison to the mosquito-bite CHMI model, consistency in the initial number of blood-stage parasites in all volunteers. The uniformity of the known starting inoculum and more datapoints available for modelling should also lead to improved confidence of the calculated PMRs and thus greater power to observe partial vaccine efficacy (141, 175, 214).

The infectious inocula used in this study were produced by Drs Gregor Lawrence, Allan Saul and colleagues at QIMR in Brisbane, Australia in 1994 (166). Procedures were designed to minimise the risk of other infectious agents in the cryopreserved samples. A volunteer with blood group O, Rhesus negative was deliberately infected with a chloroquine-sensitive strain of the *P. falciparum* clone 3D7 (homologous to the vaccine candidate antigen) via the bites of laboratory-reared *Anopheles stephensi* mosquitoes. Blood was taken from the volunteer after fever developed, when parasites were visible on thick blood film.

Initial development of the blood inoculum to be used in this study and its use in five volunteers is described by Cheng *et al.* 1997 (166). Blood was collected at the Australian Red Cross Blood Bank in an aseptic manner using standard blood bank equipment. The blood was leukocyte-depleted prior to cryopreservation and the amount of serum potentially transferred is reduced by a factor of approximately 1000 by the thawing and washing process. The volume of inoculum to be given to each volunteer contains a very small volume of red blood cells, equivalent to only 1.5 to 4 microlitres of blood. The red cells were cryopreserved using a protocol from the American Association of Blood Banks Technical Manual that is normally employed for freezing blood from patients and donors with rare blood groups.

Prior to this trial, over 100 volunteers had received the inoculum with a varied number of infected erythrocytes (from 30 to 6000). CHMI using this method has always resulted in parasitaemia as detected by PCR (140).

4.2.6 VAC054 Hypothesis

Vaccination with FMP2.1/AS01 will induce antibodies against AMA1 which will inhibit *P. falciparum* invasion of red blood cells, detectable as a reduced PMR in vaccinated volunteers compared with unvaccinated infectivity controls following a blood-stage CHMI with a homologous parasite.

4.3 VAC054 Methods

Detailed methods of the recruitment and enrolment of volunteers, as well as the assays used in this trial can be found in Chapter two: Materials and Methods.

4.3.1 VAC054 Study Design

This study was an open-label non-randomised phase I/IIa trial of the blood-stage malaria vaccine candidate FMP2.1/AS01, with efficacy assessed by blood-stage CHMI in vaccinated volunteers compared with infectivity controls (Figure 4-1). The study was conducted at the CCVTM, University of Oxford, Oxford, UK. Volunteers were also recruited and vaccinated at

two other trial sites (the NIHR WTCRF, University Hospital Southampton NHS Foundation Trust and the NIHR WTCRF, Imperial College Healthcare NHS Trust) but CHMI for all volunteers took place in Oxford. Allocation to study group was based on time of enrolment (vaccinees were enrolled before controls) and volunteer preference.

The sample size for the study was determined from power calculations performed by the Centre for Statistics in Medicine at the University of Oxford. Data were available from small studies undertaken with the same inoculum in Oxford (174, 175), as well as at the Radboud University Nijmegen Medical Centre in the Netherlands (179). These historical data suggested the coefficient of variation in the controls may range from 22% (Nijmegen where mean PMR = 10) to 33% (Oxford where mean PMR = 12). A study design (where the maximum number of volunteers = 30) with 15 controls versus 15 vaccinees consistently provided the best power (≥80%) to observe a 33% reduction in mean PMR when allowing for the coefficients of variation.

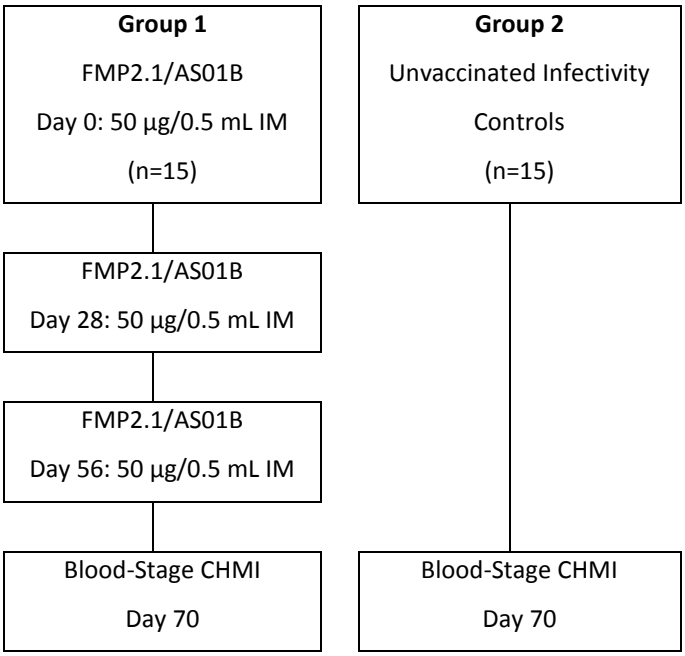


Figure 4-1: VAC054 Overview of trial groups.

IM = intramuscular; CHMI = controlled human malaria infection

4.3.2 VAC054 Ethics

The study received ethical approval from the Oxfordshire Research Ethics Committee A in the UK (Ref 13/SC/0596), and the Western Institutional Review Board (WIRB) in the USA (Ref 20131985). The study was approved by the UK MHRA (Ref 21584/0326/001-0001). The trial was registered with Clincaltrials.gov (NCT02044198) and was conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH guidelines for Good Clinical Practice.

4.3.3 VAC054 Objectives and Endpoints

Primary objective

To establish whether the FMP2.1/AS01B vaccine can demonstrate a reduced PMR in vaccinated subjects compared to infectivity controls against vaccine-homologous 3D7 clone parasites in a Phase I/IIa blood-stage CHMI model.

Primary endpoint

PCR-derived PMR was the primary study endpoint, and comparison of the endpoint between the two groups constituted the primary analysis for efficacy. The secondary analyses for efficacy were:

- Time to microscopic patency compared between the two groups.
- A test of the hypothesis that there is a relationship between *in vitro* GIA induced by the FMP2.1 vaccine and PMR.
- A test of the hypothesis that there is a relationship between anti-AMA1 antibody responses induced by the FMP2.1 vaccine and PMR.

Secondary objectives

To assess the safety of FMP2.1/AS01B in healthy malaria-naïve adults in the UK.

To assess immunological readouts for association with a reduced parasite multiplication rate.

Secondary endpoints

The safety of the FMP2.1/AS01 vaccine was assessed according to the following endpoints:

- Occurrence of each solicited AE within a 7-day follow-up period (day of vaccination and 6 subsequent days) after each vaccination.
- Occurrence of unsolicited AEs within 30 days (day of vaccination and 29 subsequent days) after each vaccination*.
- Occurrence of a serious adverse event from the first vaccination to the end of the study.

Solicited and unsolicited AE data was collected at each clinic visit from diary cards, clinical review, clinical examination (including observations) and laboratory results. These AE data were tabulated and frequency, duration and severity of AEs were compared between groups.

* The occurrence of unsolicited AEs was in fact monitored until the day 90 post-CHMI visit, but only those events occurring within 30 days of each vaccination were used for the analysis of this safety endpoint.

4.3.4 VAC054 Participants

Healthy, malaria-naïve males and non-pregnant females aged 18-45 were invited to participate in the study. All volunteers gave written informed consent prior to participation. Volunteers were recruited and vaccinated at three sites in the UK (Oxford, Southampton and London), with blood-stage CHMI and follow-up for all volunteers carried out at the CCVTM, University of Oxford. The inclusion and exclusion criteria for participation are described below.

4.3.4.1 VAC054 Inclusion Criteria

- Healthy, male or non-pregnant female adult aged 18 - 45 years.
- Subject willing and able to give written informed consent for participation in the study.
- Resident in or near Oxford for the duration of the CHMI part of the study. Or for volunteers not living in Oxford: agreement to stay in arranged accommodation close to the trial centre during a part of the study (from the day before CHMI until anti-malarial treatment is completed).

- Female subjects of child bearing potential willing to practice continuous effective contraception for the duration of the study.
- Able (in the Investigator's opinion) and willing to comply with all study requirements.
- Willing to allow his or her GP and consultant, if appropriate, to be notified of participation in the study.
- Agreement to permanently refrain from blood donation, as per current UK Blood Transfusion and Tissue Transplantation Services guidelines (221).
- Reachable (24 hours a day) by mobile phone during the period between CHMI and completion of antimalarial treatment.
- Willingness to take a curative anti-malaria regime following CHMI.
- Answer all questions on the informed consent questionnaire correctly.

4.3.4.2 VAC054 Exclusion Criteria

- History of clinical malaria (any species).
- Travel to a malaria endemic region during the study period or within the preceding six months with significant risk of malaria exposure.
- Use of systemic antibiotics with known antimalarial activity within 30 days of CHMI (e.g. trimethoprim-sulfamethoxazole, doxycycline, tetracycline, clindamycin, erythromycin, fluoroquinolones and azithromycin).
- Prior receipt of an investigational malaria vaccine or any other investigational vaccine likely to impact on interpretation of the trial data.
- Receipt of an investigational product in the 30 days preceding enrolment, or planned receipt during the study period.
- History of sickle cell anaemia, sickle cell trait, thalassaemia or thalassaemia trait or any haematological condition that could affect susceptibility to malaria infection.
- 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 or previous severe adverse reaction to a blood transfusion.

- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine (or malaria infection).
- Any history of anaphylaxis post vaccination.
- 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.
- Contraindications to the use of all three proposed anti-malarial medications; Riamet, Malarone and Chloroquine.
- Any clinical condition known to prolong the QT interval.
- Family history of congenital QT prolongation or sudden death.
- Positive family history in 1st and 2nd degree relatives < 50 years old for cardiac disease.
- History of cardiac arrhythmia, including clinically relevant bradycardia.
- An estimated, ten year risk of fatal cardiovascular disease of ≥5%, as estimated by the Systematic Coronary Risk Evaluation (SCORE) system (222).
- 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 may be requested at the discretion of the Investigator.
- 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 42 standard UK units every week.
- Suspected or known injecting drug abuse in the 5 years preceding enrolment.
- Seropositive for hepatitis B surface antigen (HBsAg).
- Seropositive for hepatitis C virus (antibodies to HCV) at screening.

- 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.
- Volunteers unable to be closely followed for social, geographic or psychological reasons.

4.3.5 VAC054 Assessment of Safety

Safety data were assessed by actively and passively collected data on adverse events (AEs) occurring throughout the VAC054 trial. Volunteers were asked to complete a paper diary card for seven days after each vaccination, recording both solicited and unsolicited AEs, as well as any medication taken. Solicited AEs included local AEs (injection site pain, redness, swelling, itch and warmth) and systemic AEs (fever, feverishness, myalgia, arthralgia, nausea, malaise, headache and fatigue). The information from the diary card was then entered into the OpenClinica eCRFs.

Adverse event data for were also collected at follow-up visits throughout the trial, until the visit 90 days after CHMI. Following this, only data on serious adverse events and adverse events of special interest were collected. The adverse events of special interest in this trial were:

- Severe hypersensitivity reactions (eg. Anaphylaxis).
- Any new, suspected auto-immune disease.
- Meningitis.

Baseline safety blood tests (FBC, U&Es, LFTs) were carried out at a volunteer's screening visit and at Days 0, 7, 14, 28, 35, 42, 56 and 63 for vaccinees, and days 69 (C-1), C+9, day of diagnosis, C+28 and at C+90 for both groups. Any laboratory AEs were graded as per site-specific grading tables, using local laboratory reference ranges (see Appendix 6). Causality relating to vaccination was assigned by the lead investigator and peer-reviewed.

Following CHMI volunteers were seen twice daily from C+2 (once on day C+1). At each visit they were asked a list of symptoms commonly associated with malaria infection ('solicited'

symptoms). These included pyrexia, low back pain, chills, rigors, feverishness, myalgia, arthralgia, headache, fatigue, nausea, sweats, vomiting and diarrhoea. In addition, following commencement of antimalarials, volunteers were also asked about the following symptoms, which may be associated with antimalarial treatment: dizziness, abdominal pain, palpitations, itchy skin, rashes, cough and insomnia. If any of these symptoms were present they were asked to grade the severity from 1 (mild) to 3 (severe), using the same severity grading criteria as used for AEs after vaccination. Any other symptoms reported by the volunteers were also recorded.

4.3.5.1 Stopping and Holding Rules

For this trial, safety stopping and holding rules were introduced to ensure participant safety during the trial. These applied to Group 1 volunteers only as Group 2 volunteers did not receive any vaccinations. The holding and stopping rules used in the VAC054 protocol are shown below.

Holding rules (only applicable to Group 1)
Solicited local adverse events:

- A Grade 3 solicited local adverse event beginning within 2 days after vaccination (day of vaccination and one subsequent day) and persisting at Grade 3 for >48 hrs in more than 3/15 of Group 1 participants.

Solicited systemic adverse events:

- A Grade 3 solicited systemic adverse event beginning within 2 days after vaccination (day of vaccination and one subsequent day) and persisting at Grade 3 for >48hrs in more than 3/15 of Group 1 participants.

Laboratory adverse events:

- Immunisation of Group 1 could be put on hold if 3/15 participants developed a Grade 2 laboratory AE which lasted for ≥ 48 hours or 2/15 participants developed any Grade 3 laboratory AE considered to be associated with immunisation.

A serious adverse event considered possibly or probably related to vaccination occurred

Individual stopping rules (for all vaccinated individuals)

In addition to the above stated group holding rules, stopping rules for individual subjects applied (i.e. indications to withdraw individuals from further vaccinations):

Local reactions:

- Injection site ulceration, abscess or necrosis.

Laboratory adverse events:

- If a participant developed a Grade 3 laboratory AE considered possibly or probably related within 2 days after vaccination (day of vaccination and one subsequent day) which persisted continuously at Grade 3 for > 72hrs, they should not continue in the trial.

Systemic solicited adverse events:

- If a participant developed a Grade 3 systemic solicited adverse event considered possibly or probably related within 2 days after vaccination (day of vaccination and one subsequent day) which persisted continuously at Grade 3 for > 72hrs, they should not continue in the trial.

Unsolicited adverse events:

- If a participant had any Grade 3 adverse event considered possibly or probably related to vaccination, persisting continuously at Grade 3 for >72hrs, they should not continue in the trial.
- If a participant had a serious adverse event considered probably related to vaccination they should not continue in the trial.
- If a participant had an acute allergic reaction or anaphylactic shock following the administration of vaccine investigational product they should not continue in the trial.

4.3.6 VAC054 Interventions

4.3.6.1 FMP2.1/AS01 vaccine preparation

The vaccine FMP2.1 was produced under GMP at the WRAIR BioProduction Facility and supplied as a lyophilised preparation. FMP2.1 was mixed with the AS01 adjuvant system from GSK immediately prior to vaccination. A 50 µg dose of FMP2.1 was administered in 0.5 mL of AS01 as an IM injection into the deltoid muscle of the non-dominant arm. The vaccine was

administered at days 0, 28 and 56 (nominal study days are used) with an allowed window between vaccinations of a minimum of 21 days and maximum of 35 days. Volunteers also attended follow-up visits on days 3, 7, 14, 31, 35, 42, 59, 63 and 69 before CHMI on day 70.

4.3.6.2 Blood-Stage CHMI

CHMI took place 2 weeks (range 14-16 days) after the final vaccination (day 70/ day of CHMI [dC]) at the CCVTM in Oxford, with the 15 infectivity controls (Group 2) inoculated at the same time as the vaccinees. Inoculation was carried out in three clinic rooms simultaneously alternating between Group 1 and Group 2 volunteers in each room. A single vial of blood-stage inoculum was thawed, washed & diluted under aseptic conditions (see Chapter two). Sequencing of the parasite's AMA1 gene confirmed 100% identity with the FMP2.1 vaccine.

The intended inoculum was 1000 parasitised erythrocytes per volunteer. A limiting dilution assay on the inoculum was set up at the time the last volunteer was infected which demonstrated 69% viability (i.e. an effective inoculum of 690 parasites per volunteer).

Following CHMI, blood samples were taken once on the day after CHMI (dC+1) and twice daily from dC+2 for *P. falciparum* qPCR and thick blood film. Diagnosis of malaria was made according to Table 4.1. The PMR was calculated from qPCR data as per the methods described in Chapter two.

	THICK FILM MICROSCOPY	
MALARIAL SYMPTOMS	**Positive**	**Negative**
Symptomatic	Positive diagnosis	Positive diagnosis if any available PCR result is ≥ 500 parasites/mL
Asymptomatic	Positive diagnosis if any available PCR result is ≥ 500 parasites/mL (Otherwise delay treatment)	Negative diagnosis

Table 4.1: VAC054 Malarial Diagnosis Criteria.

4.4 Results

4.4.1 VAC054 Participant Flow

Forty-five volunteers were screened in total and fifteen were recruited to each group, with more males recruited than females in both (Group 1 = 66.7% male; Group 2 = 73.3% male). The age range of volunteers in Group 1 was 23 – 43 years (mean 33 years) and 19 – 34 years in Group 2 (mean 22 years). Three volunteers in Group 1 withdrew from the trial prior to completing the vaccination phase, and one volunteer in Group 1 also withdrew post-CHMI (time-point dC+8.5), all for personal reasons. The trial flow diagram is shown in Figure 4-2.

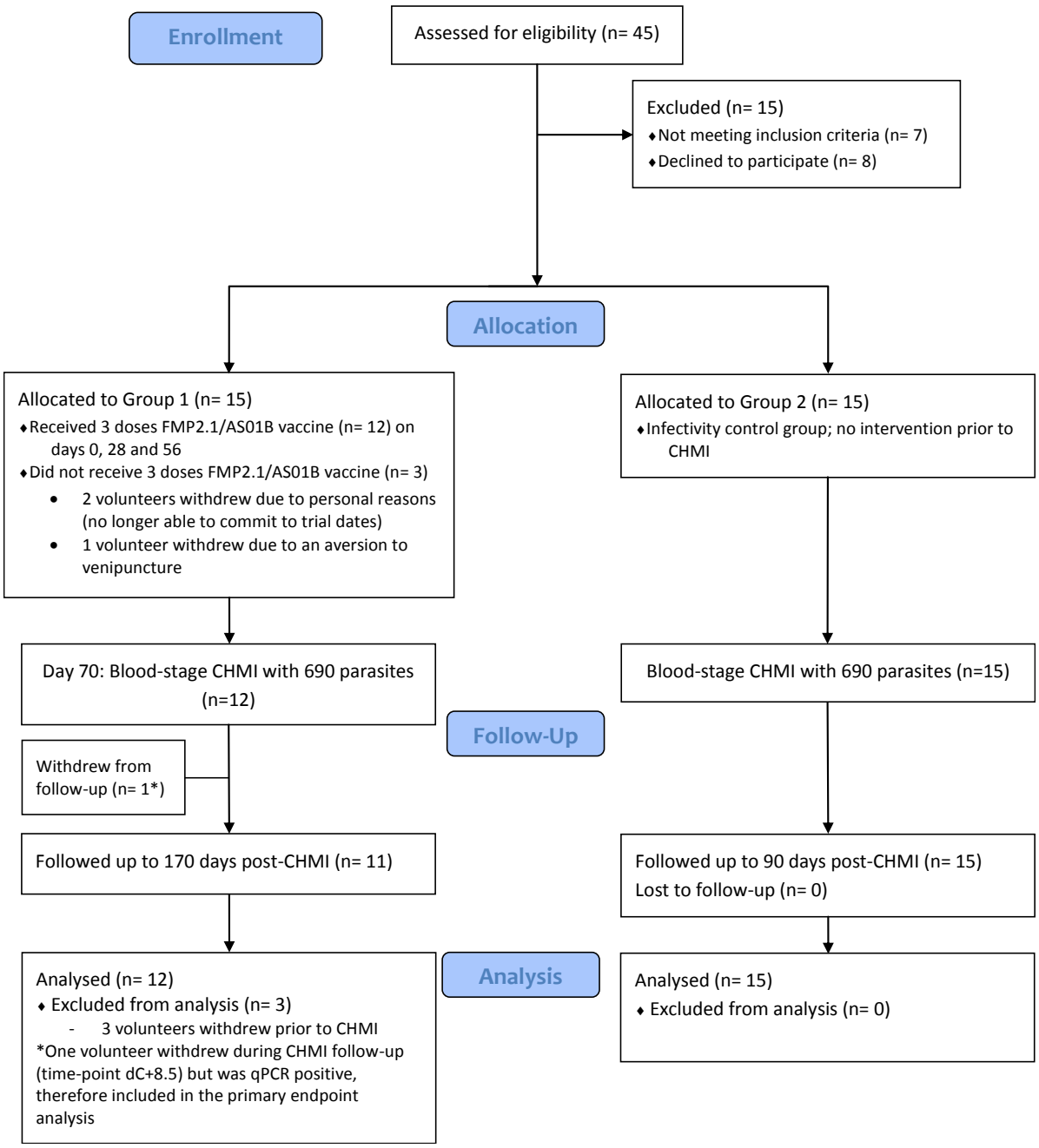


Figure 4-2: VAC054 trial flow diagram.

Vaccinations began on 28th April 2014, blood-stage CHMI occurred on 4th July 2014 and all follow-up visits were completed by 23rd December 2014. All vaccinees received their immunisations as scheduled, except for three volunteers in Group 1 who withdrew during the vaccination phase.

4.4.2 VAC054 Vaccine Safety and Reactogenicity

There were no SAEs or unexpected reactions during the course of the trial and no volunteers withdrew due to vaccine-related AEs. The safety profile of the FMP2.1/AS01 vaccine was

similar to that reported previously in healthy US adult volunteers (95), with the second and third vaccinations reported as more reactogenic than the first.

Information on solicited (expected) AEs was collected for 7 days after each vaccination by means of a diary card completed daily by volunteers and at clinic visits during this time. The percentage of volunteers experiencing these AEs at the maximum severity they reported is shown in the graph in Figures 4-3 and 4-4, and in Table 4.2. The grading of AEs is described in Chapter two.

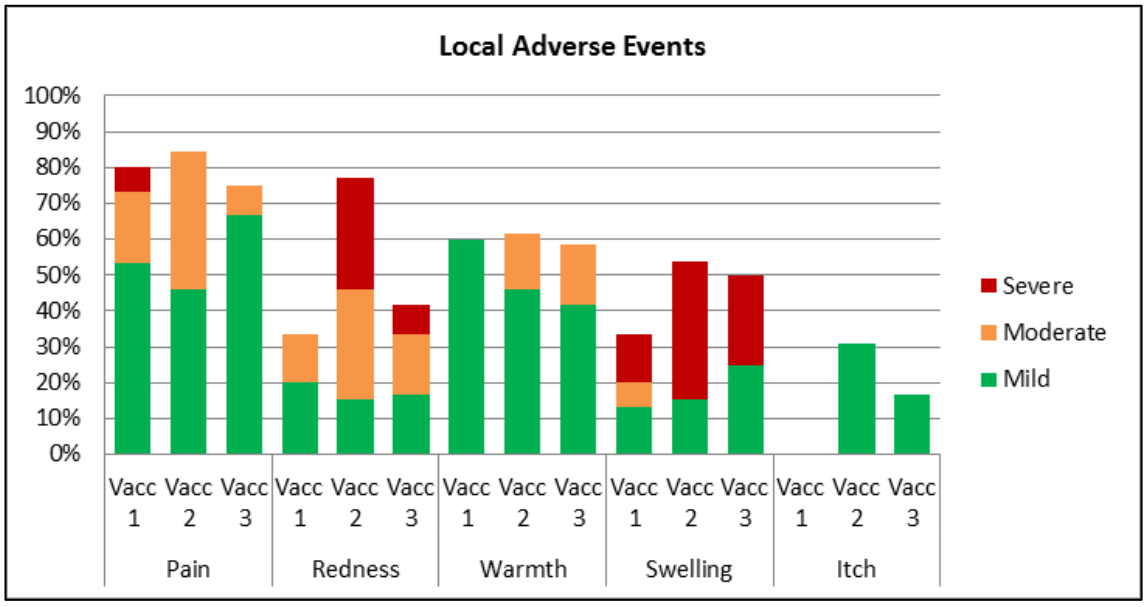


Figure 4-3: Maximum severity of Local AEs following FMP2.1/AS01B.

Only the highest intensity of each AE per subject is listed. Data were exported from the OpenClinica database into Excel and the percentages of volunteers experiencing each AE following each vaccination were calculated (Vacc 1: n= 15, Vacc 2: n=13, Vacc 3: n=12).

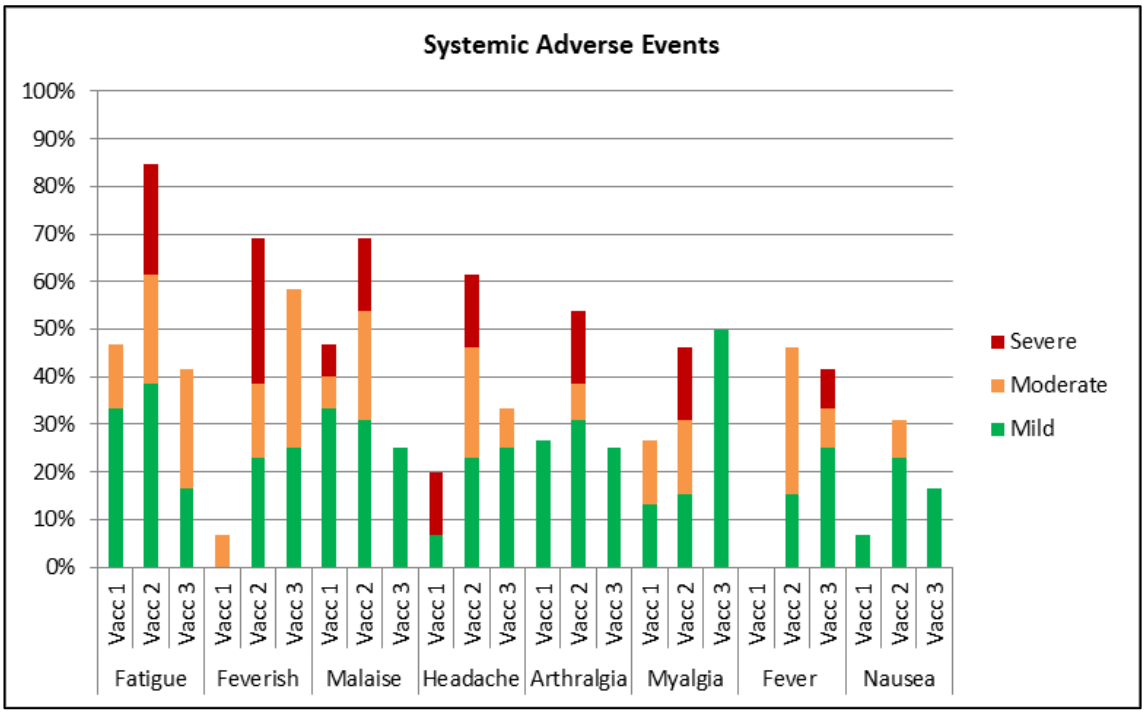


Figure 4-4: Maximum reported severity of systemic AEs following FMP2.1/AS01B.

Only the highest intensity of each AE per subject is listed. Data were exported from the OpenClinica database into Excel and the percentages of volunteers experiencing each AE following vaccinations at each timepoint were calculated (Vacc 1: n= 15, Vacc 2: n=13, Vacc 3: n=12).

		1st Vaccination FMP2.1/AS01B (*n*= 15)				2nd Vaccination FMP2.1/AS01B (*n*= 13)				3rd Vaccination FMP2.1/AS01B (*n*= 12)			
		Mild	**Moderate**	**Severe**	**Total**	**Mild**	**Moderate**	**Severe**	**Total**	**Mild**	**Moderate**	**Severe**	**Total**
Local Adverse Events	**Pain**	53.3%	20.0%	6.7%	**80.0%**	46.2%	38.5%	0.0%	**84.6%**	66.7%	8.3%	0.0%	**75.0%**
	Redness	20.0%	13.3%	0.0%	**33.3%**	15.4%	30.8%	30.8%	**76.9%**	16.7%	16.7%	8.3%	**41.7%**
	Warmth	60.0%	0.0%	0.0%	**60.0%**	46.2%	15.4%	0.0%	**61.5%**	41.7%	16.7%	0.0%	**58.3%**
	Swelling	13.3%	6.7%	13.3%	**33.3%**	15.4%	0.0%	38.5%	**53.8%**	25.0%	0.0%	25.0%	**50.0%**
	Itch	0.0%	0.0%	0.0%	**0.0%**	30.8%	0.0%	0.0%	**30.8%**	16.7%	0.0%	0.0%	**16.7%**
Systemic Adverse Events	**Fatigue**	33.3%	13.3%	0.0%	**46.7%**	38.5%	23.1%	23.1%	**84.6%**	16.7%	25.0%	0.0%	**41.7%**
	Feverish	0.0%	6.7%	0.0%	**6.7%**	23.1%	15.4%	30.8%	**69.2%**	25.0%	33.3%	0.0%	**58.3%**
	Malaise	33.3%	6.7%	6.7%	**46.7%**	30.8%	23.1%	15.4%	**69.2%**	25.0%	0.0%	0.0%	**25.0%**
	Headache	6.7%	0.0%	13.3%	**20.0%**	23.1%	23.1%	15.4%	**61.5%**	25.0%	8.3%	0.0%	**33.3%**
	Arthralgia	26.7%	0.0%	0.0%	**26.7%**	30.8%	7.7%	15.4%	**53.8%**	25.0%	0.0%	0.0%	**25.0%**
	Myalgia	13.3%	13.3%	0.0%	**26.7%**	15.4%	15.4%	15.4%	**46.2%**	50.0%	0.0%	0.0%	**50.0%**
	Fever	0.0%	0.0%	0.0%	**0.0%**	15.4%	30.8%	0.0%	**46.2%**	25.0%	8.3%	8.3%	**41.7%**
	Nausea	6.7%	0.0%	0.0%	**6.7%**	23.1%	7.7%	0.0%	**30.8%**	16.7%	0.0%	0.0%	**16.7%**

Table 4.2: Maximum Solicited Reactogenicity Summary Following FMP2.1/AS01B Vaccination.

This Table shows the maximum severity of all solicited adverse events reported by volunteers (i.e. those for which a severity score was required in the diary card for 7 days following each vaccination). Frequency is calculated as the number of subjects counted once at worst severity.

Data on unsolicited AEs were also collected. These were AEs reported either outside of the diary card period or adverse events other than those listed above reported within 7 days of vaccination. These AEs were collected throughout the trial. Unsolicited AEs were assigned a causality score, relating to vaccination, as described in Chapter two. This was agreed between myself, as lead clinician of the trial, and an independent clinician who provided peer review.

All unsolicited AEs were assigned a Medical Dictionary for Regulatory Activities (MedDRA) code. Unsolicited AEs following vaccination are shown in the tables below classed by MedDRA System Organ Class, Higher Level Term and Preferred Term. AEs occurring before CHMI and considered possibly, probably or definitely related to vaccination are shown in Table 4.3. AEs considered unlikely or unrelated to vaccination occurring before CHMI are shown in Table 4.4

Vaccination	System Organ Class	Higher Level Term	Preferred Term	No. of volunteers reporting AE (%)
1	Gastrointestinal (GI) disorders	GI and abdo pains (excl oral and throat)	Abdominal pain	1 (6.7%)
1	Nervous system disorders	Neurological signs and symptoms NEC	Dizziness	1 (6.7%)
2	General disorders and administration site conditions	Pain and discomfort NEC	Chest pain	1 (7.7%)
2	Nervous system disorders	Neurological signs and symptoms NEC	Dizziness	1 (7.7%)
2	Psychiatric disorders	Parasomnias	Nightmare	1 (7.7%)
2	Skin and subcutaneous tissue disorders	Erythemas	Erythema (arm)	1 (7.7%)
3	Skin and subcutaneous tissue disorders	Erythemas	Erythema (arm)	1 (8.3%)

Table 4.3: Related unsolicited AEs reported following vaccination with FMP2.1/AS01B.

AEs considered possibly, probably or definitely related to vaccination. The number of volunteers reporting each AE is shown; the percentage of volunteers is calculated based on the numbers vaccinated at each vaccination timepoint (i.e. 15 volunteers for the first vaccination, 13 for the second vaccination and 12 for the third vaccination).

Vaccination	System Organ Class	Higher Level Term	Preferred Term	No. of volunteers reporting AE (%)
1	Ear and labyrinth disorders	Ear disorders NEC	Ear pain	1 (6.7%)
1	Endocrine disorders	Thyroid hypofunction disorders	Hypothyroidism	1 (6.7%)
1	Eye disorders	Conjunctival infections, irritations and inflammations	Seasonal allergy	1 (6.7%)
1	General disorders and administration site conditions	Asthenic conditions	Fatigue	1 (6.7%)
			Malaise	1 (6.7%)
1	Musculoskeletal and connective tissue disorders	Musculoskeletal and connective tissue pain and discomfort	Pain in extremity	1 (6.7%)
			Musculoskeletal pain	1 (6.7%)
1	Nervous system disorders	Headaches NEC	Headache	3 (20.0%)
1	Reproductive system and breast disorders	Menstruation with increased bleeding	Menorrhagia	1 (6.7%)
1	Respiratory, thoracic and mediastinal disorders	Upper respiratory tract infections NEC	Nasopharyngitis	1 (6.7%)
			Sinus headache	1 (6.7%)
			Oropharyngeal pain	1 (6.7%)
		Nasal congestion and inflammations	Nasal congestion	1 (6.7%)
		Upper respiratory tract signs and symptoms	Sinus headache	1 (6.7%)
1	Skin and subcutaneous tissue disorders	Erythemas	Erythema (neck)	1 (6.7%)
2	Ear and labyrinth disorders	Ear disorders NEC	Ear pain	1 (7.7%)
2	Eye disorders	Lid, lash and lacrimal infections, irritations and inflammations	Erythema of eyelid	1 (7.7%)
2	Gastrointestinal disorders	Gastrointestinal and abdominal pains (excl oral and throat)	Abdominal pain	1 (7.7%)
2	Immune system disorders	Atopic disorders	Seasonal allergy	2 (15.4%)
2	Infections and infestations	Dental and oral soft tissue infections	Gingivitis	1 (7.7%)
		Upper respiratory tract infections	Nasopharyngitis	1 (7.7%)
2	Musculoskeletal and connective tissue disorders	Joint related signs and symptoms	Arthralgia	1 (7.7%)
		Musculoskeletal and connective tissue pain and discomfort	Musculoskeletal pain	1 (7.7%)
2	Nervous system disorders	Headaches NEC	Headache	1 (7.7%)
2	Psychiatric disorders	Parasomnias	Nightmare	1 (7.7%)
2	Respiratory, thoracic and mediastinal disorders	Upper respiratory tract infections NEC	Sinusitis	1 (7.7%)
		Coughing and associated symptoms	Cough	1 (7.7%)
		Bronchospasm and obstruction	Wheezing	1 (7.7%)
		Upper respiratory tract signs and symptoms	Oropharyngeal pain	1 (7.7%)
			Rhinorrhoea	1 (7.7%)
		Coughing and associated symptoms	Cough	1 (7.7%)
2	Skin and subcutaneous tissue disorders	Photosensitivity and photodermatosis conditions	Sunburn	1 (7.7%)
3	Immune system disorders	Atopic disorders	Seasonal allergy	2 (16.7%)
3	Injury, poisoning and procedural complications	Muscle, tendon and ligament injuries	Ligament sprain	1 (8.3%)
3	Nervous system disorders	Headaches NEC	Headache	1 (8.3%)
3	Skin and subcutaneous tissue disorders	Apocrine and eccrine gland disorders	Miliaria	1 (8.3%)
3	Surgical and medical procedures	Contraceptive methods female	Intra-uterine contraceptive device insertion	1 (8.3%)

Table 4.4: Unrelated unsolicited AEs reported following vaccination with FMP2.1/AS01B.

AEs considered unlikely related or unrelated to vaccination. The number of volunteers reporting each AE is shown; the percentage of volunteers is calculated based on the numbers vaccinated at each vaccination timepoint (i.e. 15 volunteers for the first vaccination, 13 for the second vaccination and 12 for the third vaccination).

Laboratory adverse events were graded as per the site-specific grading tables, using local laboratory reference ranges (Appendix 6). Causality relating to vaccination was assigned by the lead investigator and peer-reviewed. The agreed causality score is shown in Table 4.5 below.

Lab AE	Volunteer	Timepoint(s)	Max severity	Resolved by C-1	Causality	No. of volunteers with lab AE (%) (n=15)
Anaemia	1054005 1054008	Day 56 Days 0 to C-1	Mild Moderate	Yes No	1 0	2 (13.3%)
Elevated leukocytes	2054102 2054105	Day 14 Day 7	Moderate Mild	NA- withdrew before C-1 visit	2 2	2 (13.3%)
Hyperbilirubinaemia	1054002	D63	Mild	Yes	1	1 (6.7%)
Elevated alanine transaminase (ALT)	1054004 2054101	Day 35 Day 63	Mild Mild	Yes Yes	3 3	2 (13.3%)
Elevated Alkaline phosphatase	1054010	Day 14, Day 28	Mild	Yes	1	1 (6.7%)
Hypernatraemia	1054002	Day 42	Mild	Yes	1	1 (6.7%)
Hypokalaemia*	1054001 1054010 1054011 2054101	C-1 Day 56 Day 56 C-1	Mild Mild Mild Moderate	NA Yes Yes NA	0 0 0 0	4 (26.7%)
Elevated creatinine	2054103	Days 7, 56	Mild	Yes	1	1 (6.7%)

Table 4.5: Laboratory AEs following vaccination and prior to CHMI.

**Hypokalaemia is considered likely to be pseudohypokalaemia due to storage of samples before processing without centrifugation at the point of care, as described in a previous study in Oxford (223).*

4.4.3 CHMI Safety

There were no serious adverse events or withdrawals from the trial due to CHMI-related adverse events. Participants were reviewed twice daily from day 2 following CHMI. The AEs relating to CHMI are shown in Figure 4-5, with maximum severity reported at each time-point. Data on AEs relating to treatment (with Riamet in 26 volunteers and Malarone in 1 volunteer) were collected at the visits 24 and 48 hours after diagnosis. These AEs are shown in Figure 4-6.

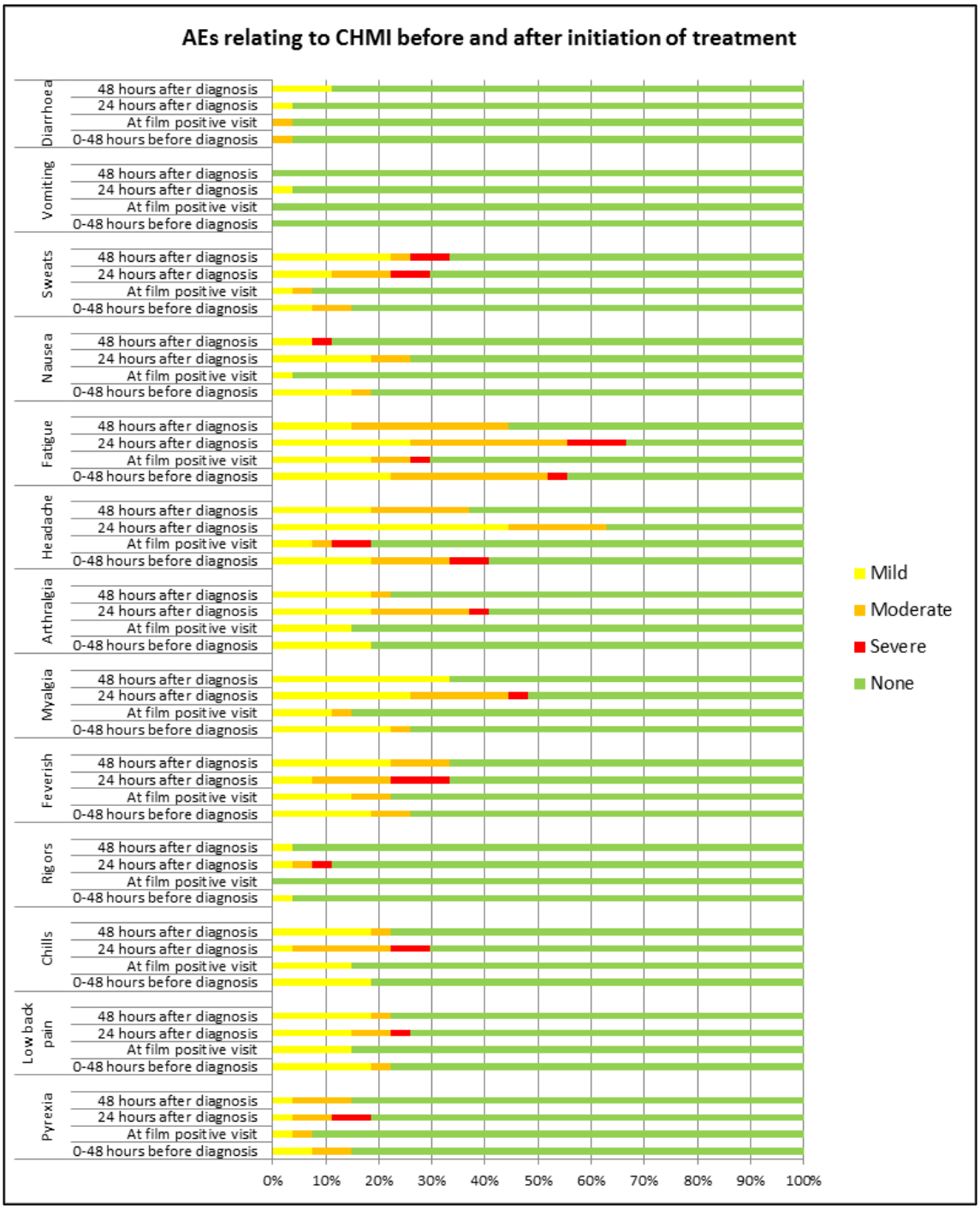


Figure 4-5: VAC054 Maximum reported severity of CHMI-related AEs before, at and after diagnosis/ treatment.

Only the highest intensity of each AE per subject is listed. Data were exported from the OpenClinica database into Excel and the percentages of volunteers experiencing each AE following CHMI were calculated (n=27).

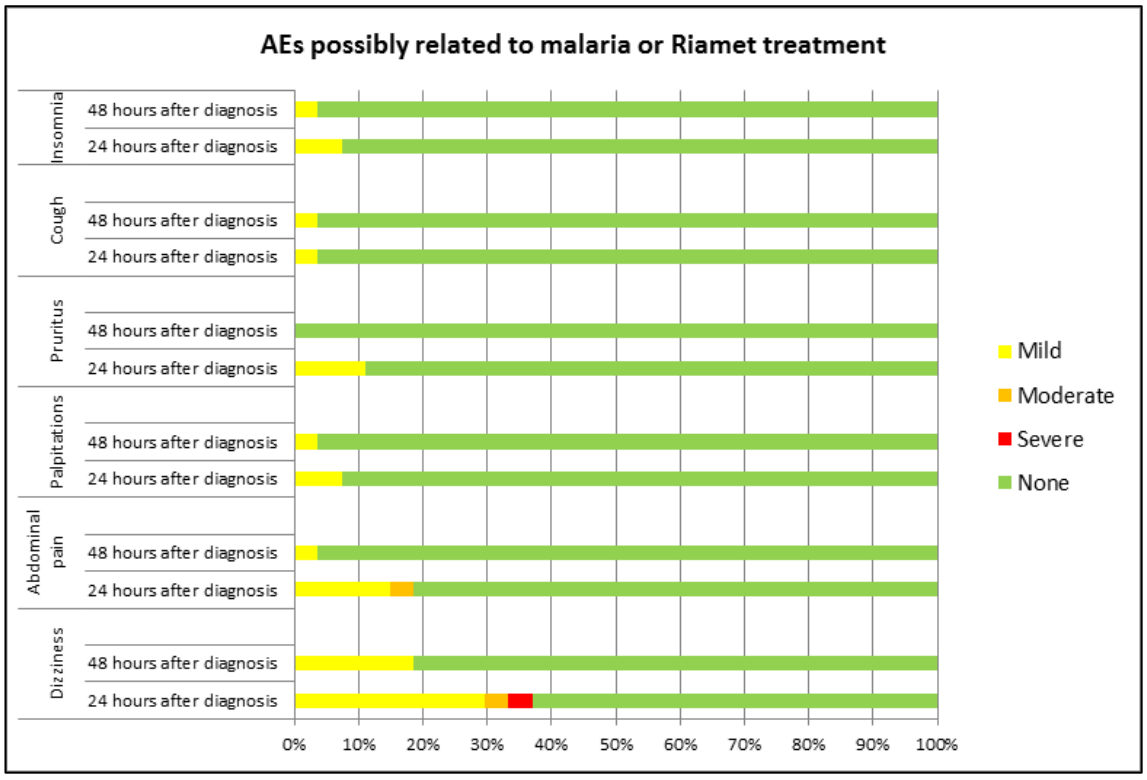


Figure 4-6: VAC054 Maximum reported severity of AEs considered possibly related to anti-malarial therapy after initiation of treatment.

These adverse events were only specifically asked about after initiation of antimalarial therapy. If these symptoms occurred before treatment they were recorded as 'unsolicited AEs'. Only the highest intensity of each AE per subject is listed (n=27). Data were exported from the OpenClinica database into Excel and the percentages of volunteers experiencing each AE following treatment for CHMI were calculated.

As well as the list of solicited adverse events collected at each visit participants were also asked to report any other adverse events that occurred, and these were recorded and assigned a MedDRA code. Any unsolicited adverse events reported by vaccinated volunteers were also assigned a causality score, as described above (causality relating to vaccination rather than CHMI). AEs occurring after CHMI are shown in Tables 4.6 and 4.7 (≤30 days and >30 days post-CHMI respectively), and include AEs occurring in both vaccinated and control volunteers. None of the AEs occurring after CHMI were considered possibly, probably or definitely related to vaccination.

Group	System Organ Class	Higher Level Term	Preferred Term	No. of volunteers reporting AE (%)
Vaccinees (Group 1)	Gastrointestinal disorders	Gastrointestinal and abdominal pains (excl oral and throat)	Abdominal pain lower	1 (3.7%)
		Diarrhoea (excl infective)	Diarrhoea	1 (3.7%)
		Gastrointestinal signs and symptoms NEC	Abdominal discomfort	1 (3.7%)
	General disorders and administration site conditions	Feelings and sensations NEC	Hangover	1 (3.7%)
		Infusion site reactions	Infusion site paraesthesia	1 (3.7%)
			Infusion site bruising	1 (3.7%)
	Immune system disorders	Atopic disorders	Seasonal allergy	1 (3.7%)
	Infections and infestations	Urinary tract infections	Urinary tract infection	1 (3.7%)
		Abdominal and gastrointestinal infections	Gastroenteritis	1 (3.7%)
		Upper respiratory tract infections	Pharyngitis	1 (3.7%)
	Musculoskeletal and connective tissue disorders	Joint related signs and symptoms	Arthralgia	1 (3.7%)
		Musculoskeletal and connective tissue pain and discomfort	Pain in extremity	1 (3.7%)
	Nervous system disorders	Neurological signs and symptoms NEC	Dizziness	1 (3.7%)
		Paraesthesias and dysaesthesias	Paraesthesia	1 (3.7%)
	Psychiatric disorders	Parasomnias	Nightmare	1 (3.7%)
	Respiratory, thoracic and mediastinal disorders	Lower respiratory tract infections NEC	Lower respiratory tract infection	1 (3.7%)
		Upper respiratory tract signs and symptoms	Oropharyngeal pain	1 (3.7%)
			Rhinorrhoea	1 (3.7%)
	Skin and subcutaneous tissue disorders	Dermatitis and eczema	Dermatitis	1 (3.7%)
		Skin injuries and mechanical dermatoses	Skin abrasion	1 (3.7%)
		Photosensitivity and photodermatosis conditions	Sunburn	1 (3.7%)
Controls (Group 2)	Gastrointestinal disorders	Gastrointestinal and abdominal pains (excl oral and throat)	Abdominal pain lower	1 (3.7%)
		Stomatitis and ulceration	Mouth ulceration	1 (3.7%)
	General disorders and administration site conditions	Infusion site reactions	Infusion site erythema	1 (3.7%)
		Administration site reactions NEC	Vessel puncture site haematoma	2 (7.4%)
			Vessel puncture site pain	1 (3.7%)
	Immune system disorders	Atopic disorders	Seasonal allergy	7.4%
		Allergic conditions NEC	Allergy to plants	1 (3.7%)
	Metabolism and nutrition disorders	Appetite disorders	Decreased appetite	1 (3.7%)
	Musculoskeletal and connective tissue disorders	Musculoskeletal and connective tissue pain and discomfort	Musculoskeletal chest pain	1 (3.7%)
			Pain in extremity	1 (3.7%)
	Nervous system disorders	Neurological signs and symptoms NEC	Dizziness	2 (7.4%)
		Paraesthesias and dysaesthesias	Paraesthesia	1 (3.7%)
		Disturbances in initiating and maintaining sleep	Insomnia	1 (3.7%)
	Renal and urinary disorders	Urinary abnormalities	Chromaturia	1 (3.7%)
	Respiratory, thoracic and mediastinal disorders	Breathing abnormalities	Dyspnoea	1 (3.7%)
		Upper respiratory tract signs and symptoms	Oropharyngeal pain	3 (11.1%)

Table 4.6: VAC054 Unsolicited AEs reported in the first 30 days following CHMI.

(n=27) CHMI occurred 2 weeks after the final vaccination. All AEs were considered unlikely related or unrelated to vaccination.

Group	System Organ Class	Higher Level Term	Preferred Term	No. of volunteers reporting AE (%)
Vaccinees (Group 1)	Infections and infestations	Neisseria infections	Gonorrhoea	1 (3.8%)
		Upper respiratory tract infections	Rhinitis	1 (3.8%)
	Investigations	Physical examination procedures and organ system status	Weight decreased	1 (3.8%)
	Nervous system disorders	Headaches NEC	Headache	2 (7.7%)
	Psychiatric disorders	Increased physical activity levels	Psychomotor hyperactivity	1 (3.8%)
	Renal and urinary disorders	Renal infections and inflammations (excl nephritis)	Kidney infection	1 (3.8%)
Controls (Group 2)	Injury, poisoning and procedural complications	Bone and joint injuries NEC	Joint injury	1 (3.8%)
	Respiratory, thoracic and mediastinal disorders	Upper respiratory tract signs and symptoms	Sneezing	1 (3.8%)
			Oropharyngeal pain	2 (7.7%)

Table 4.7: VAC054 Unsolicited AEs reported more than 30 days after CHMI.
(n=26) All AEs were considered unlikely related or unrelated to vaccination.

Volunteers had blood tests prior to CHMI (C-1) and then at C+6, diagnosis, C+28 and C+90. Blood tests were also carried out at other timepoints if clinically indicated. Causality relating to vaccination was not assigned after CHMI. AEs were as expected following malaria infection and there were none which caused clinical concern. The laboratory AEs following CHMI are shown in Table 4.8.

The donor of the infected blood for the CHMI inoculum was seropositive for EBV and CMV, so it was previously required for volunteers taking part in blood-stage CHMI studies with this inoculum to be seropositive for both viruses (174). However, this is no longer deemed to be necessary as the blood was leukodepleted prior to inoculum preparation and has since tested negative by PCR for both viruses. Several volunteers who were seronegative for one or both viruses had previously received the inoculum at other challenge centres in Australia (140) and the Netherlands (179) and none had seroconverted. To add to the safety database regarding this I checked serostatus prior to CHMI (the day before CHMI; C-1) and at the visit 90 days after CHMI (C+90). 37% of the 27 volunteers who underwent CHMI were seropositive for CMV and 89% were seropositive for EBV. None of the volunteers who were seronegative for either virus at C-1 seroconverted during the trial, as demonstrated in Table 4.9.

Laboratory AE	Volunteer	Timepoint(s)	Max severity	Resolved by C+28	Resolved by C+90	No. of volunteers with lab AE (%)
Anaemia	1054008	C+2, C+6, Diagnosis (C+10), C+28	Moderate	No	Yes	
	1054026	Diagnosis (C+9), C+28, C+90	Mild	No	No	2 (7.4%)
Neutropenia	1054022	C-1, C, C+6	Mild	Yes	Yes	1 (3.7%)
Lymphopenia	1054008	C+2	Severe	Yes	Yes	
	1054010	Diagnosis (C+10.5)	moderate	Yes	Yes	
	1054016	Diagnosis (C+10)	Severe	Yes	Yes	
	1054017	Diagnosis (C+10.5)	Mild	Yes	Yes	
	1054020	C+6, Diagnosis (C+8.5)	Mild	Yes	Yes	
	1054026	Diagnosis (C+9)	Mild	Yes	Yes	6 (22.2%_
Eosinophilia	1054012	C+90	Mild	NA	NA	
	1054018	C-1, Diagnosis (C+8.5)	Mild	Yes	Yes	
	3054208	C+6, Diagnosis (C+9), C+28, C+90	Mild	No	No	3 (11.1%)
Thrombocytopenia	1054011	Diagnosis (C+10)	Severe	Yes	Yes	
	1054016	Diagnosis (C+10)	Severe	Yes	Yes	2 (7.4%)
Hyperbilirubinaemia	1054017	C+28	Moderate	NA	Yes	
	1054007	C+90	Mild	NA	NA	
	2054106	C-1, C+6, Diagnosis (C+8.5)	Mild	Yes	No	
	3054208	C-1, C+6, Diagnosis (C+9), C+28	Mild	No	Yes	4 (14.8%)
Elevated alanine transaminase (ALT)	1054017	C+28	Mild	NA	Yes	
	3054208	C+28	Mild	NA	Yes	2 (7.4%)
Hypernatraemia	1054010	C+28	Mild	NA	Yes	
	1054013	C+28	Moderate	NA	Yes	
	1054018	C+28	Moderate	NA	Yes	
	1054026	C+28	Mild	NA	Yes	2 (7.4%)
Hypokalaemia*	1054001	Diagnosis (C+8.5)	Mild	Yes	Yes	
	1054008	C+2	Mild	Yes	Yes	
	1054016	C+28	Mild	NA	Yes	
	1054018	Diagnosis (C+8.5)	Mild	Yes	Yes	
	1054019	C-1, Diagnosis (C+8.5), C+28	Severe	No	Yes	
	2054101	Diagnosis (C+8.5)	Moderate	Withdrew before C+28	Withdrew before C+90	
	3054201	Diagnosis (C+8.5)	Moderate	Yes	Yes	
	3054202	Diagnosis (C+9)	Mild	Yes	Yes	
	3054208	C-1	Mild	Yes	Yes	9 (33.3%)
Elevated urea	2054104	C+28	Mild	NA	Yes	
	2054103	Diagnosis	Mild	Yes	Yes	2 (7.4%)

Table 4.8: VAC054 Laboratory AEs following CHMI in vaccinated and control volunteers.

**Hypokalaemia is considered likely to be pseudohypokalaemia due to storage of samples before processing without centrifugation at the point of care, as described in a previous study in Oxford (223).*

	CMV		EBV	
Volunteer	Timepoint C-1	Timepoint C+90	Timepoint C-1	Timepoint C+90
1054001	Detected		Detected	
1054002	Not Detected	Not Detected	Detected	
1054004	Not Detected	Not Detected	Detected	
1054005	Detected		Detected	
1054007	Detected		Detected	
1054008	Not Detected	Not Detected	Not Detected	Not Detected
1054010	Not Detected	Not Detected	Detected	
1054011	Not Detected	Not Detected	Detected	
1054012	Not Detected	Not Detected	Not Detected	Not Detected
1054013	Detected		Detected	
1054016	Not Detected	Not Detected	Detected	
1054017	Detected		Detected	
1054018	Not Detected	Not Detected	Not Detected	Not Detected
1054019	Not Detected	Not Detected	Detected	
1054020	Not Detected	Not Detected	Detected	
1054022	Not Detected	Not Detected	Detected	
1054024	Detected		Detected	
1054026	Not Detected	Not Detected	Detected	
1054027	Not Detected	Not Detected	Detected	
2054101	Not Detected	*	Detected	
2054103	Not Detected	Not Detected	Detected	
2054104	Detected		Detected	
2054106	Not Detected	Not Detected	Detected	
3054201	Detected		Detected	
3054202	Not Detected	Not Detected	Detected	
3054206	Detected		Detected	
3054208	Detected		Detected	

Table 4.9: VAC054 EBV IgG and CMV IgG serostatus in volunteers before and after blood-stage CHMI.

*C+90 data is shown for seronegative volunteers. *The volunteer who withdrew during the CHMI phase (2054101) was CMV seronegative and EBV seropositive at C-1; his serostatus was not rechecked after CHMI as he withdrew from the trial and declined any further blood tests.*

4.4.4 Blood-Stage CHMI and Vaccine Efficacy

All volunteers developed patent blood-stage parasitaemia following CHMI and were diagnosed by thick blood film microscopy by dC+10.5 (Table 4.10), except for the one volunteer who withdrew on dC+8.5. This volunteer was asymptomatic and thick blood film negative so had not reached the criteria for commencing treatment, but was qPCR positive at the time of withdrawal and was included in the primary analysis. There was neither a delay to diagnosis in

vaccinees compared to controls, nor any difference in parasitaemia between the groups during follow-up (Figure 4-7).

ID Number	Group	Diagnosis timepoint	Symptomatic/ asymptomatic	No. of parasites on TBF	PCR at diagnosis (p/mL)
MVT-1054001	1	8.5	Asymptomatic	2	7397
MVT-1054002	1	8.5	Asymptomatic	1	27518
MVT-1054004	1	7.5	Symptomatic	1	2371
MVT-1054005	1	9	Asymptomatic	2	16585
MVT-1054007	1	9	Asymptomatic	3	3276
MVT-1054008	1	10	Asymptomatic	2	11882
MVT-1054010	1	10.5	Symptomatic	3	29937
MVT-1054011	1	10	Asymptomatic	3	164509
MVT-2054101*	1	8.5	Asymptomatic	0	1697
MVT-2054103	1	10	Asymptomatic	2	73615
MVT-3054201	1	8.5	Asymptomatic	2	10320
MVT-3054202	1	9	Asymptomatic	2	41198
MVT-1054012	2	7.5	Asymptomatic	1	1645
MVT-1054013	2	10.5	Symptomatic	2	70367
MVT-1054016	2	10	Symptomatic	2	273247
MVT-1054017	2	10.5	Symptomatic	1	16911
MVT-1054018	2	8.5	Symptomatic	1	19670
MVT-1054019	2	8.5	Asymptomatic	1	6932
MVT-1054020	2	8.5	Asymptomatic	1	15025
MVT-1054022	2	10	Asymptomatic	1	8865
MVT-1054024	2	10	Asymptomatic	2	43707
MVT-1054026	2	9	Symptomatic	1	9132
MVT-1054027	2	9	Symptomatic	1	13421
MVT-2054104	2	9	Asymptomatic	2	54085
MVT-2054106	2	9	Symptomatic	3	14395
MVT-3054206	2	10	Symptomatic	3	185576
MVT-3054208	2	9	Symptomatic	1	1439

Table 4.10: VAC054 CHMI thick blood film and PCR results at diagnosis

Data are shown for all volunteers at diagnosis. All volunteers had a positive thick blood film (TBF), except 2054101 (*) who withdrew before diagnostic criteria were reached. Fifteen volunteers were asymptomatic at diagnosis and diagnosed on TBF and PCR criteria.

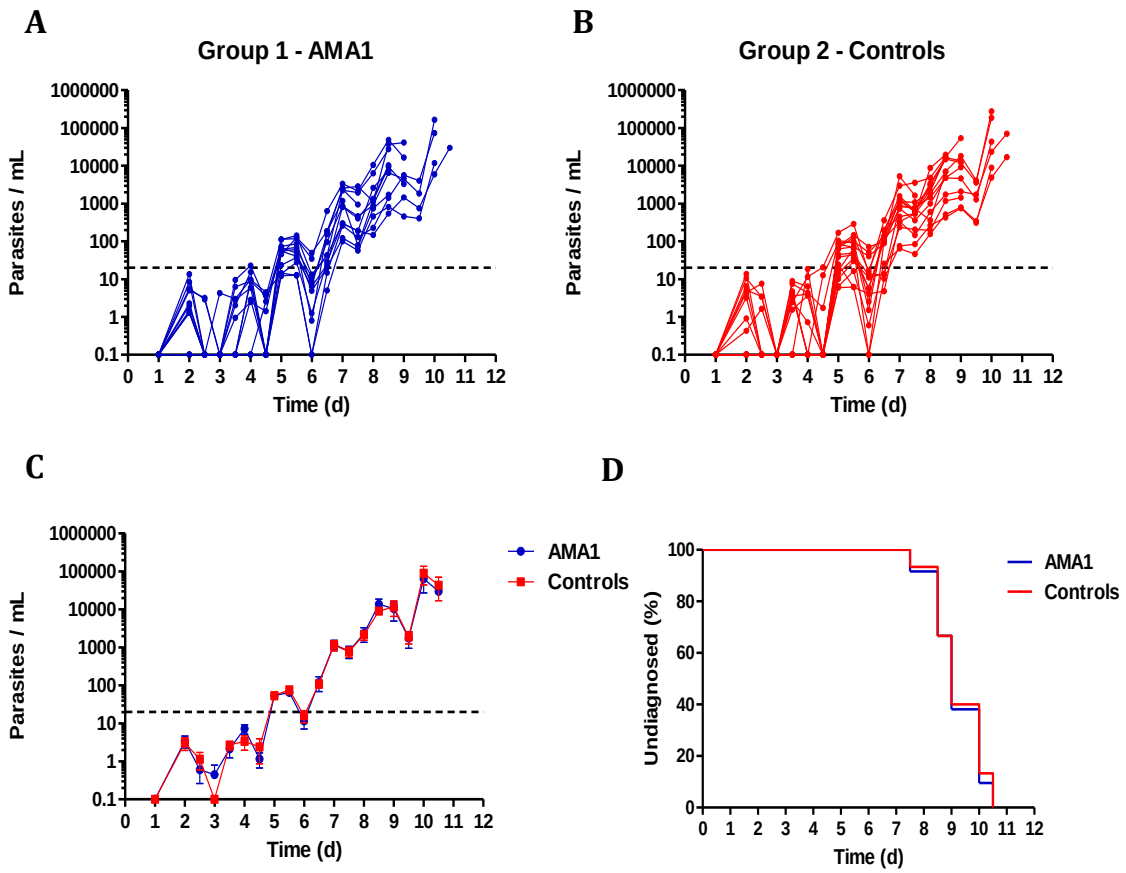


Figure 4-7: VAC054 Blood-stage CHMI efficacy outcomes.

*Individual qPCR data are shown for the VAC054 Phase IIa study including (**A**) Group 1 AMA1 vaccinees (n=12), and (**B**) the Group 2 unvaccinated infectivity controls (n=15). (**C**) The mean ± SEM parasitaemia is shown over time for each group. The lower limit of quantification is indicated by the dotted line at 20 parasites/mL. (**D**) Kaplan-Meier plot of time to patent parasitemia in days for the VAC054 study. Median time to patent parasitemia = 9.0 d for both groups. Secondary pre-specified analysis in the protocol compared time to microscopic patency between the groups; P=0.81, Mann-Witney test of time to diagnosis (excluding the volunteer in Group 1 who withdrew on dC+8.5). Time = days (d) post blood-stage CHMI. The PCR standard curve was generated automatically by the QIASymphony SP instrument. Data were imported into GraphPad Prism for graphical presentations and statistical analyses.*

The protocol pre-specified primary analysis for efficacy was comparison of PMR between the two groups. There was no difference in the mean PMRs between the two groups (Figure 4-8). The mean PMR for Group 1 was 10.32 (95% confidence interval (CI) 8.97-11.67; standard deviation (SD) =2.13) and for Group 2 was 10.31 (95% CI 9.00-11.62; SD=2.36), *P*=0.99 using two-tailed unpaired t test.

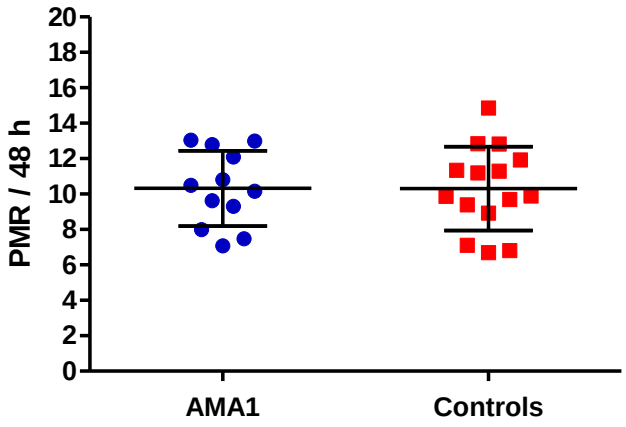


Figure 4-8: VAC054 PMR analysis.

Primary endpoint analysis of PMR, showing each individual in Group 1 (AMA1; n=12) and Group 2 (Controls; n=15), and the mean ± SD.

4.4.5 T cell Responses in Vaccinees and Controls

The FMP2.1/AS01 vaccine elicited T cell responses as assessed by *ex-vivo* IFN-γ ELISPOT, with median responses of 577 and 396 SFU/million PBMC at d42 and d69/C-1 respectively (Figure 4-9A, C). These responses did not boost post-CHMI with a median of 148 SFU/million PBMC seen at d98/C+28. Modest responses were induced in the controls (median 29 SFU/million PBMC at the same time-point), with only two volunteers showing responses > 150 SFU/million PBMC (Figure 4-9B, D-F). There was a significant increase in IFN- γ secreting T cells following the second vaccination with FMP2.1/AS01 (day 14 vs day 42) (P = 0.01, Friedman non-parametic test with Dunn's correction for multiple comparisons), but not following the third vaccination or after CHMI.

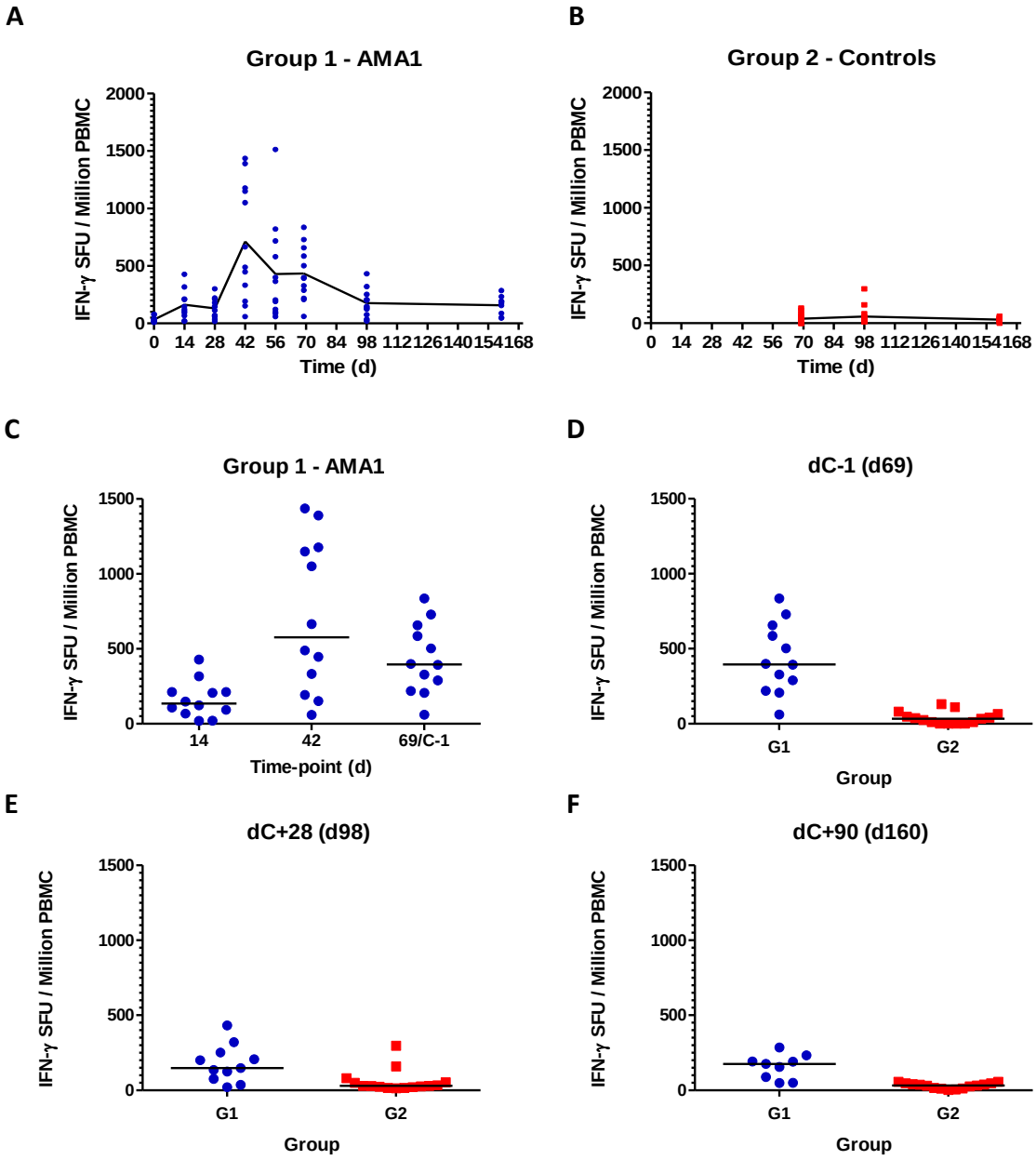


Figure 4-9. VAC054 T cell responses in vaccinees and controls.

(**A,B**) *T cell responses were assessed in each group by ex-vivo IFN-γ ELISPOT using fresh PBMC. (**C**) Median and individual responses are shown for vaccinees (Group 1; n=12) at the d14, d42 and d69/C-1 time-points. (**D-F**) Median and individual responses are shown for vaccinees (G1; n=12) and controls (G2; n=15) at the d69/C-1, d98/C+28 and d160/C+90 time-points. Spots were counted using an automated plate counter and exported into an Excel worksheet where results were obtained by subtracting any background response (from negative control wells) and then taking the average of triplicate wells. Data were then imported into GraphPad Prism for statistical analyses.*

4.4.6 Antibody Responses in Vaccinees and Controls

AMA1-specific serum IgG responses were measured by ELISA at the Jenner Institute laboratories in Oxford with median responses of 85 and 97 µg/mL at d42 and d69/C-1 respectively. These responses did not boost post-CHMI with a median of 56 µg/mL seen at

d98/C+28. Only 1 of the 15 controls showed a *de novo* anti- AMA1 IgG response at d98/C+28 (59 µg/mL) (Figure 4-10).

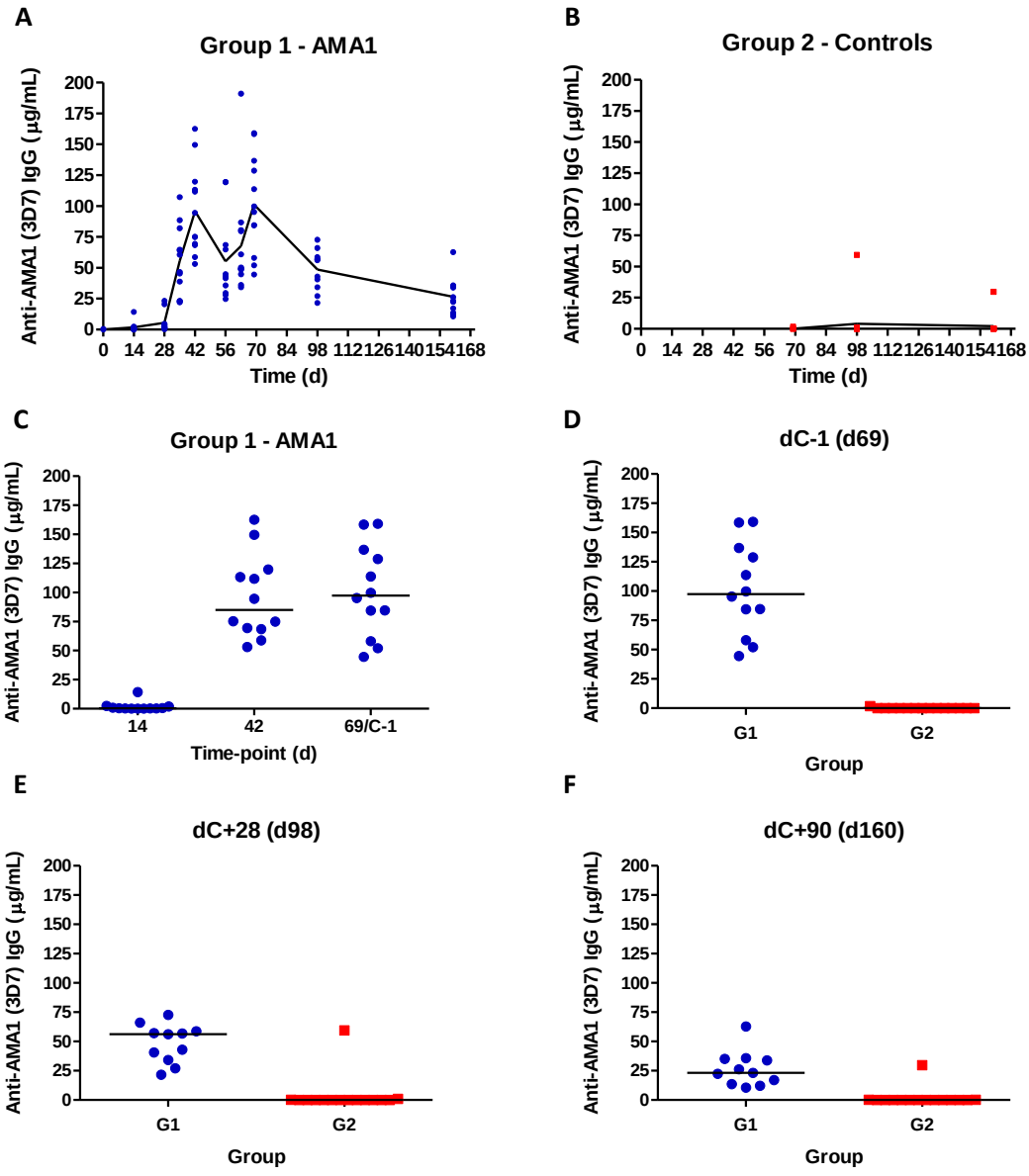


Figure 4-10: VAC054 Serum antibody responses in vaccinees and controls (Oxford).

(**A,B**) *Serum anti-AMA1 (3D7) IgG responses were assessed in Oxford for each group by ELISA. Mean and individual responses are shown over time. Blood-stage CHMI took place on D70. (**C**) Median and individual responses are shown for the vaccinees (Group 1; n=12) at the d14, d42 and d69/C-1 time-points. (**D-F**) Median and individual responses are shown for the vaccinees (G1; n=12) and controls (G2; n=15) at the d69/C-1, d98/C+28 and d160/C+90 time-points. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and values of internal controls and samples in triplicate were assessed for any aberrant readings. The results were obtained by taking an average of triplicate wells, and using the standard curve to assign ELISA arbitrary units. These were converted to µg/mL using a conversion factor as detailed in Section 2.3.4.1. Converted data were imported into GraphPad Prism for graphical presentation and analyses.*

As well as measuring the anti-AMA1 antibody response by ELISAs, the avidity of antibodies induced by vaccination and the different isotypes present were also determined by ELISA. The avidity of the anti-AMA1 IgG was similar at d42, d69/C-1 and post-CHMI in the vaccinees (Figure 4-11), and very similar to that observed with other AMA1 vaccines in humans (167, 193). The response was composed of IgG1, IgG3, IgA and IgM, and this profile was not affected by CHMI (Figure 4-12).

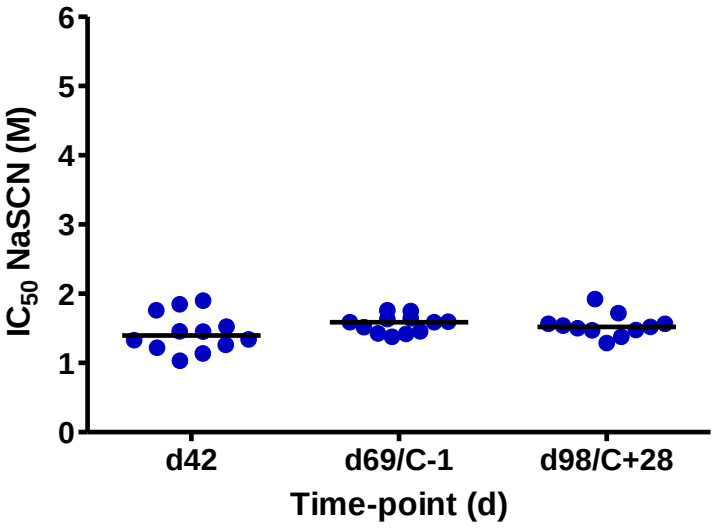


Figure 4-11.VAC054 AMA1 antibody avidity.

Avidity of serum IgG responses was assessed by NaSCN-displacement 3D7 AMA1 ELISA and is reported as the molar (M) concentration of NaSCN required to reduce the starting OD in the ELISA by 50% (IC_{50}). Individual and mean responses are shown for Group 1 volunteers (n=12). The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and the average value of duplicate readings calculated. Data were then imported into GraphPad Prism for graphical presentation and analyses.

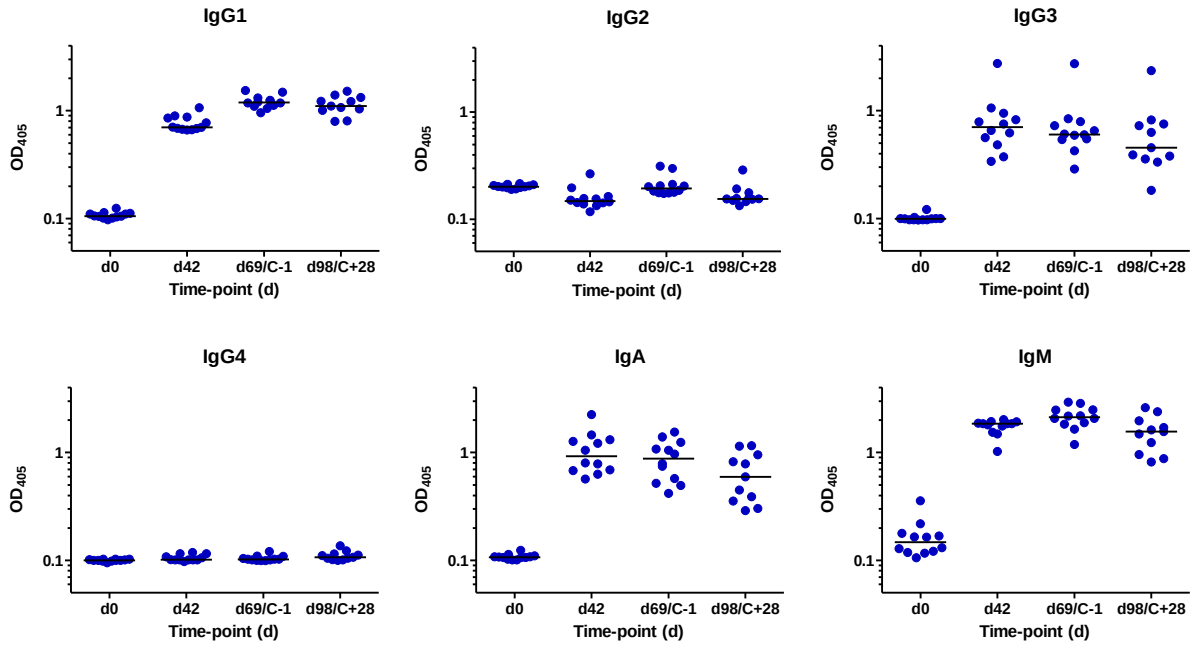


Figure 4-12 VAC054 AMA1 antibody isotype profile

Isotype profiles of serum antibody responses were assessed by 3D7 AMA1 ELISA. Responses are shown at baseline (d0), d42, d69/C-1 and post-CHMI at d98/C+28 for vaccinated volunteers in Group 1 (n=12). In all panels, individual and median responses are shown. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and the average value of duplicate readings calculated. Data were then imported into GraphPad Prism for graphical presentation and analyses.

4.4.7 Measurement of antibodies to MSP1 in vaccinees and controls

MSP1 is one of the most abundant proteins on the surface of the merozoite and antibodies against MSP1 can often be detected in those exposed to malaria in endemic areas (81). In order to see whether blood-stage CHMI induced antibodies against MSP1, sera from the control volunteers (Group 2) was analysed by ELISA for anti-MSP1 antibodies before CHMI (dC-1) and 4 weeks after CHMI (dC+28). Sera from controls from six previous CHMI studies were also analysed in the same experiment for comparison (74, 83, 138, 172, 173). These previous studies used mosquito-bite (sporozoite) CHMI so the comparative timepoint is C+35 (i.e. 4 weeks after the parasites enter the blood stream following the liver-stage of infection). This experiment demonstrated that only 4 of the 15 Group 2 volunteers seroconverted and developed antibodies against MSP1 following CHMI, whereas 30 of the 37 control volunteers across the other studies had seroconverted after CHMI (Figure 4-13).

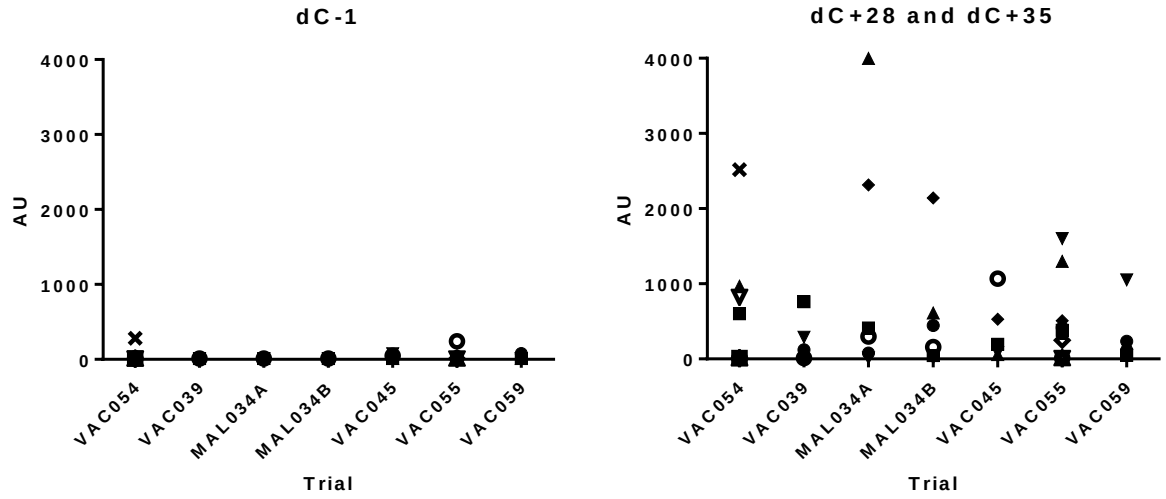


Figure 4-13 VAC054 Antibody response to MSP1 in Group 2 (control) volunteers after CHMI.

Anti-MSP1 antibodies were measured by ELISA in Group 2 volunteers (n=15) and in control volunteers from six previous CHMI studies (n=37) with samples from before CHMI (dC-1) and 4 weeks after blood stream infection (dC+28 in blood-stage CHMI and C+35 in mosquito-bite CHMI) (74, 83, 138, 172, 173)*. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and values of internal controls and samples in triplicate were assessed for any aberrant readings. The results were obtained by taking an average of triplicate wells, and using the standard curve to assign ELISA arbitrary units (AU). Data were then imported into GraphPad Prism for graphical presentation.*

4.4.8 Anti-AMA1 peripheral mBC responses following FMP2.1/AS01

Peripheral mBC responses were assessed in vaccinated volunteers (Group 1) by identifying AMA1-specific mBC-derived plasma cells by *ex vivo* ELISPOT following a 6-day polyclonal culture of PBMC (Figure 4-14). Responses were measured at d69/ dC-1 and 4 weeks after CHMI (dC+28). There was no significant difference in detectable AMA1-specific mBC-derived ASCs at the two timepoints, either as a proportion of the cultured PBMC or as a percentage of detected total IgG.

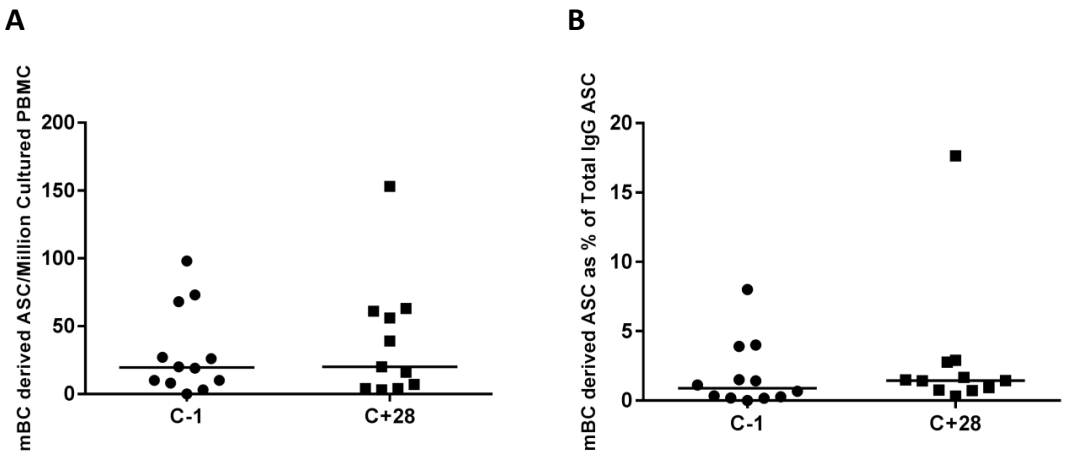


Figure 4-14: Anti-AMA1 mBC responses following FMP2.1/AS01 vaccination.

mBC- derived ASC in Group 1 volunteers (n=12) measured two weeks after final vaccination (dC-1) and four weeks after CHMI (dC+28) using ex vivo ELISPOT. (A) Comparison between timepoints dC-1 and dC+28 (mBC derived ASC per million cultured PBMC). (B) Comparison between timepoints dC-1 and dC+28 (mBC derived ASC as a % of total IgG ASC). Individual and median responses are shown. mBC ELISPOT spots were counted using an AID ELISPOT reader and automated counts were corrected by eye .Data were exported into an Excel worksheet and then imported into GraphPad Prism for graphical presentation and analyses.

4.4.9 Measurement of *in vitro* GIA

Serum was analysed at the GIA Reference Center at NIH, USA and IgG was purified from each sample. Samples from Group 1 volunteers prior to vaccination (d0) and Group 2 volunteers prior to CHMI (d69/dC-1) did not demonstrate any GIA above baseline. At a concentration of 10 mg/mL purified IgG dC-1 samples from Group 1 volunteers following three vaccinations demonstrated *in vitro* GIA of median 59.5 % (range 38.5-86.5 %) (Figure 4-15A). As the purified IgG was diluted, GIA decreased (Figure 4-15B) and was related to AMA1-specific IgG concentration (Figure 4-15C), in close agreement with other independent studies (130, 167). The EC_{50} was calculated for each purified IgG, with a median of 8.1 mg/mL. To relate these results (using a normalised concentration of purified IgG) back to the original sera, the concentration of IgG in each original serum sample was also measured. This enabled calculation of the GIA_{50} serum titre, defined previously as the dilution factor of each serum sample required to reach the concentration of purified IgG that gives 50% GIA (104). The

median GIA_{50} titre for Group 1 was 1.5, with the maximum observed being a dilution factor of 3.0 (Figure 4-15D).

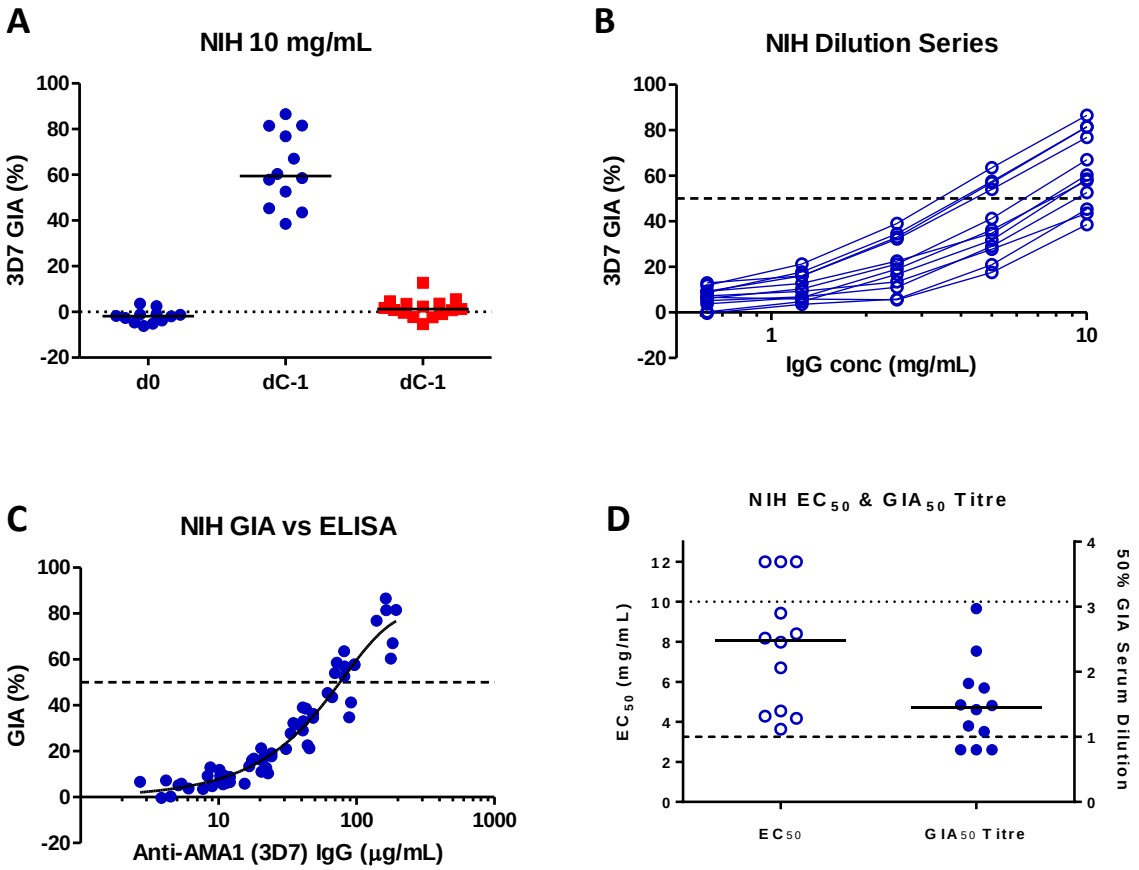


Figure 4-15: Assessment of functional GIA induced by FMP2.1/AS01.

*(**A**) In vitro GIA of purified IgG was assessed at 10 mg/mL against 3D7 clone P. falciparum parasites at the NIH GIA Reference Center. Each test IgG was incubated with synchronised human erythrocytes which contained late trophozoite and schizont stages of P. falciparum parasites prepared by Percoll gradient and/or 5% sorbitol treatment. GIA was assessed over for a single growth cycle, measuring parasite LDH after 40 hours of culture and using this to calculate relative parasitaemia levels. Individual data and medians are shown for each group (Group 1 shown in blue; n=12, Group 2 shown in red; n=15) at dC-1 as well as d0 for Group 1. Responses >12% are typically regarded as positive for 3D7. (**B**) Dilution series of purified IgG from Group 1 dC-1 samples. (**C**) Relationship between GIA and anti-3D7 AMA1 serum IgG concentrations measured by ELISA at the NIH. Non-linear regression curve is also shown (n=60). The EC_{50} (level of anti-3D7 AMA1 response in this ELISA assay that gives 50% GIA, indicated by the dotted line) was 75.5 µg/mL, (95% CI 68.3-84.2). (**D**) Individual EC_{50} of each purified IgG is shown as well as the GIA_{50} titres. Individual data and medians are shown for Group 1 dC-1.*

4.5 Discussion

AMA1 has long been considered a leading candidate antigen for a blood-stage malaria vaccine (224), but despite evidence that antibodies to AMA1 inhibit red blood cell invasion (92), significant efficacy has not been demonstrated as a primary endpoint of any Phase IIa/b clinical trial (25). Previous CHMI trials initiated by mosquito-bite have suggested some pre-erythrocytic immunity can be afforded by vaccines encoding AMA1 alone (83, 95), or in combination with the circumsporozoite protein (CSP) (225). The traditional method for assessing blood-stage vaccines has been through large field trials rather than CHMI trials. A Phase IIb field trial in Malian children with the FMP2.1/AS02 vaccine reported 64.3% efficacy (hazard ratio 0.36, 95% CI 0.08-0.86, *P*=0.03) in a pre-defined secondary analysis. This reported efficacy was against clinical malaria with 3D7-type parasites, but the number of cases meeting this definition was small (97, 98) and the reported efficacy did not extend into the second malaria season (99). The aim of this Chapter was to use the blood-stage CHMI model to assess the FMP2.1/AS01 vaccine against homologous 3D7 clone parasites, and therefore evaluate whether the vaccine is able to induce immunity active against the erythrocytic stage of the malaria parasite life cycle.

There were no safety concerns relating to vaccination with FMP2.1/AS01, and the AE profile was similar to that reported previously in healthy US adult volunteers, with injection site pain, erythema, warmth and swelling reported as the most frequent local AEs and headache, malaise and fatigue reported frequently as systemic AEs in both studies (95). The vaccine was more reactogenic than the viral vectored vaccines used in the VAC051 trial (Chapter three), with higher frequencies of volunteers reporting severe AEs, particularly following the second vaccination. Vaccine-related AEs were of short duration and all resolved spontaneously. There were also no safety concerns relating to the CHMI inoculum, and the cessation of only enrolling EBV and CMV seropositive volunteers into studies using this inoculum was further supported by the data from this trial which demonstrated no seroconversion in volunteers who were EBV and/or CMV seronegative prior to blood-stage CHMI.

The FMP2.1/AS01 vaccine was immunogenic in this trial, eliciting AMA1-specific T cell and antibody responses. IFN-γ T cell responses, measured by ELISPOT, were higher than those seen with other AMA1 protein-in-adjuvant vaccines tested using the same assay (174). This is likely to be due to the use of the AS01B adjuvant system which was specifically designed to improve Th1 responses (122). The actions of the AS01 adjuvant system are though to involve both the innate and adaptive immune systems. The MPL in the adjuvant stimulates the innate immune system via TLR4 receptors, thereby directly activating APCs which express TRLR4 (123). The response to MPL promotes production of IFN- γ by antigen specific CD4+ T cells (123, 124) and the QS21 in the adjuvant has been shown to stimulate $CD8^+$ T cells (121). As discussed in Chapter three, it is thought likely that an optimised blood-stage malaria vaccine will have both T cell and antibody-inducing properties.

The avidity and isotype profiles of the anti-AMA1 IgG antibodies were very similar to those observed following vaccination with other AMA1 vaccines in humans (167, 193), although it has been suggested that the AS01 adjuvant can aid promotion of antibody isotype switching (121). The serum antibody and GIA responses were comparable to another AMA1 vaccine candidate that failed to impact on PMR in a much smaller, and under-powered, blood-stage CHMI trial in Oxford (174). However, the anti-AMA1 antibody levels reported in this trial were substantially lower than those reported by Spring *et al* using the same vaccine at WRAIR in a previous trial (95). The reduced immunogenicity in this trial may have related to the age of the FMP2.1 protein, although the vaccine lot had passed all required testing prior to use in the VAC054 study.

In this trial, FMP2.1/AS01 vaccine did not demonstrate any efficacy, with no vaccinees protected from malaria infection and no reduction in PMR compared with the infectivity control group. The difference in efficacy between this study and the Phase IIb field study could be due to a number of possible reasons: reduced vaccine immunogenicity; the use of AS01 instead of AS02; an impact of this vaccine only at high parasite densities; a pre-erythrocytic

effect of the vaccine; or the fact that the Malian children, unlike UK adults, would have possessed pre-existing anti-malarial immune responses, including anti-AMA1 IgG, which may have acted in conjunction with the vaccine-induced anti-AMA1 responses.

Despite the lack of efficacy demonstrated in this trial, the antibodies induced by the FMP2.1/AS01 vaccine were able to inhibit parasite growth *in vitro*, as assessed by GIA. However, the level of inhibition with 10 mg/mL of purified IgG was only a median of 59.5 % (range 38.5-86.5 %). The lack of efficacy in the VAC054 trial is consistent with previously reported data in *Aotus* monkeys (104, 226), including a study showing that only vaccinated animals that achieved >60% GIA using a purified IgG concentration of 2.5 mg/mL were protected against blood-stage challenge (104). This implies that the levels of anti-AMA1 antibody required to achieve efficacy are far higher than were induced by FMP2.1/AS01. Given that the vaccine was given with a very effective adjuvant, and that the peak antibody response was median 97 μg/mL, it may not be possible to achieve high enough antibody levels in humans against AMA1 for this to be a successful vaccine candidate. Even if substantially higher levels of antibody could be induced, the polymorphism of AMA1 also raises a challenge for an effective vaccine, with only homologous efficacy demonstrated in any trial to date (97).

The VAC054 trial may not have demonstrated vaccine efficacy, but did successfully demonstrate the reproducibility of the blood-stage CHMI model, with the largest blood-stage CHMI ever trial carried out (174, 175, 227). This has important implications for assessing future blood-stage vaccine candidates as it allows modest reductions in PMR to be measured with greater power than with a sporozoite CHMI model (209). Being able to assess efficacy with smaller numbers of participants whilst retaining power to see modest effects has important implications for the ethical conduct of these studies (using the smaller numbers reduces risk) as well as financial and logistical implications. With the PMR data from this trial I was able to calculate that a similar trial design in the future (i.e. 15 vaccinees vs 15 controls with the same

inoculum) would have >80% power to see a 33% mean reduction in PMR even with a standard deviation of 4 in PMR data for vaccinated volunteers.

Given the issues with polymorphism of AMA1 and the difficulty in inducing sufficiently high levels of effective antibody, even with a leading adjuvant, a different approach will be needed if an effective blood-stage vaccine is to be developed. A multi-allele AMA1 vaccine is one approach to tackling the issue of polymorphism, such as the Diversity-Covering (DiCo) *P. falciparum* AMA1 vaccine candidate, which has recently completed a Phase I trial (NCT02014727; results not yet published). This vaccine showed cross-strain inhibition activity against three laboratory-adapted *P. falciparum* strains (FCR3, HB3 and NF54) when purified IgG from vaccinated rhesus macaques was tested in a GIA assay. Mean GIA levels for the DiCo vaccine given with the CoVaccine HT™ adjuvant were 56.3%, 57.8% and 83.7% respectively with 10 mg/ mL purified IgG (228). Efficacy against falciparum malaria was not assessed in this trial as rhesus macaques cannot be infected with *P. falciparum*, but given the results of the VAC054 trial with similar GIA levels, the levels of functional anti-AMA1 antibodies induced by the DiCo/HT vaccine may not be sufficient.

Another alternative approach is to combine the AMA1 vaccine with another antigen to try to improve efficacy. However, this approach is not necessarily an easy one as antigen combinations do not necessarily improve efficacy. The co-administration of AMA1 and MSP1, using the ChAd63 and MVA viral vectors, has previously been shown to diminish the immune response against AMA1, with a dominant response to MSP1, and corresponding decrease in GIA compared with IgG from volunteers vaccinated with AMA1 alone (83). The combination of AMA1 with the RON2 rhoptry neck protein (which AMA1 binds to during the red blood cell invasion process) is a more promising combination, with pre-clinical data demonstrating improved efficacy in mice against lethal *P. yoelii* infection, compared with vaccination with AMA1 alone (229). This approach has not yet been tested in humans against *P. falciparum* malaria, so whether this result is relevant with regards to human malaria remains to be seen,

but the potential to induce improved quality antibodies could go some way to addressing the issue of needing high antibody titres against AMA1.

The need to induce very high antibody levels for blood-stage vaccines requires very effective adjuvants. Despite the use of a leading adjuvant in the VAC054 study, the levels induced by vaccination were still insufficient to inhibit parasite growth *in vivo*. A recently re-evaluated approach for improving the immune response following malaria vaccines has been to use a delayed fractional dose. This approach was first discovered by chance due to a delay in vaccination in one of the early RTS,S vaccine trials due to safety concerns, meaning a reduced third dose was given at 7 months (following initial vaccinations at 0 and 1 month) as opposed to the planned 0, 1 and 2 month schedule. The vaccine efficacy in this CHMI trial was 86% for the RTS,S vaccine given with immune stimulants monophosphoryl lipid A and QS21 (71). This was higher than the efficacy reported in subsequent CHMI studies of RTS,S with similar adjuvants when given in a 0, 1 and 2 month regime (typically ~50%) (73). A more recent study has supported the finding of improved efficacy with a delayed fractional dose with the RTS,S/AS01 vaccine, with 86.7% vaccine efficacy reported, compared with 62.5% in volunteers who received the standard regime (230). It will be important to evaluate this with other vaccines/ target antigens and see whether this effect improves the immune response and efficacy, especially for blood-stage candidates where high antibody levels appear to be crucial for efficacy.

In this Chapter, I have demonstrated the safety, immunogenicity and efficacy results of a Phase I/IIa clinical trial of the AMA1 candidate vaccine FMP2.1/AS01 which had previously been reported to have demonstrated efficacy against homologous parasites in a Phase IIb trial in Malian children. Despite the induction of antigen-specific antibodies which demonstrated functional activity *in vitro*, the vaccine demonstrated no efficacy against a homologous blood-stage infection. The possible reasons for this have been discussed. Although the vaccine did not demonstrate any efficacy, I was able to demonstrate the reliability and reproducibility of

the blood-stage CHMI model, and its potential for the assessment of future blood-stage vaccine candidates. Researchers at the Jenner Institute, University of Oxford have been working on alternative targets for a blood-stage *P. falciparum* vaccine. An improved candidate will need to have fewer issues with polymorphism and ideally require lower antigen-specific antibody levels to be effective than the leading candidates to date (i.e. AMA1 and MSP1). The *P. falciparum* reticulocyte binding homologue 5 (PfRH5) antigen has been evaluated pre-clinically as part of this research, with very promising results (104). This has now been developed as a vaccine candidate and is discussed in Chapter five.

Chapter Five:

A Phase Ia clinical trial to assess the safety and immunogenicity of the *Plasmodium falciparum* antigen RH5 in viral vectors ChAd63 and MVA (VAC057)

5.1 Authorship statement

I set up the VAC057 clinical trial with the assistance of Alison Lawrie, Rachel Roberts, Ian Poulton, Adrian Hill and Simon Draper. This involved preparation of the study documents with submission for ethical, regulatory approval for all sites, and R&D department approval for NHS sites (in Southampton).

I screened and enrolled volunteers for the trial in Oxford. Vaccinations and follow-up visits were conducted by me and by the nursing team: Oliver Griffiths, Paula Marriott, Megan Baker, Ian Poulton, and Raquel Lopez-Ramon.

Volunteers were also enrolled at the NIHR WTCRF in Southampton. Enrolment, vaccination and follow-up of volunteers there were carried out by local study staff including: Hans de Graaf, Nicky Pugh, Prudence Miyanza and Nathan Brendish. The local PI at the site was Saul Faust.

Volunteer-reported AE data were collected on an electronic diary which was designed by me and other members of the clinical team, and developed by Sylwester Pawluk.

Laboratory assays in Oxford were carried out by me and by Sarah Silk and Sean Elias. The GIA assays were carried out at the NIH reference center laboratory in the USA by Kazutoyo Miura.

5.2 Introduction

5.2.1 Development of a new candidate blood-stage *P. falciparum* vaccine

A viral vectored *Plasmodium falciparum* vaccine has been developed at the University of Oxford using the same viral vectors as were used in the VAC051 study (Chapter three), ChAd63 and MVA. The viral vectors encode a blood-stage antigen from the *P. falciparum* parasite, reticulocyte-binding protein homologue 5 (RH5) and are therefore referred to as ChAd63 RH5 and MVA RH5. They are given in a heterologous prime-boost regime, with an eight week interval between vaccinations. The VAC057 trial was the first trial in which this vaccine and the RH5 antigen were administered to humans.

5.2.2 RH5 as an antigen

P. falciparum reticulocyte-binding protein homologue 5 (PfRH5/ RH5) is one of the reticulocyte binding-like (RBL or *P. falciparum* RBP homologue (PfRH)) proteins which are involved in parasite invasion of erythrocytes. It is expressed in merozoites and localises to the apical complex. PfRH5 is expressed in all *P. falciparum* strains tested so far, and is essential for parasite survival given two reports that the gene cannot be knocked out (100, 101). PfRH5 binds to its receptor basigin, the Ok blood group antigen, and this interaction mediates an essential interaction required for erythrocyte invasion by all tested strains of *P. falciparum* (103). Low-level antibodies to PfRH5 can be found in the pooled serum of humans from malaria-endemic countries, but not from pooled malaria-non-exposed immune sera (231, 232). These responses have also been associated with clinical protection in an endemic setting, supporting the theory that these antibodies may play a part in controlling malaria infection (233). Importantly, although antibodies have been found, it appears that PfRH5 does not come under significant immune pressure. This may account for the limited polymorphism in PfRH5 which was reported from the malaria whole genome sequencing project (234) (using >220 field isolates from Africa, Asia and Oceania) where only 12 single nucleotide polymorphisms were identified in this 526 amino acid antigen, and of these only 7 showed a frequency of >5% (235). The basigin binding site on PfRH5 may also be functionally constrained, thus limiting polymorphism, given just a few amino acid substitutions have been reported to affect basigin recognition and thus host erythrocyte tropism (101, 236, 237). Blocking of the PfRH5-basigin interaction has also been shown to be an important contributor of anti-PfRH5 antibody action (238).

Polyclonal antibodies induced by PfRH5 vaccination (or by natural infection) overcome two of the major difficulties for blood-stage vaccination: firstly, antibodies can block erythrocyte invasion to high efficiency (requiring lower antibody concentrations than previously studied targets, such as PfAMA1) (235), and secondly, and even more importantly, these antibodies cross-inhibit *in vitro* all *P. falciparum* lines and field isolates tested to date (158, 233, 235, 239,

240). Importantly, high-level efficacy induced by PfRH5 vaccination against heterologous strain challenge in an *in vivo Aotus* monkey *P. falciparum* challenge model has also been demonstrated (104). This means that the PfRH5 antigen is a substantial step forward in the blood-stage malaria vaccine field.

5.3 VAC057 Methods

Detailed methods of the recruitment and enrolment of volunteers, as well as the assays used in this trial can be found in Chapter two: Materials and Methods.

5.3.1 VAC057 Study Design

VAC057 was a first-in-human, open-label, non-randomised, dose escalation Phase Ia clinical trial evaluating the safety and immunogenicity of the viral vectored vaccines ChAd63 Rh5 and MVA RH5 in a heterologous prime-boost regime with an eight week interval (Figure 5-1). The study was conducted in the UK at the Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, and the NIHR WTCRF in Southampton. Healthy, malaria-naïve males and non-pregnant females aged 18-50 were invited to participate in the study. Allocation to study groups occurred at screening based on sequential recruitment of groups and volunteer preference. The sample size for this study was chosen to allow determination of the magnitude of the outcome measures, especially of serious and severe adverse events, rather than aiming to obtain statistical significance. The decision to enrol eight volunteers into Groups 2B and 2C was based on a power calculation to have 76% power (1-β) to detect a mean two-fold improvement (two-tailed) at a significance level (α) of $P = 0.05$ in immunogenicity (IgG and T cell response) between Groups 2B and 2C following MVA RH5 boost vaccination.

Group Number		Number of volunteers	Dose ChAd63 RH5	Dose MVA RH5
1		4	5 x 10^9 vp IM	--
2	**A**	4	5 x 10^{10} vp IM	**--**
	B	8	5 x 10^{10} vp IM	1 x 10^8 pfu IM
	C	8	5 x 10^{10} vp IM	2 x 10^8 pfu IM

Figure 5-1: VAC057 study groups

5.3.2 VAC057 Ethics

The trial was registered with Clincaltrials.gov (NCT 02181088) and was conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH guidelines for Good Clinical Practice. The study received approvals from the UK NHS Research Ethics Service (Oxfordshire Research Ethics Committee A, Ref 14/SC/0120) and the UK MHRA (Ref 21584/0331/001-0001).

5.3.3 VAC057 Objectives and Endpoints

Primary Objective

To assess the safety of ChAd63 RH5 when administered alone and in a heterologous prime-boost regime with MVA RH5.

Primary Outcome Measures

The specific endpoints for safety and reactogenicity were actively and passively collected data on AEs. These data were collected using electronic diary cards which were completed by volunteers for 28 days after each vaccination, as well as recording any AEs reported by

volunteers or detected by study staff (e.g. laboratory abnormalities) at each clinic visit during this period. After 28 days only SAEs were recorded.

Secondary Objective

To assess the cellular and humoral immunogenicity of ChAd63 RH5 when administered alone, and in a heterologous prime-boost regime with MVA RH5 in healthy volunteers.

Secondary Outcome Measures

Immunogenicity outcome measures following ChAd63/MVA RH5 included:

- Induction of antigen-specific T cells (assessed by *ex-vivo* IFN-γ ELISPOT);
- Induction of antigen-specific IgG (assessed by anti-RH5 ELISA);
- Measurement of antigen-specific antibody secreting cells (ASCs) and memory B cells (mBCs) following vaccination;
- Functional activity of antigen-specific IgG (assessed by GIA assay).

T cell ELISPOT assays, RH5 ELISA assays, ASC ELISPOT assays and mBC ELISPOT assays were carried out in the Jenner Institute laboratories in Oxford using the methods described in Chapter two. Sarah Silk and Sean Elias carried out the T cell ELISPOT s and the ASC and mBC ELISPOT assays; Sarah Silk and I carried out the ELISA assays. Samples were sent to the NIH reference center in the USA for GIA assays conducted by Kazutoyo Miura. Volunteers were consented for this.

5.3.4 ChAd63 RH5 and MVA RH5 Vaccines

The ChAd63 and MVA viral vectors are the same as those described in the VAC051 trial (Chapter three). Both viral vectors express a synthetic gene insert encoding the *Plasmodium falciparum* antigen RH5. The RH5 insert is a codon-optimised transgene encoding the RH5 sequence from the 3D7 clone of *Plasmodium falciparum*.

ChAd63 RH5 was manufactured under GMP conditions by Okairos, Italy (this company is now ADVENT S.r.l. since being acquired by GSK). MVA RH5 was manufactured under GMP

conditions by IDT, Germany. Both vaccines were labelled and released to trial by a QP at the CBF, University of Oxford following regulatory approval.

5.3.5 VAC057 Participants

Participants for this clinical trial were enrolled at two sites in the UK, the CCVTM in Oxford and the NIHR WTCRF in Southampton. Twenty four volunteers were enrolled in total and all completed study follow-up. All volunteers gave written informed consent prior to participation, and ongoing consent was confirmed before each vaccination. The inclusion and exclusion criteria for participation are described below:

5.3.5.1 VAC057 Inclusion Criteria

The volunteer had to satisfy all of the following criteria to be eligible for the study:

- Healthy adult aged 18 to 50 years.
- Able and willing (in the Investigator's opinion) to comply with all study requirements.
- Willing to allow the discussion of their medical history with their GP.
- For females only, willingness to practice continuous effective contraception during the study and a negative pregnancy test on the days of screening and vaccination.
- Agreement to refrain from blood donation during the course of the study.
- Provision of written informed consent.

5.3.5.2 VAC057 Exclusion Criteria

The volunteer could not enter the study if any of the following applied:

- Participation in another research study involving receipt of an investigational product in the 30 days preceding enrolment or during the study period.
- Prior receipt of an investigational malaria vaccine or any other investigational vaccine likely to impact on interpretation of the trial data.
- Administration of immunoglobulins and/or any blood products within the three months preceding vaccination.
- Any confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days)

immunosuppressant medication within 6 months preceding vaccination (inhaled and topical steroids were allowed).

- History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, e.g. egg products.
- Any history of anaphylaxis in relation to vaccination.
- Pregnancy, lactation or willingness/intention to become pregnant during the study.
- History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ).
- History of serious psychiatric condition likely to 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 42 units every week.
- Suspected or known injecting drug abuse in the 5 years preceding enrolment.
- Seropositive for hepatitis B surface antigen (HBsAg).
- Seropositive for hepatitis C virus (antibodies to HCV).
- History of clinical malaria (any species).
- Travel to a malaria endemic region during the study period or within the previous six months.
- Any clinically significant abnormal finding on screening biochemistry or haematology blood tests or urinalysis.
- 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.
- Inability of the study team to contact the volunteer's GP to confirm medical history and safety to participate.

5.3.6 VAC057 Assessment of safety

Following each vaccination, volunteers completed an electronic diary card for 28 days with any adverse event data. Further details about the scoring systems for the diary card can be found in Chapter two. The electronic diary was developed by Sylwester Pawluk in consultation with me, to provide a system which enabled participants to log their AE data in real time. The diary

was accessed over the internet and was designed so that it could be used on computers, tablets or smart phones. Participants were given a one-time login link and then had to set a password to access their diary. They were shown how to use the diary on the day of vaccination, and entries were checked at all visits during the 28 day diary period. Study staff were able to see all AE data for participants as soon as each day had been completed, and were able to edit entries if required (e.g. if further detail was desired). All changes were recorded on an audit log so that if changes had been made by study staff this was evident.

Observations (heart rate, temperature and blood measure) were measured at the clinic visits from the day of vaccination until the 28 day follow-up visit. These were recorded directly into the OpenClinica eCRFs. Blood samples for safety (full blood count, liver function, urea and electrolytes) were carried out at screening, day 0, day 7 and day 28 for all groups, as well as on days 56, 63 and 84 for Groups 2B and 2C. These were processed at the NHS laboratories in Oxford and Southampton.

5.3.6.1 VAC057 Stopping and holding rules

Safety stopping and holding rules were used in this study to ensure participant safety, particularly given that this was a first-in-human dose escalation study. For safety reasons the first volunteer who received a new vaccine dose was vaccinated alone and there was at least a 48 hour gap before subsequent volunteers were vaccinated. A further two further volunteers could be vaccinated 48 hours after the first, and then at least another 48 hours gap had to elapse before the rest of the volunteers receiving that dose of vaccine could be vaccinated.

Group holding rules

The study would have been put on hold if any of the following criteria were reached:

Solicited local adverse events:

- If more than 25% of doses of a vaccine were followed by Grade 3 solicited local adverse event beginning within 2 days after vaccination (day of vaccination and one subsequent day) and persisting at Grade 3 for >48 hrs.

Solicited systemic adverse events:

- If more than 25% of doses of a vaccine were followed by Grade 3 solicited systemic adverse event beginning within 2 days after vaccination (day of vaccination and one subsequent day) and persisting at Grade 3 for >48hrs.

Unsolicited adverse events:

- If more than 25% of volunteers developed a Grade 3 unsolicited adverse event (including the same laboratory adverse event) that was considered possibly, probably or definitely related to vaccination and persisted at Grade 3 for > 48hrs. For the ChAd63 RH5 vaccination Groups 2A and 2B were considered as one group as they were enrolled simultaneously and received the same vaccine dose.

A serious adverse event considered possibly, probably or definitely related to vaccination, a death or a life-threatening reaction occurred.

Individual stopping rules (applied to all vaccinated individuals)

In addition to the above stated group holding rules, stopping rules for individual volunteers applied (i.e. indications to withdraw individuals from further vaccinations). Volunteers would have been withdrawn from further vaccinations if any of the events listed below occurred and were considered possibly, probably or definitely related to vaccination.

Local reactions:

- Injection site ulceration, abscess or necrosis.

Laboratory AEs:

- If the volunteer developed a Grade 3 laboratory adverse event considered possibly, probably or definitely related within 7 days after vaccination which persisted continuously at Grade 3 for > 72hrs.

Systemic solicited adverse events:

- If the volunteer developed a Grade 3 systemic solicited adverse event considered possibly, probably or definitely related within 2 days after vaccination (day of vaccination and one subsequent day) which persisted continuously at Grade 3 for > 72hrs.

Unsolicited adverse events:

- If the volunteer had a Grade 3 adverse event, which persisted continuously at Grade 3 for >72hrs.
- If the volunteer had a serious adverse event.
- If the volunteer had an acute allergic reaction or anaphylactic shock following the administration of the vaccine investigational product.

5.3.7 VAC057 Interventions

Vaccination of volunteers was carried out at the CCVTM in Oxford and NIHR WTCRF in Southampton. Vaccines were stored at -80°C in a locked freezer. Administration of vaccines was carried out by two members of the local study team (clinician or nurse). One of the clinical study staff was required to give the vaccine and the other to check the volume drawn and countersign the procedure. In Oxford I checked eligibility and consent prior to vaccination; this was done by the trial clinician in Southampton. Vaccines were all administered IM into the deltoid muscle, preferentially into the non-dominant arm unless there was a contraindication (or the volunteer stated a preference for the other arm). The Investigator administering the vaccine wore an apron, gloves and eye protection. The vaccines are GMOs and therefore all waste from a vaccination procedure was autoclaved to minimise dissemination of the recombinant vectored vaccine virus into the environment, in accordance with UK Genetically Modified Organisms (Contained Use) Regulations (2000).

Volunteers were required to remain at the CCVTM for an hour after vaccination. Their vital signs and the vaccination site were checked at 30 minutes (at which time the vaccination site dressing was removed and discarded as GMO waste) and 60 minutes to check for evidence of any immediate reactions to the vaccine.

Blood tests for exploratory immunology were taken at all visits except those occurring 2 days after each vaccination (i.e. days 2 and 58). The methods used to assess vaccine

immunogenicity (T cell ELISPOT, ASC and mBC ELISPOT, IgG ELISA, avidity ELISA, isotype ELISA and GIA) are all described in Chapter 2: Materials and methods.

The four volunteers in Group 1 were vaccinated with 5 x 10^9 vp of ChAd63 RH5. Following a safety review, twelve volunteers (Groups 2A and 2B) were vaccinated with the 'full dose' of ChAd63 RH5 (5 x 10^{10} vp), eight of whom (Group 2B) went on to receive MVA RH5 1 x 10^8 pfu eight weeks later. A further eight volunteers were enrolled into the final group (Group 2C), and a safety review was carried out prior to the full dose MVA RH5 vaccinations (2 x 10^8 pfu) given eight weeks after ChAd63 RH5 prime with 5 x 10^{10} vp.

Vaccination visits occurred on days 0 (all groups) and 56 (Groups 2B and 2C) (nominal study days are used throughout). Volunteers also attended follow-up visits on days 2, 7, 14, 28, 56 and 84 in Group 1, on days 2, 7, 10 14, 28, 56, 63, 84 and 140 in Group 2A and on days 2, 7, 10, 14, 28, 58, 63, 84 and 140 in Groups 2B and 2C, with a final follow-up phone call for these groups on day 240.

5.4 Results

5.4.1 VAC057 Participant Flow

Thirty two volunteers were screened, of whom 24 were enrolled and 8 were excluded (Figure 5-2). Vaccinations began on 18th August 2014 and all follow-up visits were completed by 28th October 2015. All vaccinees received their immunisations as scheduled and there were no withdrawals from the study. Similar numbers of males and females were enrolled (13 females, 11 males). The mean age of volunteers was 28 years (range 19 – 48 years). The four Group 1 volunteers received 5 x 10^9 vp of the ChAd63 RH5 vaccine. Following a safety review, the dose of ChAd63 RH5 was increased for Group 2 and volunteers received 4.26 – 4.77 x 10^{10} vp (a nominal figure of 5 x 10^{10} vp will be used throughout the rest of this Chapter). Four volunteers in Group 2A received ChAd63 RH5 alone. Eight volunteers were enrolled into Group 2B and a further eight into Group 2C. These volunteers received ChAd63 RH5 followed 8 weeks later with a 'boost' vaccination of MVA RH5 at a dose of 1 x 10^8 pfu (Group 2B) or 2 x 10^8 pfu (Group

2C). There was a 2 week interval between the final vaccination in Group 2B with MVA RH5 at the lower dose of 1 x 10^8 pfu and the first vaccination with MVA RH5 at the full dose of 2 x 10^8 pfu in Group 2C, with a safety review prior to dose escalation.

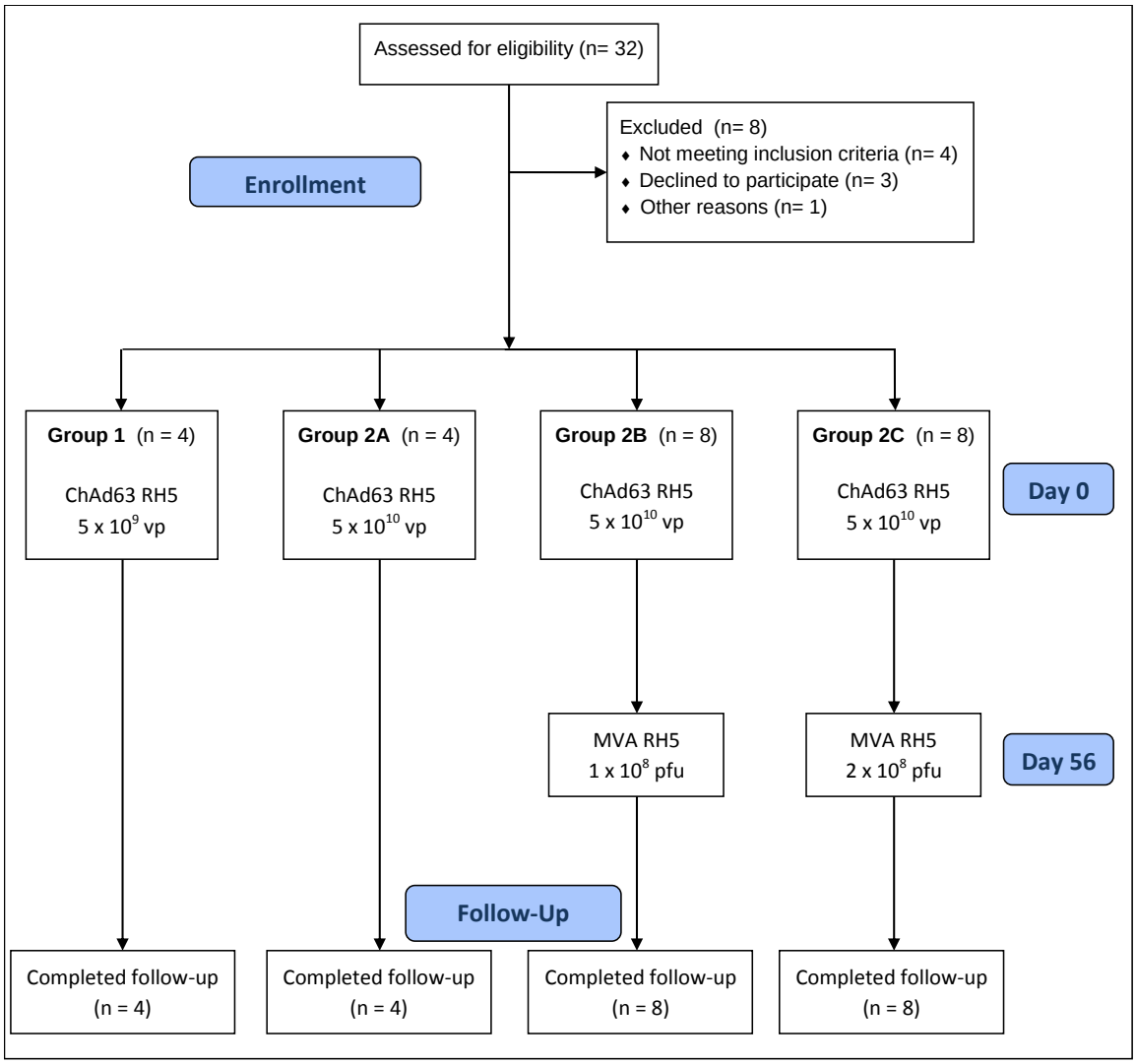


Figure 5-2: VAC057 Study Flow

5.4.2 VAC057 Vaccine Safety and Reactogenicity

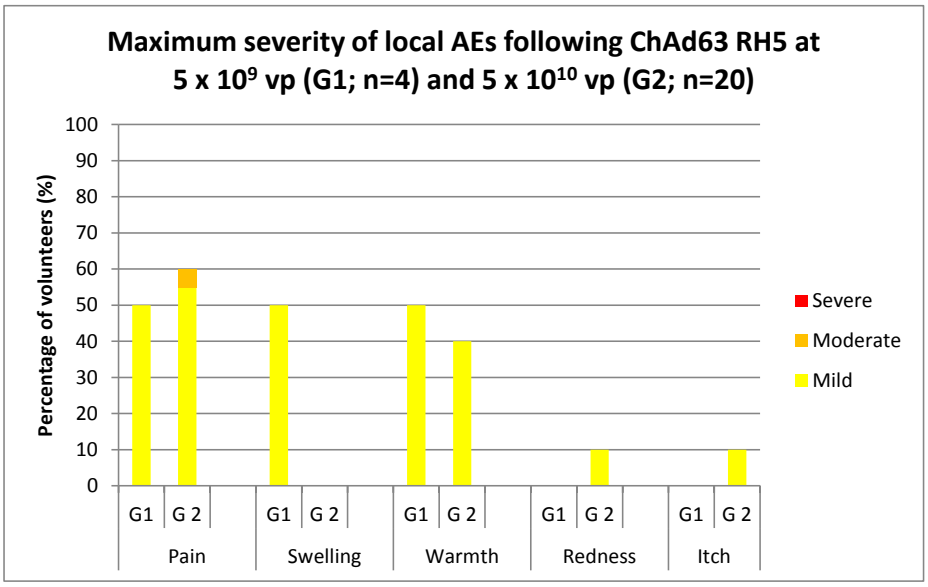
There were no SAEs or unexpected reactions and no safety concerns during the course of the trial. No volunteers withdrew from the study. The reactogenicity of the vaccines was similar to that seen in previous malaria vaccine trials using the same viral vectors at similar doses in healthy adults (84, 155), with the higher doses of both vaccines associated with an increased number and higher severity of reported adverse events. The reactogenicity was also similar to

that seen with the *P. vivax* viral vectored vaccines using the same vectors described in Chapter three.

5.4.2.1 Solicited Adverse Events

The maximum reported severity of solicited AEs following ChAd63 RH5 are shown in Figure 5-3A (local AEs) and Figure 5-3B (systemic AEs). The majority of AEs were mild but moderate AEs were reported by some volunteers in both groups, and two volunteers who received the full dose of ChAd63 RH5 reported severe AEs on the day of vaccination which resolved within 24 hours. Similarly, solicited AEs following MVA RH5 are shown in Figures 5-4A (local AEs) and 5-4B (systemic AEs). All moderate or severe solicited systemic AEs following MVA RH5 occurred in volunteers who had received the higher dose of vaccine. The majority of solicited AEs occurred within the first 2 days after vaccination (Figure 5-5) and the median duration of each systemic AE was between 1 and 2 days following either vaccine.

A



B

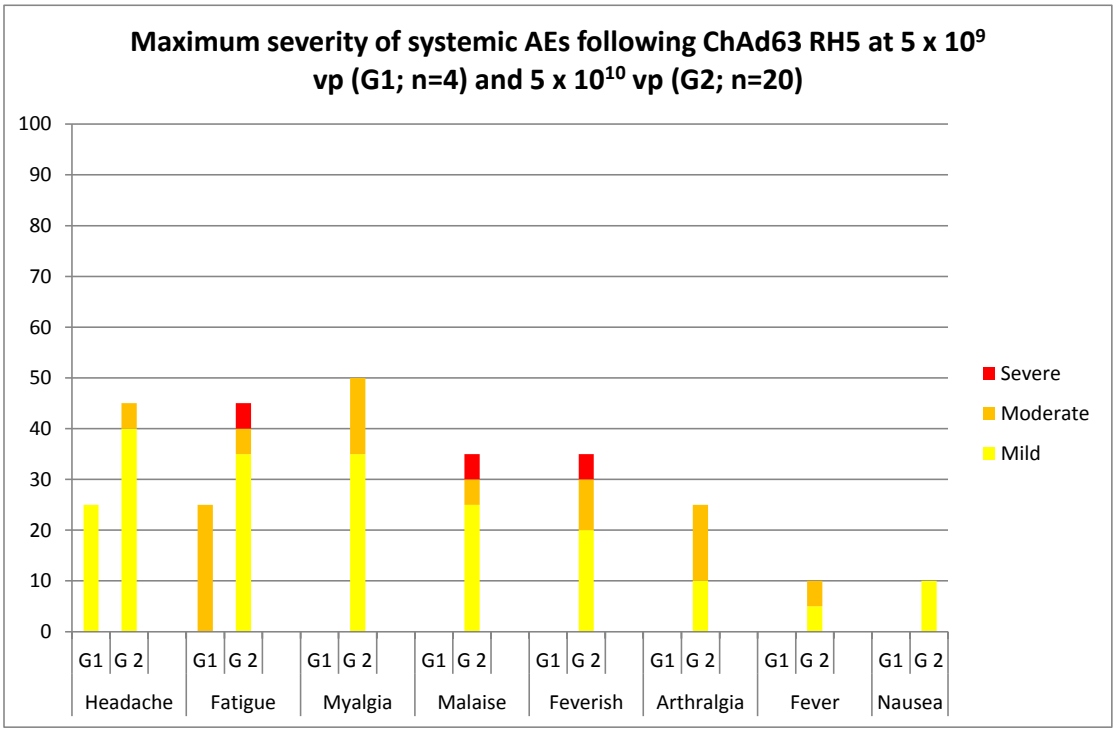
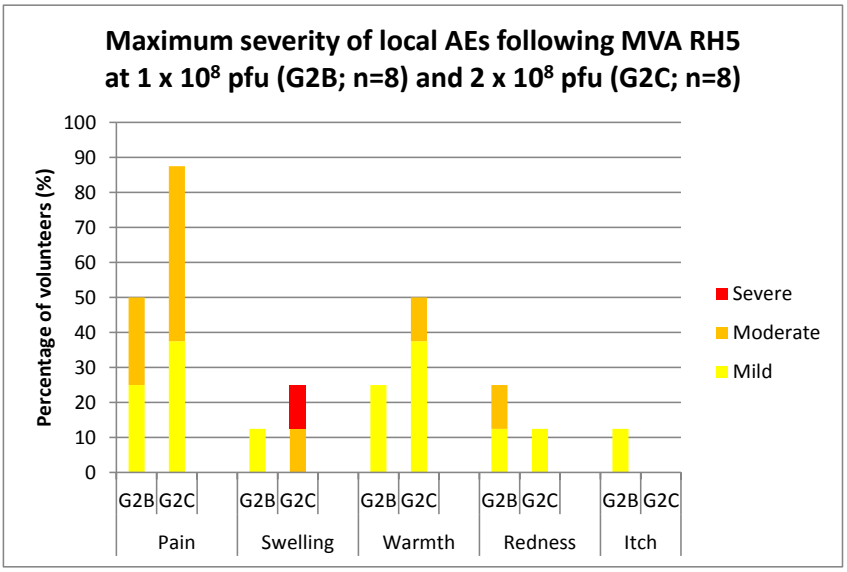


Figure 5-3: Solicited local and systemic AEs following ChAd63 RH5.

Only the highest intensity of each AE per subject is listed. Data are combined for all AEs for all volunteers receiving the same vaccine at the stated dose. ***(A)*** *Local AEs post ChAd63 RH5 in Group 1 (G1) at the 5 x 10^9 vp dose (4 volunteers) and Group 2 (G2) at the 5 x 10^{10} vp dose (20 volunteers).* ***(B)*** *Systemic AEs post ChAd63 RH5 in G1 and G2 at doses of 5 x 10^9 vp and 5 x 10^{10} vp respectively. Data were exported from the OpenClinica database and the eDiary into Excel worksheets, and data were combined to calculate the percentages of volunteers experiencing each AE following vaccinations at different doses.*

A



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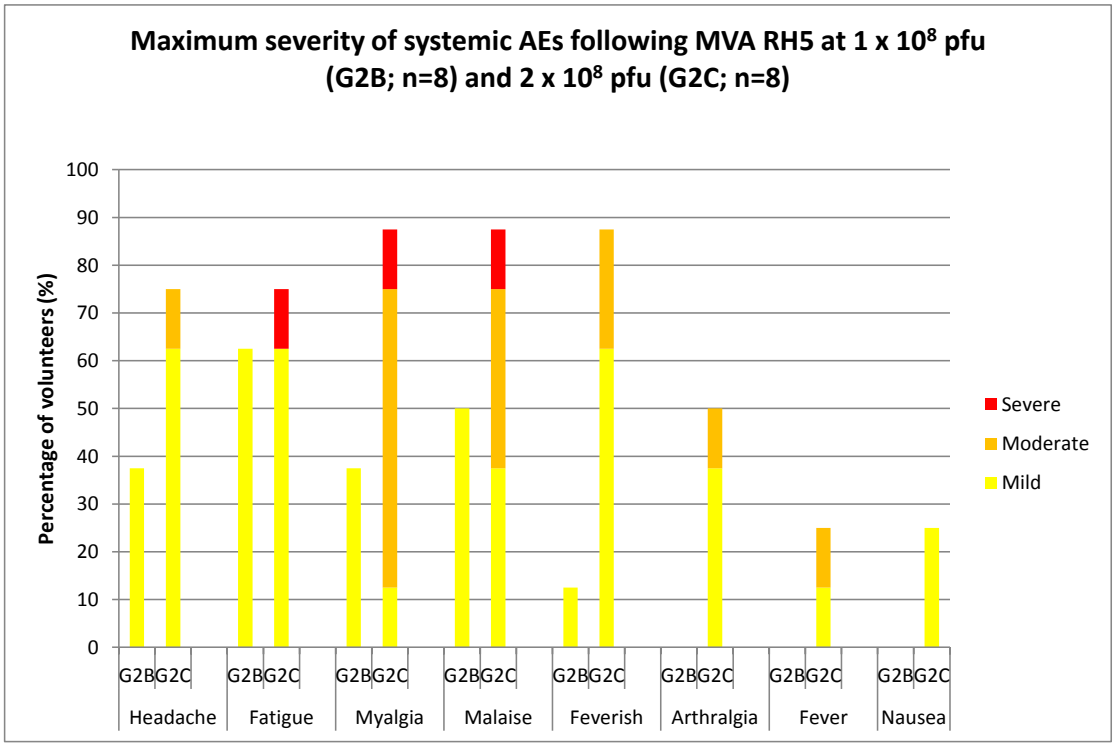

Figure 5-4: Solicited local and systemic AEs following MVA RH5.

Only the highest intensity of each AE per subject is listed. Data are combined for all AEs for all volunteers receiving the same vaccine at the stated dose. ***(A)*** *Local AEs post MVA RH5 in Group 2B (G2B) at the 1 x 10^8 pfu dose (8 volunteers) and Group 2C (G2C) at the 2 x 10^8 pfu dose (8 volunteers).* ***(B)*** *Systemic AEs post MVA RH5 in G2B and G2C at doses of 1 x 10^8 pfu and 2 x 10^8 pfu respectively. Data were exported from the OpenClinica database and the eDiary into Excel worksheets, and data were combined to calculate the percentages of volunteers experiencing each AE following vaccinations at different doses.*

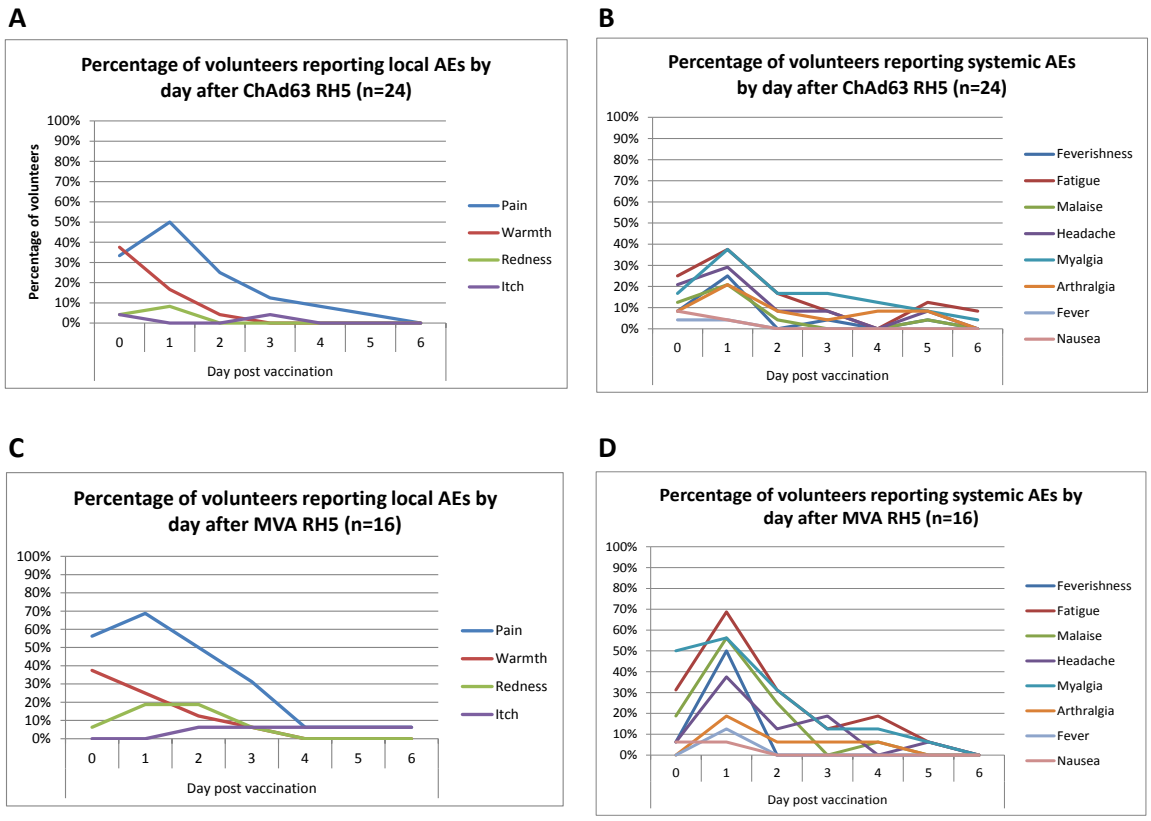

Figure 5-5: VAC057 Percentage of volunteers reporting solicited local and systemic AEs by day

Data are shown for 7 days after ChAd63 RH5 vaccination (n=24) (A, B) and MVA RH5 (n=16) (C, D) for all volunteers receiving each vaccine, regardless of dose. Data were exported from the Openclinica database and eDiary into Excel and the percentages of volunteers reporting each solicited AE by day post vaccination were calculated.

5.4.2.1 Unsolicited Adverse Events

Unsolicited AE data were collected for 28 days after each vaccination (SAE data were collected until the end of follow-up). Causality was assigned by the lead clinician and peer-reviewed by an independent clinician with an agreed final causality score. Causality was assessed as described in 'Materials and Methods' (Chapter two). One volunteer reported a severe unsolicited adverse event which was considered probably related to vaccination as it occurred the day after ChAd63 RH5 5 x 10^9 vp. This was abdominal cramping lasting around 30 minutes which resolved spontaneously and did not recur. There were no severe unsolicited adverse events reported after MVA RH5 vaccination. Unsolicited adverse events considered possibly, probably or definitely related to vaccination are shown in Table 5.1.

As well as the unsolicited adverse events listed below, five volunteers had mild hypertension recorded during the trial. This was recorded as a single reading in two volunteers, recorded twice in one volunteer and recorded intermittently for a further two volunteers, both of whom were noted to have mild hypertension at screening. One volunteer had a mild tachycardia recorded at two clinic visits (days 2 and 28) but this was not sustained.

Unsolicited AEs following ChAd63 RH5				
Group	**Subject ID**	**Timepoint(s)**	**Symptom**	**Severity**
1	MVT-0571005	1	Abdominal cramping	3
2A	MVT-0571003	0	Numbness around the lips	1
2A	MVT-0571003	9, 11	Headache	2
2A	MVT-0571003	9	Fatigue	1
2A	MVT-0571009	9	painful injection site	1
2A	MVT-0571010	1 to 2	sore throat	1
2B	MVT-0571015	10	fatigue	2
2C	MVT-0571014	7 to 8	Temp 37.2; feeling unwell	2
2C	MVT-0571014	8	Weakness	2
2C	MVT-0571014	8	Fatigue	2
2C	MVT-0571014	11	Headache	1
2C	MVT-0572211	2	Felt light headed and dizzy after going for a run.	1
2C	MVT-0572205	1	Palpitations	2
2C	MVT-0572205	1	Insomnia	2
2C	MVT-0572210	7 to 8	Sore throat	1
2C	MVT-0572210	9 to 12	mild sinus/nasal congestion	1
Unsolicited AEs following MVA RH5				
Group	**Subject ID**	**Timepoint(s)**	**Symptom**	**Severity**
2B	MVT-0571012	1 to 11	Coryzal symptoms, cough and congestion	1
2B	MVT-0571018	0 to 1	abdominal pain	1
2B	MVT-0571020	4 to 5	Coryzal symptoms	1
2B	MVT-0572204	10	Headache	1
2C	MVT-0571014	8	Vomited x 1	2
2C	MVT-0572211	0 to 3	Disturbed sleep	1
2C	MVT-0572205	1	Tinnitus	1
2C	MVT-0571017	0 to 1	Small swelling/haematoma at vaccination site	1

Table 5.1: VAC057 Unsolicited adverse events considered possibly, probably or definitely related to vaccination.

Maximum reported severity shown. Timepoint(s) = days post vaccination.

5.4.2.3 Laboratory Adverse Events

There were no severe laboratory AEs following ChAd63/MVA RH5 vaccination. One volunteer had a moderately raised ALT at day 7 following ChAd63 RH5 which had resolved fully by day

28. One volunteer had moderate thrombocytopenia and mild leukopenia at day 28 following ChAd63 RH5 but had commenced post-exposure prophylaxis for HIV exposure the day before these bloods were taken, so these findings may have been due to the antiretroviral medication rather than the vaccination. All other laboratory AEs were mild and had resolved fully by day 84 except for one volunteer who had a persistent mild anaemia. This had been present at screening and had not worsened over the course of the study so was not considered significant.

5.4.3 ChAd63/MVA RH5 T cell immunogenicity assessed by *ex-vivo* IFN-γ ELISPOT

The ChAd63/MVA RH5 vaccines elicited T cell responses as assessed by *ex-vivo* IFN-γ ELISPOT, with peak responses after the MVA boost (Figure 5-6). The median peak responses (day 63) were 2092 SFU/million PBMC for Group 2B and 2281 SFU/million PBMC for Group 2C. This difference was not statistically significantly different (P = 0.32; analysed by Mann Whitney test). The response at day 140 (final immunology timepoint) was also similar in both groups with a median response of 458 and 663 SFU/million PBMC in Groups 2B and 2C respectively.

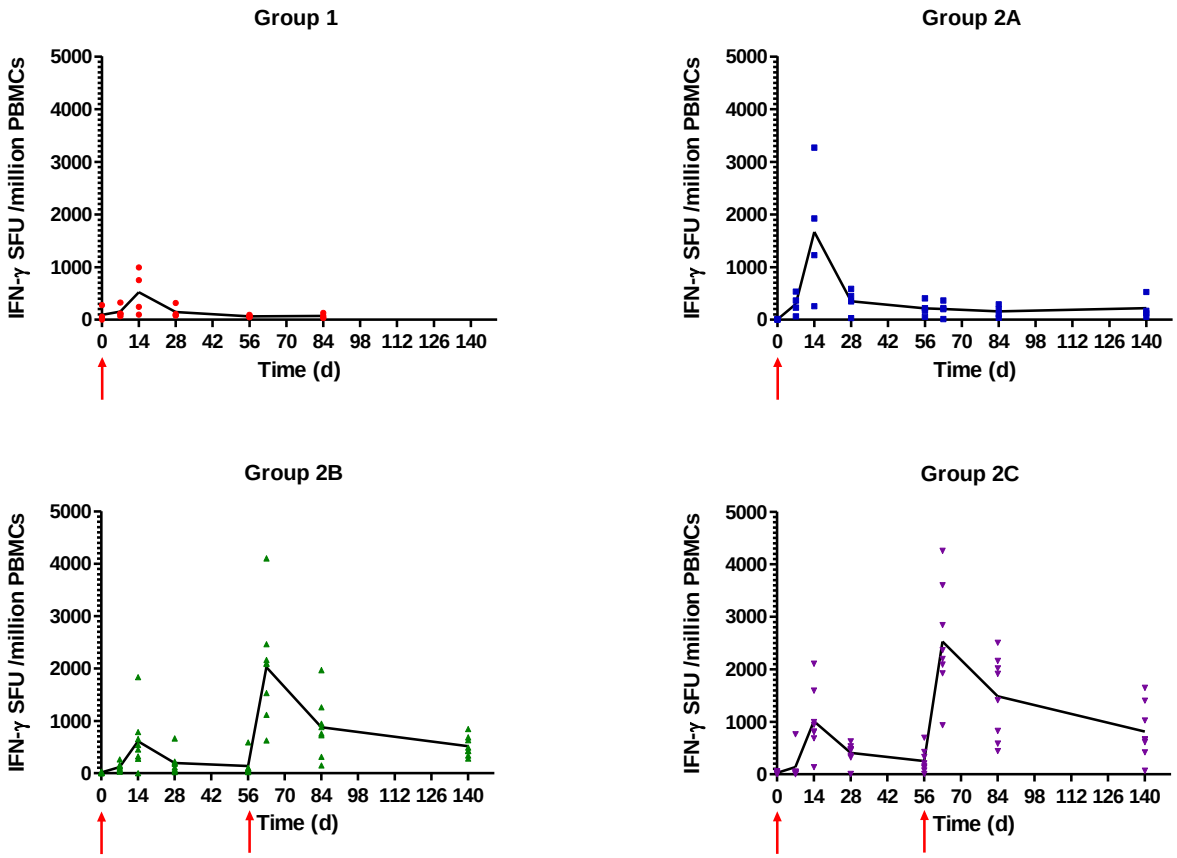

Figure 5-6: VAC057 T cell responses as assessed by ex vivo IFN- γ ELISPOT

Individual and median group results are shown for volunteers vaccinated with ChAd63 RH5 (all groups) and boosted with MVA RH5 (Groups 2B and 2C). Arrows indicate vaccination timepoints. Spots were counted using an automated plate counter and exported into an Excel worksheet where results were obtained by subtracting any background response (from negative control wells) and then taking the average of triplicate wells. Data were then imported into GraphPad Prism for statistical analyses.

5.4.4 ChAd63/MVA RH5 antibody response assessed by ELISA

The kinetics and magnitude of the serum IgG antibody responses were assessed over time by an ELISA to recombinant PfRH5 protein (Figure 5-7). The IgG response was higher in the group vaccinated with full dose (5 x 10^{10} vp) ChAd63 RH5 and peaked after MVA boost with similar IgG responses in both boosted groups (Groups 2B and 2C). The median peak response, seen four weeks after MVA RH5 (i.e. day 84) was 720 AU in Group 2B and 1696 AU in Group 2C. Most volunteers who received the lead-in dose of ChAd63 RH5 alone did not seroconvert (Group 1; Figure 5-7B), whereas those who received full dose did. The difference in peak median responses between Groups 2B and 2C at day 84 was not calculated to be significant

but the response in Group 2C was significantly higher than that in the unboosted volunteers in Group 2A (*P* value = 0.01; analysed by Kruskal-Wallis test).

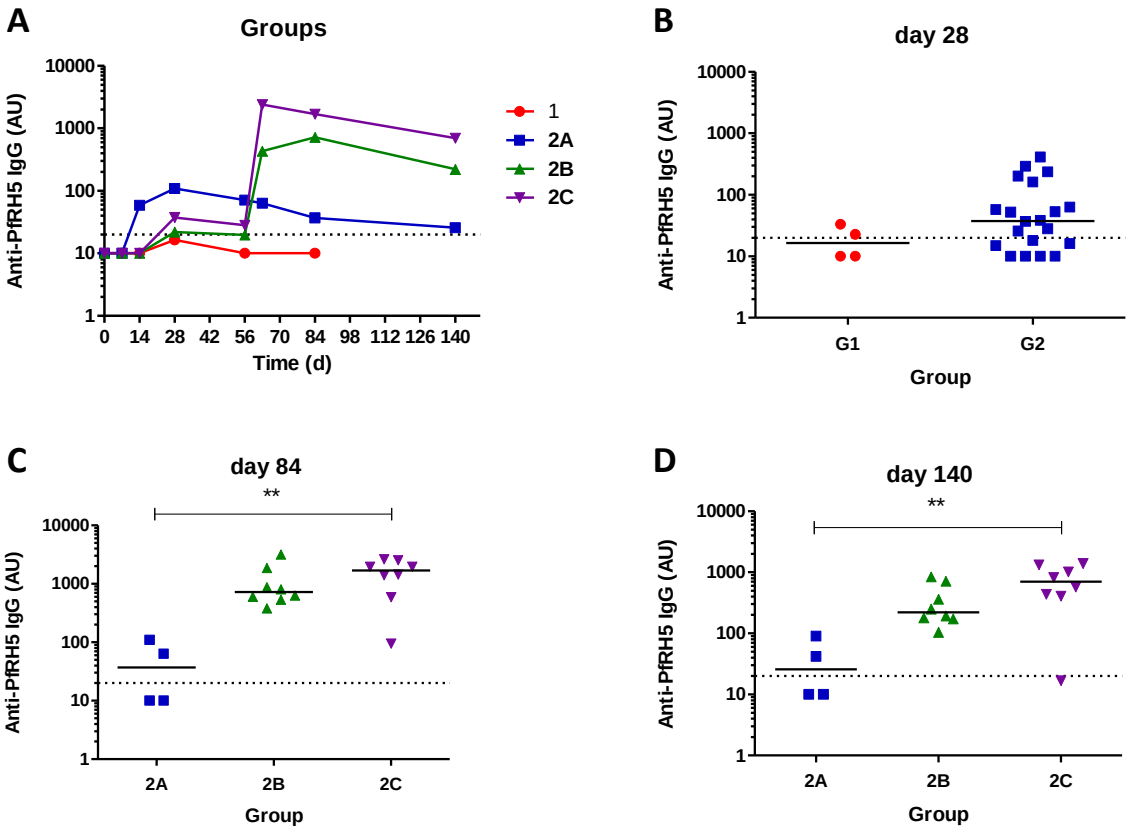

Figure 5-7: VAC057 Anti-RH5 IgG responses measured by ELISA

Median responses for all groups (A), and individual and median responses following ChAd63 RH5 (B) (G1, n=4; G2, n=20) and following MVA RH5 in G2B (n=8) and G2C (n=8) (C and D) are shown. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and values of internal controls and samples in triplicate were assessed for any aberrant readings. The results were obtained by taking an average of triplicate wells, and using the standard curve to assign ELISA arbitrary units (AU). The limit of the assay is 20 AU (marked by a dotted line), below which is classed as negative. Data were then imported into GraphPad Prism for statistical analyses.

5.4.5 Anti-RH5 IgG avidity and antibody response profile

A NaSCN-displacement ELISA was carried out to assess the avidity of the serum IgG responses following ChAd63/MVA RH5 vaccination (Figure 5-8) and is reported as the molar concentration of NaSCN required to reduce the OD 405 to 50% of that without NaSCN (IC50). Avidity for samples negative for Total IgG ELISA cannot be analysed. The avidity of the anti-RH5 IgG was significantly but marginally higher at the peak of the IgG response after MVA boost

than following ChAd63 RH5 alone. Avidity was lower with ChAd63/MVA RH5 than seen following ChAd63/MVA PvDBP (Chapter three).

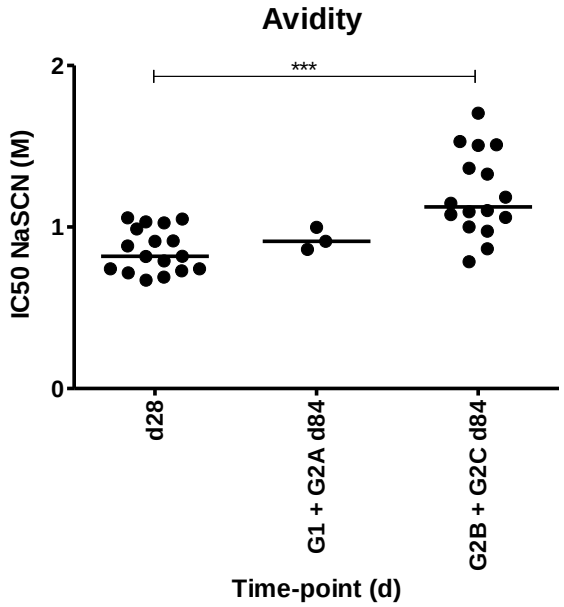

Figure 5-8: IgG Avidity assessment following ChAd63/MVA RH5 vaccinations.

*Individual and median responses at four weeks (d28) post ChAd63 PfRH5, twelve weeks post ChAd63 PfRH5 (G1 and G2A d84) and four weeks post MVA PfRH5 boost (d84 G2B and G2C) are shown. Avidity for samples negative for Total IgG ELISA could not be measured. The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and the average value of duplicate readings calculated. Data were then imported into GraphPad Prism for statistical analyses. ***P<0.001 Kruskal-Wallis test with Dunn's correction for multiple comparison.*

An isotype ELISA (Figure 5-9) demonstrated a similar antibody profile following ChAd63/MVA RH5 as was seen following ChAd63/MVA PvDBP (Chapter three) with a predominant IgG1 and IgG3 response. There was also a significant increase in IgA seen following ChAd63/MVA RH5 vaccination. Although there was a significant increase in IgM, many of the baseline results were positive, suggesting there was some interference or background reactivity in the assay for this isotype.

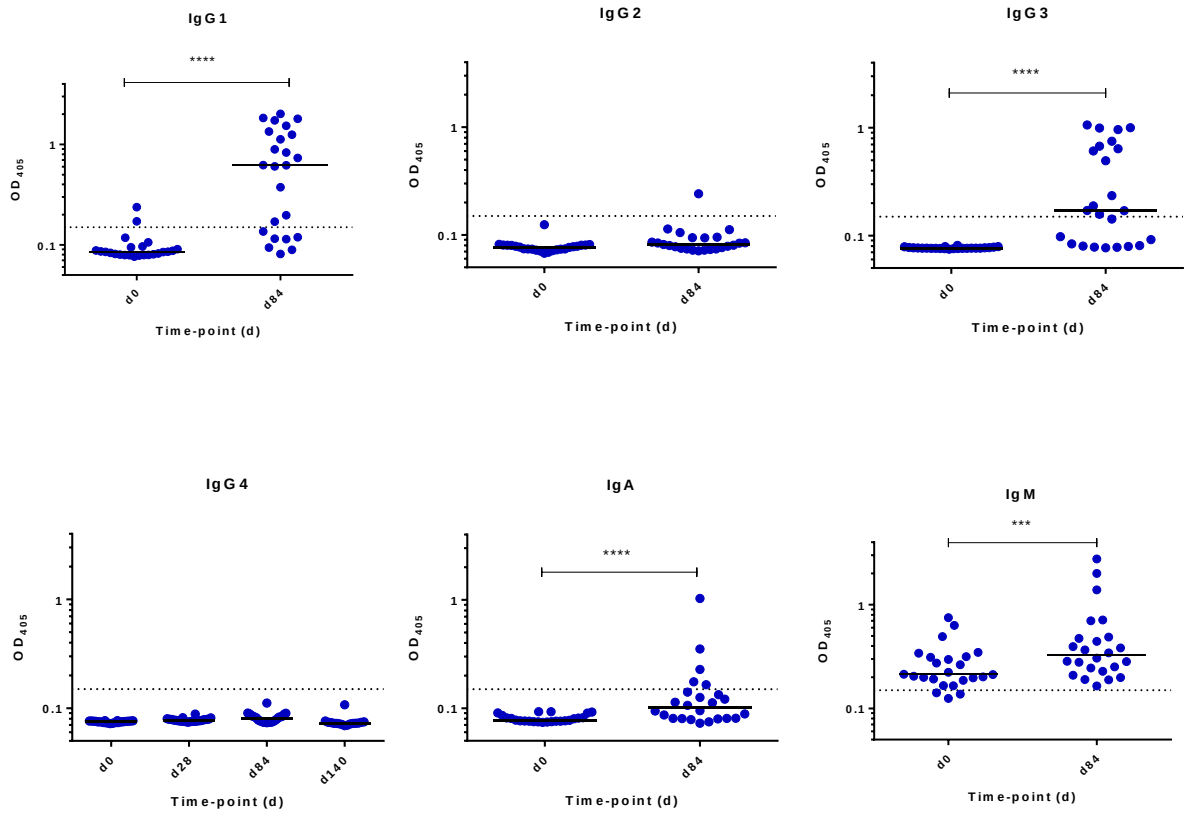

Figure 5-9: Antibody isotype profile following ChAd63/MVA RH5 vaccination.

*Antibody isotypes were assessed by ELISA. Baseline (d0) response, and response twelve weeks post ChAd63 RH5 for G1 (n=4) and G2A (n=4) and four weeks post MVA RH5 boost for G2B (n=8) and G2C (n=8) (d84) are shown. Individual and median responses are shown for every isotype. Dotted line represents ELISA cut-off (OD of 0.15, below which samples were considered negative). The absorbance at 405nm (OD_{405}) was read using a Biotek ELx800 microplate reader with Gen5 software. Data were exported into an Excel worksheet and the average OD values of duplicate samples calculated. Data were then imported into GraphPad Prism for statistical analyses ***P<0.001, ****P<0.0001 Wilcoxon matched-pairs signed rank test.*

5.4.6 Detection of anti-RH5 ASCs and mBCs following ChAd63/MVA RH5

Samples from boosted volunteers (G2B and G2C) were assessed by *ex-vivo* ASC ELISPOT at the d63 timepoint using fresh PBMC, and at a later timepoint using frozen PBMC for comparison. There was no significant difference between Groups 2B and 2C using either fresh PBMC (P = 0.15; Mann Whitney test) (Figure 5-10A) or frozen PBMC (P = 0.27; Mann Whitney test) (Figure 5-10B). There was a trend towards RH5-specific ASC making up a higher percentage of the total IgG-secreting ASC in Group 2C but this was not statistically significant (P = 0.80; Mann Whitney test) (Figure 5-10C).

Peripheral mBC responses were assessed by identifying RH5-specific mBC-derived plasma cells by *ex-vivo* ELISPOT following a 6-day polyclonal culture of PBMC (Figure 5-11). The responses were measured at the peak IgG response (d84) and at the final timepoint (d140). There was no significant difference between the median response in G2B and G2C, both as a measure of mBC-derived ASC.

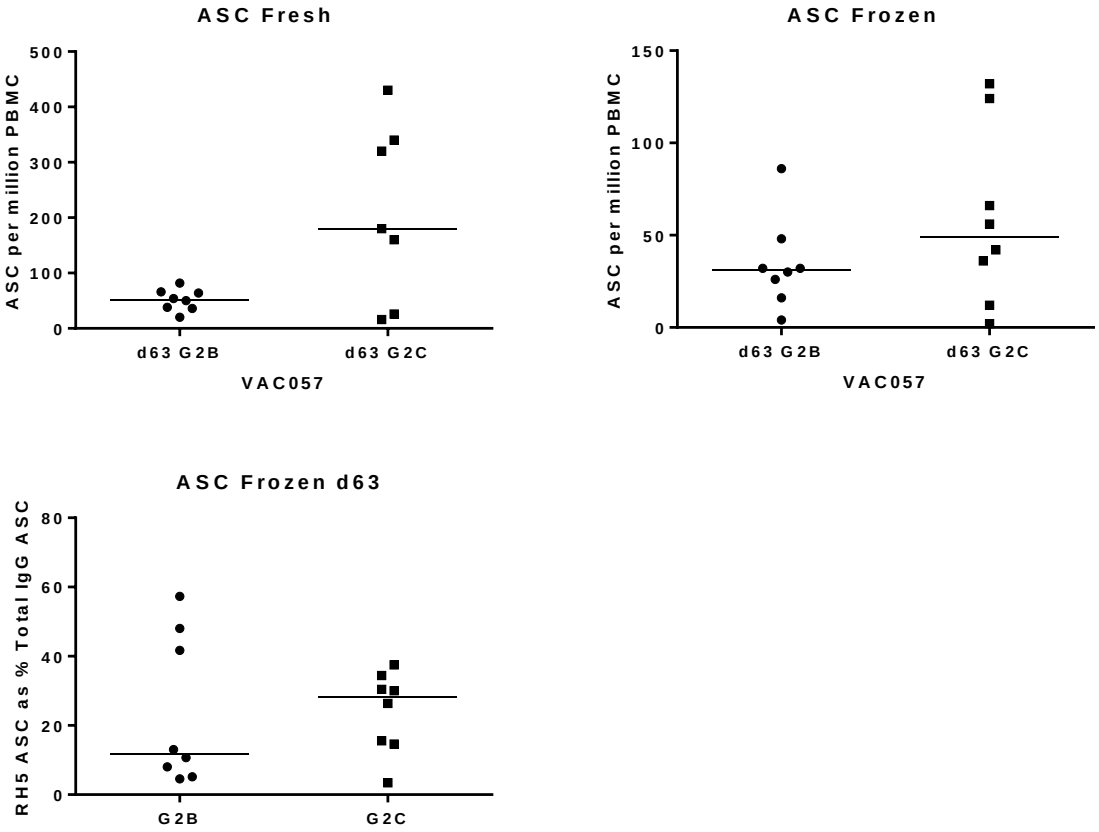

Figure 5-10: ASC responses following ChAd63/MVA RH5 vaccination.

RH5-specific peripheral blood ASCs measured from fresh PBMC (A) and frozen PBMC (B) isolated 7 days following MVA RH5 vaccination (d63) using ex-vivo ELISPOT. (C) RH5-specific ASC as a percentage of total IgG detected from frozen PBMC at d63. Data are shown for G2B (n=8) and G2C (n=8). Spots were counted using an AID ELISPOT reader and automated counts were corrected by eye to ensure only spots consistent with IgG secreting ASCs were counted. Data were exported into an Excel worksheet and then imported into GraphPad Prism for graphical presentation and statistical analyses. There were no significant differences between Groups 2B and 2C using the Mann Whitney test.

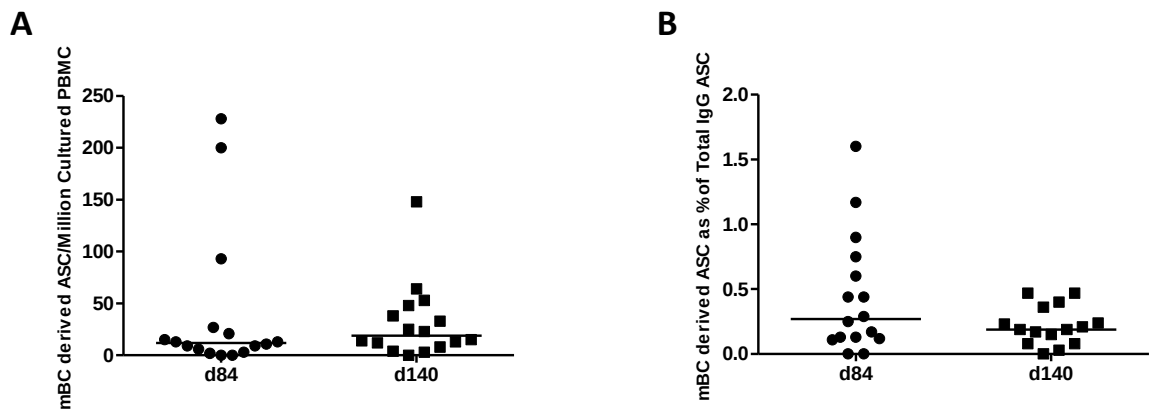

Figure 5-11: mBC responses following ChAd63/MVA RH5 vaccination

mBC derived ASC measured at the peak of the response at d84 and at the final timepoint (d140) using ex-vivo ELISPOT for Groups 2B (n=8) and 2C (n=8). Individual and median results for both groups are shown as (A) mBC derived ASC per million cultured PBMC and (B) as a percentage of total IgG ASC. mBC ELISPOT spots were counted using an AID ELISPOT reader and automated counts were corrected by eye .Data were exported into an Excel worksheet and then imported into GraphPad Prism for statistical analyses. There was no significant difference between the median response in G2B and G2C (assessed by Mann Whitney test).

5.4.7 VAC057 Measures of *in vitro* GIA

Serum was sent to the GIA Reference Center at NIH, USA and IgG purified from each sample. GIA was assessed against the vaccine homologous clone (3D7) for all volunteers who received both vaccines (Groups 2B and 2C). IgG was initially assessed at 10 mg/mL (Figure 5-12A) and then using a serial dilution. As expected, GIA decreased as the IgG was diluted (Figure 5-13). As discussed in Chapter four, a GIA of >60% with 2.5 mg/mL purified IgG was required in the *Aotus* animal model to correlate with protection against *P. falciparum* malaria (104) and none of the volunteers in VAC054 achieved greater than 40% GIA at this concentration of IgG. Therefore we expect that GIA levels are required to be >40% (and possibly >60%) at 2.5 mg/mL purified IgG if protection against *P. falciparum* blood-stage CHMI is likely to be seen. Figure 5-12B demonstrates that none of the volunteers in VAC057 achieved >40% GIA at 2.5 mg/mL purified IgG after ChAd63/MVA RH5 vaccination. Nonetheless, the result was encouraging as the level of GIA achieved with the ChAd63/MVA RH5 viral vectors in this trial was similar to that seen with the FMP2.1 protein vaccine given with the potent AS01 adjuvant (209).

GIA was also assessed against a range of other *P. falciparum* strains (Figure 5-14). These data show that the antibodies induced by vaccination were able to inhibit the growth of all strains of *P. falciparum* tested, although the levels of GIA were not equal for all strains.

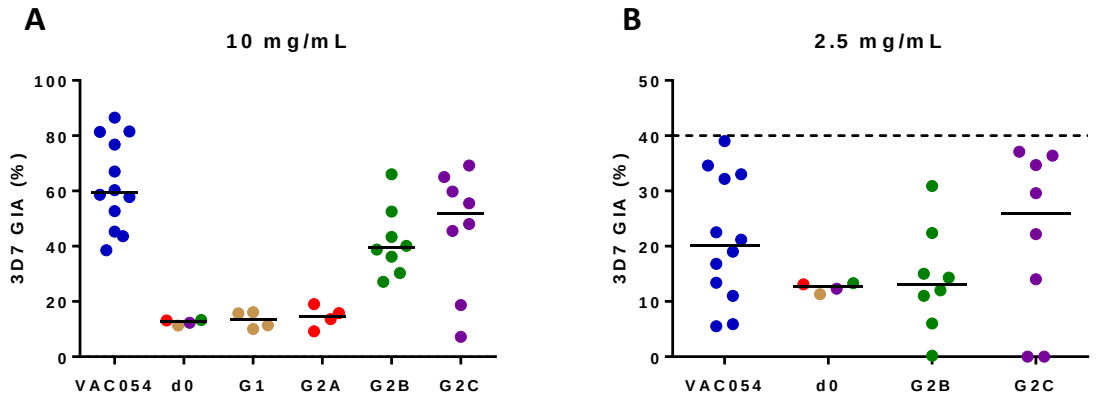

Figure 5-12: VAC057 GIA against reference clone P. falciparum 3D7 at 10 mg/mL and 2.5 mg/mL purified IgG.

GIA for each group with individual and median responses are shown at a purified IgG concentration of 10 mg/mL (A) and 2.5 mg/mL (B). Results are compared with purified IgG from vaccinees at the same dilution from the VAC054 trial (Chapter four). A single-cycle assay was used, with the readout measured after 48 hours. Data were received from the NIH reference center in an Excel worksheet and were imported into GraphPad Prism for graphical presentation and analyses.

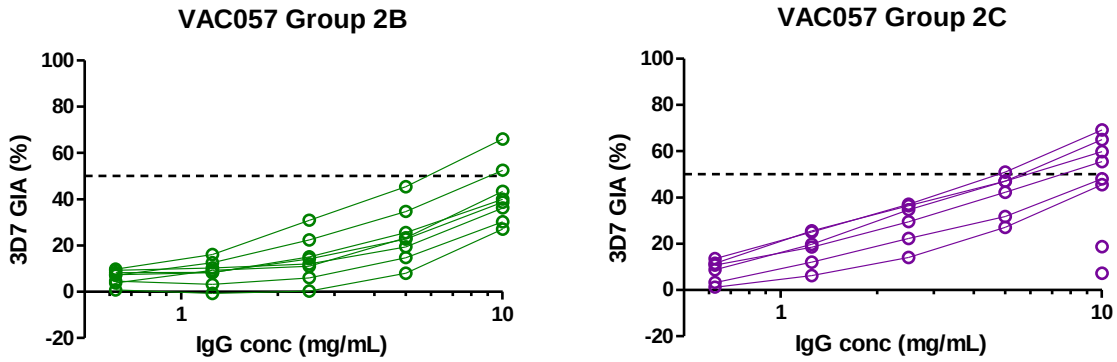

Figure 5-13: VAC057 GIA against reference clone P. falciparum 3D7 at 10 mg/mL purified IgG and serial dilutions.

A single-cycle assay was used, with the readout measured after 48 hours in samples from volunteers in Groups 2B (n=8) and 2C (n=8). Data were received from the NIH reference center in an Excel worksheet and were imported into GraphPad Prism for graphical presentation and analyses.

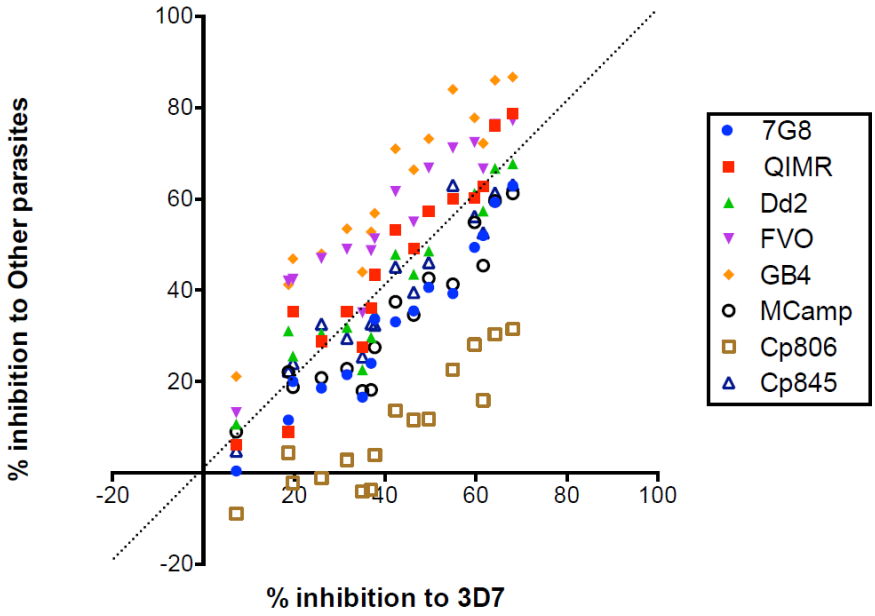

Figure 5-14: VAC057 Growth inhibition activity of purified IgG at 10mg/mL against a range of P. falciparum strains compared with GIA against the reference 3D7 clone.

5.5 Discussion

As discussed in Chapter four, the development of vaccines against *P. falciparum*, and especially against the blood-stage parasite has proved extremely challenging, with no blood-stage candidate vaccine to date demonstrating significant efficacy against infection. The reasons for the lack of efficacy include difficulty in achieving adequate antibody concentrations (given the very short time interval in which the merozoite is exposed before erythrocyte invasion) and antigen polymorphism. The antibody levels required to block invasion differ according to the antigen, for example, higher concentrations of anti-MSP1 antibody are required to block invasion in a GIA assay compared with anti-AMA1 antibodies (130). The polymorphisms described for both MSP1 (87) and AMA1 (241-243) also create issues for vaccine development. One approach is to develop a multi-allele vaccine (244, 245), but covering all antigen variations would be difficult and it is possible that parasites may find other mechanisms for invasion.

The PfRH5 antigen has important advantages over blood-stage antigens such as AMA1 and MSP1. Firstly, there is only limited polymorphism (235), presumably because PfRH5 appears not to come under significant immune pressure. Secondly, anti-RH5 antibodies are able to

block erythrocyte invasion with lower antibody concentrations than anti-AMA1 and anti-MSP1 antibodies (130, 235) which means that lower antibody levels are potentially needed for an effective vaccine than for these candidate antigens. As discussed at the start of this Chapter, anti-RH5 antibodies induced by vaccination have been shown to inhibit multiple strains of *P. falciparum in vitro* (158, 235, 239, 240) and vaccination with RH5 vaccines protected *Aotus* monkeys against a heterologous strain challenge *in vivo* (104).

In this Chapter, I have decribed the safety and immunogenicity of the candidate vaccines ChAd63/MVA RH5 in a first-in-human Phase Ia clinical trial. The vaccines were well tolerated with an acceptable reactogenicity profile, no SAEs and all volunteers completed follow-up in the study. In this study, as opposed to VAC051 (Chapter three) and VAC054 (Chapter four), I implemented an electronic diary to collect volunteer-reported AEs. This enabled the safety data to be monitored remotely for all enrolled volunteers in real time, as well as reducing the risk of data errors in transcribing from paper diaries to a database. Furthermore, use of the electonic diaries meant I collected data daily for 28 days after each vaccination rather than 7 or 14, improving the accuracy of the data as data collected after day 7 did not rely purely on recall at clinic visits. The change of duration for collecting all AEs in this study also meant that the most relevant information regarding AEs was collected, i.e. those occurring within the first four weeks after vaccination, and any serious AEs. Blood samples for haematological and biochemical analyses were taken only over the same period as it was felt that any abnormalities attributable to vaccination would be detectable within this timeframe.

The ChAd63/MVA RH5 vaccines were immunogenic, eliciting RH5-specific T cell and IgG reponses in vaccinees who received the higher dose of ChAd63 RH5 and peaking after MVA RH5 boost. There was a trend towards higher anti-RH5 IgG responses in Group 2C, who received the full dose of both vaccines, but the difference was not statistically significant. However, the number of RH5-specific ASCs measured at 7 days post-MVA RH5 were significantly higher following full dose MVA RH5 than the 1 x 10^8 pfu dose. There was also a

significant increase in IgG avidity after MVA RH5 boost. The RH5-specific T cell response was similar in both boosted groups, with a peak response seen 1 week after the MVA RH5 vaccinations. Although it is likely that protection against blood-stage malaria is mainly antibody-mediated, T cells are important in providing B cell help and also in activating Th1 responses which help mediate effector mechanisms of both antibodies and cell-mediated immunity (246).

Antibodies induced by vaccination demonstrated *in vitro* efficacy against *P. falciparum* in a GIA assay. As discussed in Chapter three, the induction of functional antigen-specific antibodies provides evidence that the RH5 protein was produced as a correctly folded protein and secreted successfully following ChAd63/MVA RH5 vaccination. Most importantly, the purified IgG from vaccinated volunteers was able to block not only the strain homologous with the PfRH5 in the ChAd63/MVA RH5 vaccines, but also every other strain tested in this assay. This is the first time antibodies against a blood-stage antigen have demonstrated cross-strain inhibition following vaccination of humans with a monovalent *P. falciparum* candidate vaccine. The level of GIA differed according to strain, with some demonstrating greater inhibition than against the 3D7 reference clone whilst others had lower levels. This may be due to several reasons including: polymorphisms within the RH5 protein leading to slight differences in the binding site with basigin and therefore differences in activity of anti-RH5 antibodies; differences in the amount of RH5 expressed by the various strains; or differences in the release kinetics of RH5 between strains.

The level of GIA seen against the 3D7 reference clone was similar to that seen following the FMP2.1/AS01 vaccine discussed in Chapter four, with a median GIA at 10 mg/mL purified IgG of 40% in Group 2B and around 50% in Group 2C (compared with around 60% in the VAC054 trial). At 2.5 mg/mL purified IgG the GIA levels fell to a median of 14% for Group 2B and 26% for Group 2C (compared with 20% in the VAC054 volunteers). It is therefore unlikely that this vaccine regime would be protective against *P. falciparum* infection in a blood-stage CHMI trial.

However, the result is still encouraging as the levels of antibody induced by viral vectored vaccines are typically lower than those after protein-in-adjuvant vaccine regimes. A previous Phase Ia trial in Oxford demonstrated that purified IgG at 10 mg/mL from volunteers vaccinated with AMA1 given in viral vectored vaccines using the same vectors as were used in this trial gave less than 20% GIA (167). In this same study, a protein-in-adjuvant AMA1 vaccine was given following a ChAd63 AMA1 priming vaccine and this gave similar levels of GIA at 10 mg/mL purified IgG to those seen in the VAC054 study with the AMA1 vaccine FMP2.1/AS01B (167, 209). This implies that antibody levels could be improved upon with a vaccine which was tailored to induce a stronger B cell response as the T cell response against this antigen is likely to have less of a role. Having said that, the GIA assay only assesses the functional activity of IgG and the response against *P. falciparum* infection may be better than predicted if T cells do play a role.

To conclude, this study marked a major milestone in *P. falciparum* blood-stage vaccine development, showing that induction of antibodies active against multiple parasite strains is possible in humans. Future work will aim to develop a vaccine which is better at inducing antibodies than viral vectored vaccines, and this will hopefully lead to demonstrable efficacy against the parasite.

Chapter six:

Concluding Remarks and Future Directions

6.1 Summary

Malaria remains a significant global health burden, affecting millions of individuals each year. Most cases of malaria are due to *P. falciparum*, but a significant proportion of those in Asia and South America are due to *P. vivax*. The development of effective malaria vaccines has proved extremely challenging, with only one vaccine candidate (GSK's *P. falciparum* pre-erythrocytic vaccine, RTS,S) reaching Phase III trials, and demonstrating only moderate efficacy (57). The most recent update to the Malaria Vaccine Technology Roadmap calls for a vaccine effective against both falciparum and vivax malaria by 2030 (7). Blood-stage malaria vaccines for *P. falciparum* have faced particular difficulties with the need for very high antibody levels and issues with polymorphism of candidate antigens. No blood-stage vaccine has demonstrated significant efficacy against infection to date, but the concept of a blood-stage vaccine is supported by the development of natural immunity in endemic areas with a corresponding rise in serum titres of antibodies against blood-stage malaria proteins (247). However, in natural immunity responses to a broad range of antigens are required (248) and strain-specificity has been demonstrated (249). Even in those who acquire natural immunity, protection is far from complete with a reduction in disease severity but ongoing risk of infection (247, 250). There had not previously been any blood-stage *P. vivax* vaccine to reach clinical trial, and there are reasons to believe that a vaccine against this parasite may be easier to achieve as in almost all cases *P. vivax* invades erythrocytes via DARC, making this an essential interaction for the parasite. Furthermore, there is evidence that antibodies against PvDBP are able to block multiple strains of *P. vivax*, meaning heterologous as well as homologous protection may be possible with a PvDBP-based vaccine (251).

This thesis has described three clinical early-phase vaccine trials carried out in healthy adult volunteers at the Jenner Institute in Oxford. Two of the trials investigated *P. falciparum* candidate blood-stage vaccines, and one trial examined a *P. vivax* vaccine. One of the *P. falciparum* blood-stage vaccine trials also led to the development of a human blood-stage CHMI model to assess vaccine efficacy. All vaccines tested were safe and immunogenic.

However, despite functional AMA1-specific antibodies being induced by the FMP2.1/AS01 vaccine, efficacy against homologous blood-stage CHMI was not seen. Similarly, functional antibodies against RH5 were seen following the ChAd63/MVA RH5 heterologous prime-boost regimen, but the level of GIA seen was thought unlikely to lead to protection against *P. falciparum* infection, although this currently remains untested. Importantly though, the functional activity seen was strain-transcending, a novel finding following vaccination of humans with a blood-stage vaccine candidate. The ChAd63/MVA PvDBP vaccine regimen was the first blood-stage *P. vivax* vaccine candidate ever to be tested in a clinical trial. Again, anti-PvDBP antibodies were induced which demonstrated blocking of the PvDBP-DARC interaction in an *in vitro* assay, but as blood-stage vaccine efficacy has never been tested for *P. vivax* it is unknown whether this would be likely to translate into protection against infection *in vivo*. These studies have therefore highlighted some of the difficulties in blood-stage malaria vaccine development but have also made important progress in this field.

6.2 Development of the CHMI model for assessing blood-stage *P. falciparum* vaccines

The best method for assessing vaccine efficacy for blood-stage *P. falciparum* vaccines has been widely debated (214). It may be that efficacy only occurs at high parasite densities, which do not occur in CHMI trials as participants are treated as soon as malaria is diagnosed on thick blood film, even if asymptomatic. However, an effective vaccine against malaria will need to be able to control parasitaemia before the onset of symptoms if it is to be useful and acceptable. In order to assess early effects on parasitaemia, the effect on PMR following vaccination can be analysed as an efficacy endpoint. This allows for even small changes in the growth rate of the parasite to be detected, demonstrating partial efficacy as well as the potential for demonstrating sterile efficacy if a volunteer is protected from developing malaria. Demonstration of partial efficacy would still be an important finding as it infers that protection is possible and allows further development to produce an improved vaccine going forwards.

The vaccine tested in the VAC054 trial (Chapter four) had previously been reported as demonstrating evidence of strain-specific efficacy in Malian children. The trial reported 64.3% efficacy (hazard ratio 0.36, 95% CI 0.08-0.86, *P*=0.03) in a pre-defined secondary analysis against clinical malaria with 3D7-type parasites (defined by eight immunologically important AMA1 polymorphisms in the cluster 1 loop of domain I), although the number of cases meeting this definition was small (97, 98) and protection did not extend into the second season of follow-up (99). This vaccine was therefore selected to be used in developing the CHMI model, as the AMA1 clone (3D7) used in the vaccine was the same as that in the CHMI inoculum. Although the trial did not demonstrate any vaccine efficacy, the reproducibility of the CHMI model was successfully demonstrated, and the potential improvement in measuring modest changes in PMR compared with sporozoite CHMI was also shown (209). The lack of FMP2.1/AS01 efficacy in this trial, in contrast to the study in Malian children, could be due to a number of possible reasons. Firstly, there may have been reduced immunogenicity of the vaccine in the VAC054 trial. ELISA and functional GIA analysis were performed at WRAIR (where the vaccine was developed) as well as in Oxford (209) and these showed the responses to be modestly, but significantly, lower than those reported in a trial of FMP2.1 administered with AS01 or AS02 in healthy US adults (95). Secondly, the use of AS01 instead of AS02 may have impacted the immunogenicity and efficacy. Thirdly, the vaccine may only have an impact at high parasite densities which would not be detected in a CHMI trial where participants are treated before high parasite densities are allowed to develop. Fourthly, the vaccine may have had a pre-erythrocytic effect which would not be detected with blood-stage CHMI. Finally, Malian children, unlike UK adults, would have possessed pre-existing anti-malarial immune responses, including anti-AMA1 IgG, which may have acted in conjunction with the vaccine-induced anti-AMA1 responses.

This was the largest blood-stage CHMI trial conducted to date and the model should accelerate proof-of-concept testing of new blood-stage malaria vaccines as the study can be powered to detect relatively low efficacy even with a small number of participants (fifteen vaccinees and

fifteen infectivity controls). This will enable much faster and more cost-effective testing of new candidate vaccines compared with the traditional field trial approach, and requires fewer participants than the sporozoite model in order to achieve the same power, which is an important ethical and logistical consideration.

It will be very important to the field to correlate any vaccine efficacy seen with future blood-stage candidate vaccines with levels of GIA. This has been shown to correlate in the *Aotus* monkey model (104, 226, 252) and the rhesus macaque monkey model using *P. knowlesi* parasites (253), but as no blood-stage *P. falciparum* vaccine has demonstrated efficacy with this model it is not definitively known whether the same can be said for a correlation in humans between GIA and vaccine efficacy.

6.3 Development of safety data collection and analysis for early-phase clinical trials

Over the course of the clinical trials described in this thesis, which commenced in May 2013 (VAC051) and were completed by December 2015 (VAC057) I made several changes to the way safety data were collected and analysed to improve accuracy and reduce bias. These changes included the use of an eCRF for live entry of data in clinic, which removed the risk of transcription errors and allowed data to be viewed in real time as there was no delay between a participant being seen and the data being available in the database. I also led the development of an eDiary system for volunteers to enter AE data into directly, rather than using paper diaries which then had to be transcribed into the OpenClinica database. This system was developed in conjunction with the other clinicians working across many different trials in the Jenner Institute by Sylwester Pawluk. This system enabled me to see when volunteers were completing the diary cards and meant I had access to AE data in real time, unlike with paper diary cards which were only reviewed when participants were seen in clinic. This meant that any severe AEs could be noted quickly and further review of volunteers could be planned if necessary, as well as ensuring that the safety stopping and holding rules were

adhered to. From anecdotal evidence, as a study team we were aware that volunteers sometimes completed paper diary cards on the day they were seen in clinic, which could be seven or fourteen days after vaccination. This meant the data were inaccurate as data was subject to substantial recall bias, as was the collection of AE data in clinic after the paper diary cards had been completed. By introducing a 28 day diary and only collecting SAE data after this (as was done in VAC057) I believe I have improved the quality and reliability of AE data collected. As safety of the vaccines is a primary endpoint for any Phase I trial, this is extremely important. As well as improving data accuracy, the eDiaries have substantially reduced the amount of time required for data entry and analysis as the data are already processed when the Excel spreadsheet extraction is done; unlike with OpenClinica data extracts which require further processing.

The analysis of laboratory AE data is another area in which I sought to improve accuracy and comparability of data. Having recognised that the different study sites in which trials were being carried out all had some variability in normal reference ranges for several of the parameters we were measuring, I sought to develop site-specific laboratory AE grading tables which took these into account. This was done in collaboration with other Jenner and study site clinicians and these tables are now used across all Jenner Institute trials. This has improved the quality of these safety data as it ensures that laboratory results are correctly defined as an AE where necessary, and are graded at an appropriate severity. I believe that for early-phase vaccine trials this is extremely important as any haematological or biochemical abnormalities need to be assessed and the relationship to vaccination determined. I also introduced day 0 safety bloods after the VAC051 trial as this is a more accurate baseline than using the bloods checked at screening, which could be up to 90 days before vaccination. This means the causality of any laboratory AEs noted after vaccination can be more accurately assigned.

Finally, the other change made to safety data analysis since I started this work is the assignment of causality. This was originally done in real time for all AEs by the local site

clinician (me) for VAC051, but I was concerned that this meant there was potential for significant bias in these data and that rare events may be incorrectly assigned as 'not related' or 'unlikely to be related', especially in multi-site trials. I therefore suggested to the Sponsor (University of Oxford) that the process be changed so that causality was assigned at interim analyses and at the end of the study by the lead clinician (me). I also changed the causality assignment procedure to state that any solicited AEs occurring within the first seven days should be considered at least possibly related to vaccination. Furthermore, causality is now peer-reviewed by a clinician from another group within the Jenner Institute and any discrepancies discussed by the lead clinician for the trial and the clinician who performed the peer review. This enables rare events to be assessed more accurately, especially if they occur at different trial sites, and reduces bias in the causality assignment.

There is a need for standardisation across early-phase vaccine trials, with consensus on severity grading so that accurate comparisons of vaccines in different populations and settings can be made. Some progress has been made in this area by the Brighton Collaboration Methods Working Group (254, 255) but there is no globally accepted standard for measuring vaccine reactions. The changes mentioned above have enabled standardisation across the trials conducted at the Jenner Institute, but global standards would be a further improvement.

6.4 Future work

6.4.1 Improving vaccine immunogenicity

As highlighted in the trials described in this thesis, improvements in vaccine design are needed to improve the immunogenicity, specificity and efficacy of blood-stage vaccines. There are several methods being evaluated with this goal in mind. These either work to improve the immunogenicity of the vaccine itself by improving antigen presentation, for example through the use of virus-like particles (VLPs) or molecular adjuvants (e.g. IMX313), improve immunogenicity through use of a more potent adjuvant, or design vaccines with specific

epitopes known to be involved in antigen binding. This section will discuss the progress being made in these areas for blood-stage vaccines in particular.

6.4.1.1 Viral vectored vaccines

Two of the trials I conducted used heterologous prime-boost with the ChAd63 adenoviral vector followed eight weeks later with an MVA boost. The advantages of these types of vaccines are that they can be made to GMP relatively easily and quickly, are extremely good at inducing T cell responses and do not require an adjuvant to be co-administered. As access to adjuvants has historically been limited, requiring collaboration with pharmaceutical companies for access to the most effective, viral vectors were an attractive means of developing vaccines. The use of the ChAd63/MVA platform meant that the world's first blood-stage *P. vivax* blood-stage vaccine reached clinical trial. Although the PvDBP antigen had been of interest as a vaccine candidate for many years GMP production of the protein had proved difficult (202) but use of viral vectors meant a vaccine suitable for human use was successfully produced (157). Viral vectors induce moderate antibody responses, and this is a disadvantage for antigens that require high antibody levels in order to be effective. Antibody responses are generally lower than those seen following protein-in-adjuvant vaccine formulations (167) and there are logistical implications for the required storage conditions (stored at -80^{o}C). Historically there have been concerns about difficulties in scaling up the production process to produce large amounts of vaccine but this has been addressed to some extent recently with the rapid manufacture of thousands of doses of the ChAd3 ZEBOV Ebola vaccine candidate in response to the 2014 – 2015 Ebola outbreak in West Africa (153). Recent work in Oxford has also looked at other ways of boosting the response to antigens encoded within viral vectors, including the use of a molecular adjuvant IMX313, which is currently being evaluated with the viral vectors in a Phase I trial (NCT02532049) of a novel transmission blocking vaccine (ChAd63/MVA Pfs25-IMX313) after preclinical studies showed improved immunogenicity compared with viral vectors encoding the Pfs25 protein alone. The IMX313 causes the Pfs25 antigen to heptamerise so that a nanoparticle is expressed from the viral vectors rather than a Pfs25

monomer. In preclinical studies, the heptamer induced a significantly higher percentage of germinal centre B cells in the draining lymph nodes compared with monomeric Pfs25, which was thought to account for the difference in antibody responses (119).

In 2013 GSK acquired Okairos, the company which owned the ChAd63 viral vector platform (256), so trials in Oxford since then have required collaboration with GSK to use this vector. This collaboration has led to the Jenner Institute having improved access to GSK adjuvants and vaccines (172, 209) for clinical trials, which has broadened the types of vaccines developed and tested here.

6.4.1.2 Recombinant protein vaccines

The VAC054 trial (Chapter four) used a recombinant protein vaccine given with the Adjuvant System AS01. This approach was chosen to try to achieve as high an anti-AMA1 antibody concentration as possible, as this is deemed necessary for protection. Recombinant protein vaccines are typically better at inducing an antibody response than a cell-mediated response, and are therefore an attractive option for blood-stage vaccine candidates. The main disadvantages of recombinant protein subunit vaccines are the difficulties in producing soluble, conformationally correct proteins in sufficient quantity to GMP standards (257) and access to effective adjuvants, which are essential for inducing a substantial immune response.

Significant improvements have been made in recombinant protein vaccine production in the past few years (258). New expression systems have been developed, for example, the S2 *Drosophila* insect-cell system (169, 259), which enables expression of proteins that have not been successfully expressed using bacterial or yeast systems. The use of fusion proteins, such as the IMX313 carrier protein, can improve immunogenicity of candidate vaccines when expressed as a protein nanoparticle (119). Long synthetic peptides enable multi-epitope vaccines which can include several B, CD4+ and CD8+ T lymphocyte epitopes capable of binding different MHC class I and II molecules in the same peptide(s), overcoming MHC restriction. They also allow rapid production of stable proteins (260). Protein complexes of two

antigens may also act synergistically to improve the immune response, for example, the AMA1-RON2 complex (229), compared with a single antigen. The vaccine candidate GMZ2 consists of a fusion protein of the blood-stage antigens Glutamate Rich Protein (GLURP) and MSP3. This has been assessed in Phase Ia, Ib and IIb trials, in adults and children, and demonstrated low level efficacy against malaria in an endemic setting (261-264). The use of VLPs which express antigens on their surface are another successful method for improving vaccine production and immunogenicity; these will be discussed further in the next section.

Although these techniques may improve the ease of production of recombinant proteins, it is also essential to focus on the quality of the antibody response to ensure that as high a percentage as possible of the antibodies induced are functional. Ways of improving antibody quality include structure-based immunogen design, where vaccines are designed based on how antibodies successfully inhibit the parasite. This potentially enables the identification of conserved epitopes which could be utilised in vaccine development (265). Other future prospects are the discovery of new antigens (ideally those which are not under significant immune pressure in endemic settings but required for invasion), the use of new antigen combinations, improved methods for vaccine production and delivery, and improvements in adjuvants which augment the immune response (258).

6.4.1.3 Virus-like particles

The only malaria vaccine to have reached Phase III trials, RTS,S, is based on a recombinant VLP of Hepatitis B surface antigen (HBsAg) displaying repeats from the *P. falciparum* CS protein (57). This enabled a significant improvement in vaccine efficacy compared with recombinant CS protein alone (266). VLPs have several advantages as vaccine platforms. Firstly, they are a similar size to pathogenic organisms, and are therefore more easily recognised and taken up by antigen-presenting cells at the vaccination site (267, 268). Secondly, they typically have a high density of repetitive epitopes on the particle surface, which are recognised by pattern recognition receptors, triggering the innate immune system, and subsequently the adaptive

immune system and leading to higher affinity antibodies. Thirdly, there may be a 'depot' effect with gradual release of the target antigen from the particle and, finally, the particle may allow immunostimulatory adjuvants (e.g. TLR agonists) to be delivered to the same APC as the antigen, ensuring a specific APC activation (268).

Few blood-stage vaccine candidates have been developed using VLP platforms to date. There was a suggestion of a protective effect with a virosome-based VLP conjugated with PfAMA1 and PfCS in children in a Phase Ib trial (269) although the AMA1-virosome and CS-virosome did not show any efficacy when assessed individually in a Phase IIa CHMI trial (268). The GLURP-MSP3 fusion protein has been assessed pre-clinically in a virosome-based vaccine, and induced similar antibody levels to the recombinant fusion protein (GMZ2) administered with alum or Montanide ISA 20 adjuvants (270). There is therefore potential to further develop these platforms to improve blood-stage vaccine production, delivery and immunogenicity, and thereby, efficacy.

6.4.1.4 Whole parasite blood-stage vaccines

The most advanced whole parasite vaccine to date is the pre-erythrocytic vaccine developed by Sanaria, PfSPZ, which involves injection of radiation-attenuated sporozoites intravenously, and has demonstrated high-level efficacy, particularly in malaria-naïve individuals (68). A pre-erythrocytic whole parasite vaccine needs to be 100% effective as even a single parasite transmitted by an infected mosquito after vaccination can cause malaria disease if it escapes through to the blood-stage. In contrast, a blood-stage whole parasite vaccine could potentially be beneficial even if only partially effective, mimicking natural infection and allowing asymptomatic infection with lower parasitaemias (271). A vaccine with these qualities would need to ensure that parasites were no able to grow above a disease 'threshold' 100% of the time in order to be safe for use.

The whole-parasite blood-stage vaccine approach was thought to be supported by a study conducted at QIMR, Brisbane, Australia in which four volunteers were exposed to very low

doses of red blood cells infected with malaria parasites (using the same inoculum as was used for CHMI in VAC054) and treated when malaria DNA was detectable in blood by PCR but before they became symptomatic. They received a total of three rounds of submicroscopic malaria infections for which they were treated before CHMI, following which three of the four were sterilely protected and one had a delay in parasitaemia and asymptomatic infection (272). This study was flawed however, as participants were treated with atovaquone-proguanil after each malaria infection, and this has subsequently been shown to inhibit parasite development for up to six weeks after treatment. This implies that the suppression of malaria in these individuals was likely due to residual atovaqone rather than development of immunity (273).

Techniques investigating this approach are ongoing, and involve assessment of regimes using killed blood-stage parasites, live radiation-attenuated parasites, chemically attenuated parasites or genetically attenuated parasites. There are various challenges to be overcome if this approach is to be used, however, including issues relating to the use of human blood products (e.g. transmission of other infections), possible underattenuation of parasites or reversion of parasites to wild-type, the need for a potent adjuvant if killed parasites are used and the logisitical challenges of culturing large quantities of *P. falciparum* and producing a vaccine to GMP (271).

6.4.1.5 P. vivax blood-stage vaccines

The ChAd63/MVA PvDBP vaccines (157) in the Phase Ia clinical trial (VAC051) described in Chapter three were the first blood-stage *P. vivax* vaccines ever to reach clinical trial. The results from this trial were encouraging with inhibition of PvDBP-DARC binding *in vitro*. The efficacy of this regime remains to be seen, however, and the *in vitro* results will need to be correlated with efficacy results when data are available. It is likely that a recombinant protein platform will induce higher antibody levels than the viral vectors, as discussed previously. Other techniques to improve vaccine immunogenicity include using a TLR-agonist in the

vaccine formulation, which improved the range of response against polymorphic variants (111) and designing a vaccine with fewer of the polymorphic variant epitopes to focus the immune response towards more conserved epitopes (DBPII-DEK^{null}). It is hoped that this might improve the breadth of response against PvDBP variants and avoid the development of strain-specific immunity (274).

Although the PvDBP antigen is considered the leading blood-stage vaccine candidate for *P. vivax*, it has now been established that the parasite is able to infect Duffy-negative individuals, albeit with a significantly reduced prevalence (187). A highly conserved duplication in the PvDBP gene has been identified in parasites in some of the Duffy-negative individuals infected with vivax malaria, implying a recent evolutionary change in the parasite (275). These findings suggest that other blood-stage antigens may also need to be targeted if a vaccine is going to be widely effective, and in order to reduce the risk of the parasite developing resistance to the vaccine through use of an alternative invasion pathway.

P. vivax MSP1 has been evaluated pre-clinically alone and in combination with PvDBP, but has not entered clinical testing. Similarly, PvAMA1 has been studied in both a viral vectored vaccine and a recombinant protein pre-clinically. The anti-PvAMA1 antibodies were functional against diverse *P. vivax* strains (276). The use of these antigens as vaccine candidates may be more effective in combination with, for example, PvDBP, but that will require further clinical development and testing to ascertain.

6.4.2 Assessing vaccine efficacy

Malaria vaccine efficacy has traditionally been assessed either in large field trials in endemic countries or in small CHMI studies in healthy volunteers, the majority of which have been conducted in non-endemic settings. CHMI trials are used for proof-of-concept early-phase testing and aid decisions in vaccine development but ultimately a malaria vaccine will have to be effective in an endemic setting if it is to be useful. Many studies have demonstrated that there are significant differences in the efficacy seen in these different populations. RTS,S, for

example, had an efficacy of between 50-75% in CHMI trials (73, 172) but only 18-28% in young infants and children in Africa (57). The differences in immune response following repeated malaria exposure is an area that would benefit from further research, and potentially incorporated into vaccine design. Recently a few CHMI trials have taken place in endemic populations (139, 277), with the aim of developing a model for early assessment of candidate vaccines in endemic settings. These studies also allow a deeper understanding of the mechanisms of infection and the immune response in those with previous exposure and potentially a degree of natural immunity.

Chapter four described the development of a CHMI model to assess blood-stage vaccine efficacy for *P. falciparum* with power to see a fairly small effect on parasite multiplication rate. The consistency of the model has provided further data for using this model with even greater confidence of being able to detect an effect in the future with other blood-stage vaccine candidates. Although the ChAd63/MVA RH5 vaccines discussed in Chapter five did not elicit an antibody response that was likely to lead to efficacy, a recombinant protein vaccine with this antigen has been developed in Oxford (169) and will shortly commence clinical testing. It is anticipated that the efficacy of this vaccine will be assessed with blood-stage CHMI. If this vaccine demonstrates a reduction in PMR compared with infectivity controls it will be the first blood-stage vaccine in humans to do so. The blood-stage CHMI model could also be used to further evaluate the response to malaria infection, especially if repeated infections were given, mimicking the repeated exposure that occurs naturally in endemic settings.

As discussed in Chapter three, CHMI models for *P. vivax* are being developed, with one pre-erythrocytic vaccine candidate having been assessed in a Phase IIa trial (77). The recently developed blood-stage inocula for vivax CHMI should allow for blood-stage vaccine candidates to be assessed relatively rapidly in proof-of-concept trials before larger, more expensive field trials. This should enable the development of vaccines to progress efficiently with only candidates which demonstrate efficacy being taken forward. Similarly, the use of *in vitro*

assays alongside efficacy data could help to inform decisions about vaccine development and the likelihood of a novel vaccine succeeding in preventing or reducing infection. It is planned to take forward the ChAd63/MVA PvDBP vaccines to a Phase IIa trial where efficacy can be assessed against heterologous blood-stage infection using an infected blood inoculum for CHMI.

6.4.3 Closing remarks

To conclude, this thesis has demonstrated the early-phase testing of three blood-stage malaria vaccine candidates, all of which were safe and immunogenic, but also highlighted some of the challenges in developing and assessing new vaccines. In recent years there have been substantial improvements in the development and production of candidate vaccines, and in the adjuvants available meaning that real progress has been made in this field. However, despite this, relatively few vaccines have reached efficacy testing, and none of the blood-stage vaccine candidates to date have demonstrated significant levels of efficacy. Nonetheless, new antigens are being discovered, and as described in this thesis, new vaccines have reached clinical trial. There is still much to be understood about the mechanisms used by *Plasmodium* parasites to invade cells and evade the immune system. Further research into these areas is likely to aid the design of improved vaccines.

The clinical trials I led were important milestones in the blood-stage malaria vaccine field. VAC051 (Chapter 3) was the first, and only blood-stage *P. vivax* vaccine to reach clinical trial to date. This trial demonstrated that antibodies induced by a vaccine against PvDBP are able to block binding between the antigen and its receptor, DARC. This vaccine regime needs to be further assessed in an efficacy trial, and a Phase IIa CHMI trial is planned to assess this in the future. The VAC054 trial (Chapter 4) was the largest blood-stage CHMI trial ever conducted and enabled the development of a proof-of-concept efficacy model which can be used to assess other blood-stage vaccine candidates in the future.

An effective malaria vaccine, either for *P. falciparum, P. vivax* or both parasites, is likely to require a multi-component approach. This may involve targeting different stages of the life cycle or targeting multiple antigens in the same stage. Once a blood-stage vaccine has been developed which demonstrates efficacy, this can then be assessed in combination with other antigens to look for evidence of synergy as has been suggested pre-clinically for some antigen combinations. Early proof-of-concept efficacy testing of candidate vaccines using CHMI models should allow more rapid development, especially if these trials are also conducted in previously exposed individuals in endemic settings and information from these studies is used to inform vaccine design.

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Appendices

Appendix 1: Participant information sheets (PIS)

VAC051 Participant information sheet v3.0

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PARTICIPANT INFORMATION SHEET: VAC051

A study to assess new *Plasmodium vivax* malaria vaccines; ChAd63 PvDBP and MVA PvDBP

A phase Ia clinical trial to assess the safety and immunogenicity of new Plasmodium vivax malaria vaccine candidates ChAd63 PvDBP alone and with MVA PvDBP

We would like to invite you to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully and discuss it with friends, relatives and your General Practitioner (GP) if you wish.

- Part 1 tells you the purpose of the study and what will happen to you if you take part.
- Part 2 tells you more information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

Malaria is caused by 5 different types of malaria parasite. The most common 2 types are *Plasmodium falciparum* and *Plasmodium vivax*. Although *Plasmodium falciparum* causes the most severe disease and deaths, *Plasmodium vivax* is geographically the most widespread, and accounts for up to 50% of malaria cases in South and South East Asia, and up to 81% in South America. There are estimated to be between 106 and 313 million cases of vivax malaria per year. Although vivax malaria does not cause as many deaths as falciparum malaria it does still cause significant levels of illness, and can cause severe illness and death in some cases. It is therefore a major problem for those who live in affected areas and for travellers. There is a great need for a safe, effective malaria vaccine. Researchers around the world, including members of Professor Hill's group at the University of Oxford, have been investigating malaria for over 15 years. Over the last 10 years, we have been conducting clinical studies of new malaria vaccines. So far these studies have been looking at vaccines for falciparum malaria, but if malaria infection is to be controlled worldwide, it is vital to develop a vaccine for vivax malaria as well.

The purpose of this study is to examine the safety and immune response to two new vivax malaria vaccines; **ChAd63 PvDBP** and **MVA PvDBP** administered either as **ChAd63 PvDBP** alone or in combination with **MVA PvDBP**. These vaccines are made from viruses which are inactivated so that they are unable to multiply within the body. The viruses contain genetic information (DNA) from the vivax malaria parasite. This genetic material is a protein named PvDBP, and relates to a part of the parasite which it needs to infect human blood cells. PvDBP has not been used to

vaccinate humans before but it is believed to be a very important target for vivax malaria. This study is therefore the first time these vaccines have been used in humans. The aim is to use these vaccines to help the body make an immune response against this part of the vivax malaria parasite, and therefore prevent the parasite from being able to infect blood cells.

The viruses used in these vaccines have been used before many times in clinical trials for malaria and other infections, and have been safe. We do not expect the side effect profiles of these vaccines to be significantly different from previous trials where these viruses have been used with different malaria proteins.

The **ChAd63 PvDBP** vaccine is based on a weakened version of an adenovirus (a common cold virus). The strain of adenovirus we use for this vaccine usually affects chimpanzees. The **MVA PvDBP** vaccine is based on the modified vaccinia virus Ankara (MVA), which is a safer form of the vaccine virus previously widely used for smallpox vaccination.

The purpose of this study is to assess the two vaccines at different doses and alone or in combination. This study will enable us to assess:

1. The safety of the vaccine schedules in healthy volunteers.
2. The response of the human immune system to different vaccine schedules.

We will do this by giving volunteers one or two vaccinations and doing blood tests to assess the response of the immune system to the vaccines. We hope to recruit 24 volunteers to be vaccinated.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason but you may be asked to return to the clinic for follow up for safety reasons.

What will happen if I decide to take part?

This study involves having one or two vaccinations and then being followed up with blood tests. Volunteers are enrolled in groups depending on which vaccine schedule they will receive. You will be able to choose which group you are enrolled in, although as groups fill up there will be less choice.

Length of research

If you decide to take part in this study, you will be involved in the trial for approximately 3 to 5 months depending on what group you are in.

Am I eligible to be involved in the trial?
In order to be involved in the study you **must**:

- Be a healthy adult aged between 18 and 50 years.
- Be able and willing (in the investigators' opinion) to comply with all study requirements.
- Allow the investigators to discuss your medical history with your GP.
- Practice continuous effective contraception for the duration of the study (women only).
- Refrain from blood donation during the course of the study and for 6 months after the end of your involvement in the study.

You cannot participate in this study if:

- You have had malaria before.
- You have travelled to a malaria endemic region in the six months preceding your involvement in the study or are intending to travel to a malaria endemic region during the study.

- You have participated in another research study in the 30 days preceding involvement in this study.
- You are planning to participate in another study at the same time as participating in this study.
- You have previously received an investigational malaria vaccine.
- You have had immunoglobulins and/or any blood products in the three months preceding your involvement this trial.
- You have problems with your immune system.
- You are pregnant, breast feeding or intend to become pregnant during the study.
- You have a history of a severe allergic reaction to a vaccination.
- You have an allergy to eggs or Kathon (a biocide added to body washes, conditioners, liquid soaps, shampoos and wipes as a preservative)
- You have a history of cancer.
- You have a history of a serious psychiatric condition that may affect participation in the study.
- You have any other serious chronic illnesses requiring hospital follow-up.
- You drink on average more than 42 units of alcohol a week (a pint of beer is two units, a small glass of wine 1 unit and a shot of spirits one unit).
- You have injected drugs at any time in the last 5 years.
- You have hepatitis B, hepatitis C or HIV infection.
- You have a history of autoimmune disease.

Mild conditions, such as childhood asthma, which are well-controlled would not automatically exclude you from participating. If you are unclear whether you are eligible to be involved in the study you can contact the study team who will be able to advise you.

CONSIDERATIONS BEFORE TAKING PART IN THIS STUDY

Screening Visit: This takes place up to 3 months before the study starts at the Centre for Clinical Vaccinology and Tropical Medicine in Oxford and lasts up to one and a half hours. The purpose of the screening visit is for you to discuss the trial with us and decide if you still wish to enter the study. If you decide to participate, you will be asked to sign a consent form. We then need to check that you are eligible to participate. You will be asked some medical questions and a doctor will examine you. Some blood tests will be taken to check your red and white cells, your liver and your kidney function. These tests need to be normal for you to be enrolled in the study. Your blood will also be tested for infection with hepatitis B, hepatitis C or HIV. These viruses are transmitted by infected blood or sexually and can affect the immune response to infection. If you test positive to any of these infections, we will inform you of the result and offer referral for medical review and treatment (with your permission).

All participants are asked for urine samples at screening to check for glucose (to exclude diabetes), protein and blood (which can indicate kidney disease). For women, a urine pregnancy test will also be performed. Pregnancy tests will also be repeated prior to each vaccination if you go on to take part in the study. To avoid repeated testing, if you are not enrolled into this study and apply to enter another study conducted by the Jenner Clinical Trials Group based at the Centre for Clinical Vaccinology & Tropical Medicine (CCVTM) the screening blood results may be used in that study, where appropriate.

Blood Donation: Under current UK regulations, volunteers will not be able to donate blood during the study or for 6 months after the end of the trial.

Private Medical Insurance: If you have private medical insurance you are advised to contact your insurance company before participating in this trial as involvement in the trial may affect the cover provided by private insurance.

Malaria Prophylaxis: You should note that the vaccines being tested in this trial are experimental. If you travel to a malaria endemic region in the future you should not assume that the vaccines you received in this study have given you any protection against malaria. Make sure you visit your GP or a travel clinic before travelling to a malaria endemic region and use prophylactic anti-malarial medications, bed nets and insect repellent during your trip as directed by your GP or the clinic.

Contraception: The vaccines being tested in this study are at an early stage of development and it is currently unknown as to whether they are safe in pregnancy. For this reason, it is important that all women use adequate contraception for the duration of the trial.

VACCINATIONS

What are the vaccines that are being tested?

We are testing two vaccines; ChAd63 PvDBP and MVA PvDBP These vaccines will be given into the muscle of your upper arm(s). Once these vaccinations have been given they cannot be undone, so it is important you are clear of the potential risks of the vaccines before you agree to be involved in the study.

1. ChAd63 PvDBP

ChAd63 PvDBP is based on a virus that infects chimpanzees called chimpanzee adenovirus 63 (ChAd63). We have genetically disabled the virus so that it is impossible for it to grow in humans, and added a gene encoding a protein from the vivax malaria parasite (the "PvDBP" part of the vaccine). We want to try and make the body develop an immune response to this malaria protein. This protein has not been given to humans before, but side effects from these types of vaccines are usually due to the viruses used rather than the proteins. We have given ChAd63 encoding other malaria proteins to over 250 volunteers, and it has been safe and well-tolerated. It can however, cause some short-lived side effects.

Expected Side Effects:

Volunteers receiving ChAd63 PvDBP in this trial may experience injection site pain. This is most likely to be mild, however there is a chance this could be moderate or severe in intensity. Volunteers may also experience redness, swelling, itching and warmth at the vaccine site, although these symptoms are likely to be mild if present. Generally volunteers report a transient 'flu-like illness within 24 hours of vaccination which resolves within 48 hours. This can include headache, muscle aches, joint aches, feverishness, tiredness and feeling generally unwell. The majority of general symptoms are likely to be mild but there is a possibility of moderate or severe headache or feeling unwell.

2. MVA PvDBP

MVA PvDBP is based on a virus called modified vaccinia virus Ankara (MVA) and contains the gene encoding the same malaria protein as ChAd63 PvDBP (PvDBP). We have found that giving the MVA vaccine after a ChAd63 vaccine produces the best immune response. This regimen is termed a 'prime-boost' regimen. MVA encoding malaria antigens has been given to over 950 individuals, including children, in sub-Saharan Africa with no serious side effects. An MVA 'boost' vaccine has been given to over 160 healthy UK adults following a 'prime' vaccination with ChAd63 encoding the same malaria protein. It appears safe and well tolerated but can cause short-lived side-effects.

Expected Side Effects:

Volunteers receiving MVA PvDBP in this trial may experience injection site pain. This is most likely to be mild, but there is a chance this could be moderate in intensity. Volunteers may also experience redness, swelling, itching and warmth at the vaccine site, although these symptoms are likely to be mild if present. Generally volunteers report a transient 'flu like' illness within 24 hours of vaccination which resolves within 48 hours. This can include headache, muscle ache, joint ache, feverishness, tiredness

and feeling generally unwell. The majority of general symptoms are likely to be mild but there is a possibility of moderate headache, tiredness, muscle aches or feeling unwell.

MVA tends to cause more reaction than ChAd63 vaccines so we have used a lower dose in this trial compared with previous studies. Part of this study will involve comparing a lose dose of MVA with a slightly higher dose to look at the effects on the immune response generated, and also compare the reaction to the vaccine.

It is important to remember these are vaccines in the early stage of development; therefore the amount of safety data available is limited. The malaria protein (PvDBP) has not previously been used in this type of vaccine in humans before. For this reason there is a chance you could experience a side effect that is more severe than that described above, or that has not been seen before with these vaccines. You are encouraged to consider taking over the counter medications such as paracetamol or ibuprofen if you experience symptoms post vaccination as this is likely to reduce the intensity of any symptoms you have.

All volunteers will be given one or two vaccinations. The number and doses of the vaccinations will vary between groups. The ChAd63 vaccine dose is measured in 'viral particles' (vp) and the MVA vaccine dose is measured in 'plaque forming units' (pfu). All of the virus doses to be used in this study have been used safely in previous trials with the same viruses encoding different malaria proteins.

The vaccination groups are summarised in the following table:

Group Number		Number of volunteers	Dose ChAd63 PvDBP Day 0	Dose MVA PvDBP Day 56
1		4	5 x 10^9 vp IM	--
2	**A**	4	5 x 10^{10} vp IM	**--**
	B	8	5 x 10^{10} vp IM	1 x 10^8 pfu IM
	C	8	5 x 10^{10} vp IM	2 x 10^8 pfu IM

Severe Reactions

With any vaccination there is a risk of rare serious adverse events, such as an allergic reaction, which may be related to the nervous system or the immune system. Severe allergic reactions to vaccines (anaphylaxis) are also rare but can be fatal. Doctors qualified in the management of anaphylaxis will be present at each vaccination. Reactions in the nervous system are also extremely rare following vaccination and can cause an illness called Guillain-Barré syndrome. Guillain-Barré syndrome is an illness in which people can develop severe weakness and can also be fatal. These adverse events have not previously been seen with the types of vaccines used in this study. If you experience unexpected events, or become in any way concerned you should contact one of the Investigators (who are available 24 hours a day) using the contact details at the end of Part 2.

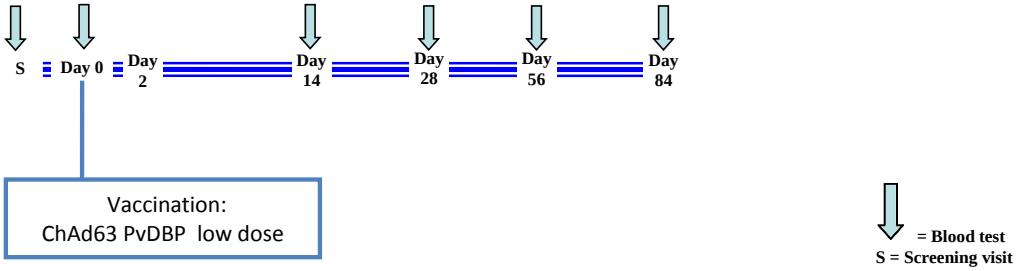
Vaccination Days
If you are the first volunteer to receive a new dose of either vaccine we will ask you to wait for 2 hours after the vaccination with that new dose of vaccine, to make sure there are no immediate problems. For all other volunteers in the groups, we will ask you to wait for 1 hour after each vaccination. You will be assessed again before leaving and given a diary card, thermometer and tape measure to take away. We will ask you to record your symptoms and the size of any redness or swelling every day for 7-14 days after each vaccination. Your diary card will be collected from you at your next visit.

We may ask to photograph your vaccination site and you can choose whether or not to agree to this when you sign the consent form. You will not be identifiable in these photographs, as only the vaccination site and your unique trial number will be visible. These photographs may be shown to other professional staff, used for educational purposes or included in a scientific publication.

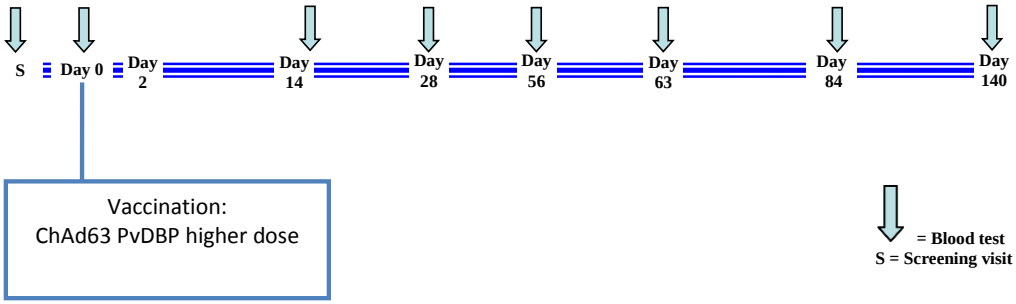
Number, timing and purpose of visits
You will receive either 1 or 2 vaccinations and attend between 7 and 10 visits in total. Visits may include a medical assessment, temperature, pulse and blood pressure readings, examination by a doctor if needed and blood tests. All visits will take place at the Centre for Clinical Vaccinology & Tropical Medicine (CCVTM), on the Churchill Hospital site in Oxford (OX3 7LE).

Timeline for vaccinations

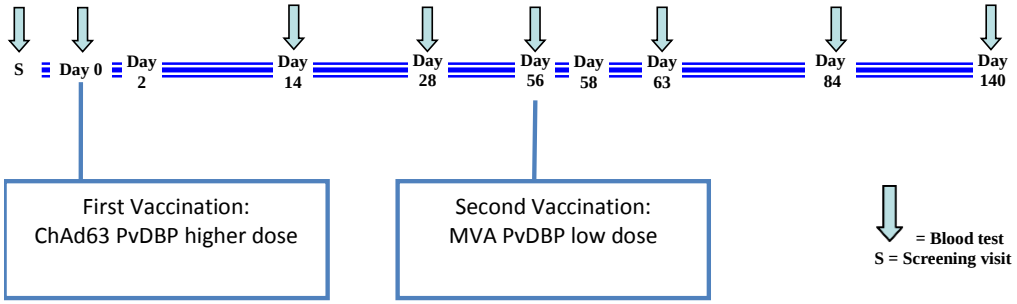
Group 1



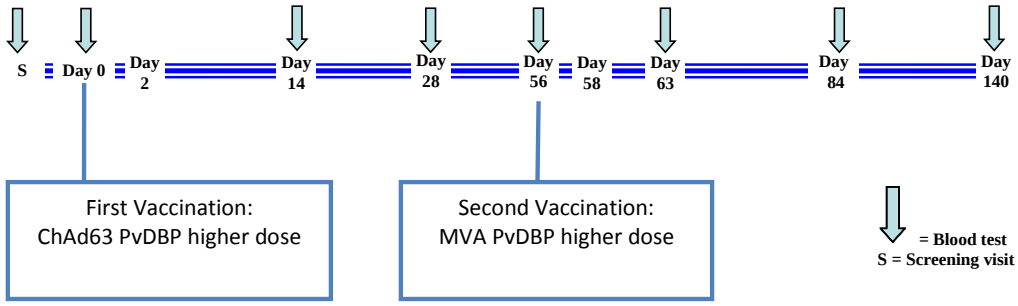
Group 2A



Group 2B



Group 2C



OTHER INFORMATION

Blood Tests

We take blood tests as part of the screening visit to help us to assess your general health. Blood tests are also taken at the study visits in order for us to assess your immune response to the vaccine and for safety reasons. If you would like them, we will give you the results of the blood tests. We only send the results to your GP if you wish us to and will not report them to anyone without your permission. The total volume of blood taken during the study varies according to which group you are enrolled in but will not exceed 500 mL over five months.

The following blood tests will be performed;

- Tests for Hepatitis B, Hepatitis C and HIV are done at the screening visit.
- HLA typing, a test of a component of the body's immune system may be done at the first vaccination visit.
- Tests of red and white blood cells and tests of liver and kidney function are done at the screening visit and most of the other visits when you come for vaccination or follow up after vaccination in order to check the vaccines are safe.
- Tests of the immune responses to vaccines are done at most of the visits.
- The volume of blood taken at each visit ranges from 5 to 75 mL.

The blood samples we collect will be stored after testing, and may be used in future malaria research. Samples will be anonymised. You can request that your samples are destroyed at any time. As part of our study we may send samples to collaborators in other countries, including countries outside Europe. Again, these samples would be anonymised. You will be asked to consent specifically for blood to be stored, and for samples and anonymised data to be shared with research collaborators.

Discomfort related to blood tests

Drawing blood may cause slight pain and occasionally bruising at the site where blood tests are taken. Sometimes, people feel light-headed or even faint. The total volume of blood taken during the study (334-474mL) should not cause any problems

for healthy people, and is about the same amount that may be given during one donation to the National Blood Transfusion Service (typically 470mL).

Urine Tests

- A urine sample will be tested at screening in order to check for glucose (to exclude diabetes), protein and blood (which can indicate kidney disease).
- All women will have urinary pregnancy testing at screening and before each vaccination.

Abnormal Results

If abnormal results or undiagnosed conditions are found in the course of the study these will be discussed with you and, if you agree, your GP will be informed. For example, a new diagnosis of high blood pressure might be made. Any newly diagnosed conditions will be looked after by your GP within the NHS.

Expenses and Payments

You will be compensated for:

- Travel expenses: £10 per visit
- Time required for visit: £20 per hour
- Inconvenience of blood tests: £10 per blood donation

Group No	Time in Trial (approx.)	No. of Visits	No. of Blood Tests	Maximum Volume of Blood Taken
1	3 months	7	6	334mls
2A	5 months	9	8	464mls
2B	5 months	10	8	474mls
2C	5 months	10	8	474mls

The total compensation for taking part in the study, if all follow-up is completed, will be between £330- £520. If you choose to leave the study early or are withdrawn from the study, you will be compensated according to the length of your participation based on the figures above. You should note that compensation payments received in this trial may have an impact on your entitlement to benefits.

What do I have to do?

- You **must** provide a name and 24 hour phone number for someone who lives near to you and who will know where you are for the duration of the study.
- You **must** attend all the visits that are outlined above
- You should record in the study diary all the things you notice about injection sites, any other change in your health or the way you feel after each injection.
- Women **must** use effective contraception for the duration of the study.
- Women will be asked to provide urine for pregnancy testing when required. If you become pregnant during the study, you **must** inform us **immediately**.
- You **must not** donate blood during the study or for 6 months after the end of the trial (current National Blood Service guidelines).
- You **must not** enrol in another clinical trial for the duration of this study.

What alternatives are present?

At present, there is no malaria vaccine licensed anywhere in the world. There are other malaria vaccines in various stages of development, but very few targeting vivax malaria. This study may help develop an effective vivax malaria vaccine.

What are the possible benefits of taking part?

This study will not benefit you, but the information gained from the study might help to prevent vivax malaria infection and disease in those who live in areas where malaria is common and in travellers.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

What happens when the research study stops?
If you have any queries or concerns once the study is over please do not hesitate to get in touch with us.

Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the vaccine that is being studied, for example if unexpected side effects occur. If this happens, we will tell you about it and discuss whether you want to or should continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. On receiving new information, we may consider it to be in your best interests to withdraw you from the study.

What will happen if I don't want to carry on with the study?
If, at any time after agreeing to participate you change your mind about being involved with this study, you are free to withdraw without giving a reason**.** Your compensation would be paid as a proportion of the total compensation according to the length of your participation. Unless you state otherwise any blood taken whilst you have been in the study will continue to be stored and used for research as detailed above. You are free to request that your blood samples are destroyed at any time during or after the study.

What if there is a problem?

Complaints:
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you wish to complain formally about any aspect of the way you have been approached or treated during the course of this study you should approach the University's Clinical Trials and Research Governance office (Clinical Trials and Research Governance, Joint Research Office, University of Oxford, Block 60, Churchill Hospital, Old road, Oxford, OX3 7LE. Telephone: 01865 572224, or the head of CTRG- email: ctrg@admin.ox.ac.uk).

Harm:
The investigators recognise the important contribution that volunteers make to medical research, and will make every effort to ensure your safety and well-being. In the unlikely event of harm during the research, the University has arrangements in place to provide for harm arising from participation in the study for which the University is the Research Sponsor. In the unlikely event of harm being suffered, while the University will cooperate with any claim, you may wish to seek independent legal advice to ensure that you are properly represented in pursuing any complaint. At any time during the study you will be entirely free to change your mind about taking part, and to withdraw from the study. This will not affect your subsequent medical care in any way.

Will my taking part in this study be kept confidential?
All information that is collected about you during the course of the research will be coded with a study number and kept strictly confidential. The information is available to the study team, the safety monitors, the ethical review committee, the sponsors, government regulatory agencies and external monitors who can ask to audit or monitor the study. Any information about you that leaves the hospital or clinic will have your name and address removed so that you cannot be identified from it. Your information is stored on a secure server and any paper notes will be kept in a locked filing cabinet.

Involvement of the General Practitioner/Family doctor (GP)
In order to enrol into this study, you will be required to sign a form, documenting that you consent for us to contact your GP. This is to inform them that you are interested in being involved in the study and to ensure there are no medical reasons that they are aware of why this would not be safe. The researchers will not enrol you in the trial if they have any concerns about your eligibility or safety; therefore we need to have communication from the GP confirming this before you can be enrolled. We will write to your GP to let them know whether or not you are finally enrolled in the study so they can update your medical records accordingly.

Prevention of 'Over Volunteering'
Volunteers participating in this study must not be concurrently involved in another study. In order to check this, you will be asked to provide your National Insurance or Passport number (if you are not entitled to a NI number). This will be entered on to a national database which helps prevent volunteers from taking part in too many clinical trials. More information can be found at *www.tops.org.uk*. Your national insurance or passport number is also required to allow processing of compensation payments.

What will happen to any samples I give?
If you consent, some of your leftover blood samples will be stored and may be used for further studies of the human body's immune response to malaria and vaccination. Any such tests will have an appropriate ethical review. Tests that may be performed include measurements of antibody levels, white blood cell activity and the ability of blood to inhibit the growth of malaria parasites in the laboratory. Samples may also be used to assess what genes are expressed by cells following vaccination. Upon your request at any time, your remaining blood samples will be destroyed. Your participation in this study will not be affected by your decision to allow or not allow storage and future use of your leftover blood samples. The blood tests mentioned in part 1 will be analysed in the hospital laboratory, and Oxford University research laboratories. Other blood tests to look at the response of your body to the vaccine may be done with collaborating laboratories in other countries. Any samples or data sent to them would be anonymous.

Will any genetic tests be done?
Yes. Some blood may be used to look at the pattern of your genes that can affect the immune system (including the Human Leukocyte Antigen or HLA genes). The immune response to vaccines is in part genetically controlled, so knowing your pattern of genes that regulate immune responses (such as HLA type) may help us to understand the responses to vaccination. We will also look at the expression of certain genes which relate specifically to the immune response to the vaccines and may also perform DNA sequencing on your blood, so we can understand how people respond to the vaccines – you can opt out of DNA testing if you wish, without any effect on your participation in the trial.

What will happen to the results of the research study?

The results of this research study may be published in a scientific medical journal. This may not happen until 1 or 2 years after the study is completed. If you contact the researchers in the future you can obtain a copy of the results. You will not be identified in any report or publication.

Data from this study may be used as part of a student post-graduate degree, for example a MD.

Who is organising and funding the research?
The study is funded by Okairòs Srl (a clinical-stage biopharmaceutical company), the Wellcome Trust, the National Institute of Health Research Biomedical Research Centre and the Medical Research Council.

Neither your GP, nor the researchers are paid for recruiting you into this study.

The study is organised by a research team at The Jenner Institute at the University of Oxford, headed by Dr Simon Draper.

Who has reviewed the study?
This study has been reviewed by Oxford Research Ethics Committee and has been given a favourable ethical opinion. The Medicines and Healthcare products Regulatory Agency (MHRA) which regulates the use of all medicines in the UK has reviewed the study design and has granted permission to use these unlicensed vaccines in this clinical study.

Thank you for reading this information sheet. If you are interested in being involved in the study please contact the study team at the Centre of Clinical Vaccinology and Tropical Medicine (details below) to arrange a screening appointment.

Contact details for further information:
E-mail:vaccinetrials@well.ox.ac.uk

Telephone: 01865 857401

Centre of Clinical Vaccinology and Tropical Medicine,
Churchill Hospital, Old Road, Headington, Oxford, OX3 7LE

Out of hours mobile number for emergencies: 07917882967

Professor Adrian V.S. Hill (DM FRCP)

E-mail: vaccinetrials@ndm.ox.ac.uk
Tel: +44 1865 857406 (volunteer co-ordinator)

NRES Committee South Central - Oxford A
Reference number: 13/SC/0596

PARTICIPANT INFORMATION SHEET: VAC054

A study to assess the safety and effectiveness of an experimental malaria vaccine by infecting vaccinated volunteers with malaria parasites using malaria-infected red blood cells

A Phase I/IIa Study of the Safety, Immunogenicity and Efficacy of FMP2.1/AS01B, an Asexual Blood-Stage Vaccine for Plasmodium falciparum Malaria

We would like to invite you to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully and discuss it with friends, relatives and your General Practitioner (GP) if you wish.

- Part 1 tells you the purpose of the study and what will happen to you if you take part.
- Part 2 tells you more information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?
Malaria remains a major public health problem accounting for an estimated 219 million cases of malaria and 660,000 deaths worldwide in 2010. It is a major problem for those who live in affected areas and for travellers. There is a great need for a safe, effective malaria vaccine as the range of effective medicines for treating malaria is limited and resistance to commonly used medicines is increasing. Researchers around the world, including the University of Oxford, have been investigating malaria vaccines for many years. Unfortunately there is currently no licensed malaria vaccine available.

The purpose of this study is to evaluate an experimental malaria vaccine for its ability to prevent malaria infection or disease in a blood-stage challenge model (when volunteers are infected with malaria parasites using malaria-infected red blood cells). The vaccine we are testing is a protein called FMP2.1, which is given with an adjuvant (a substance to improve the body's response to a vaccination) called AS01B. The protein has been given to over 340 people, including over 200 children with no vaccine-related serious adverse events. The AS01B adjuvant has been given to several hundred adults, in combination with other proteins, with no major concerns, such as illness. The FMP2.1/AS01B combination has been given to 20 adults without any serious adverse events.

The aim is to use this protein and adjuvant to help the body make an immune response against parts of the malaria parasite. This study will enable us to assess:

1. The ability of the vaccine to prevent malaria infection.

2. The safety of the vaccine in healthy participants.
3. The response of the human immune system to the vaccine.

We will do this by giving participants three vaccinations and then exposing them to malaria infection by transfusing a small number of red blood cells infected with malaria under carefully regulated conditions. We will follow participants closely to observe if and when they develop malaria. If the vaccine provides some protection against malaria, participants will take longer to develop malaria than usual or will not develop malaria at all. We hope to recruit 15 participants to be vaccinated and then challenged with malaria.

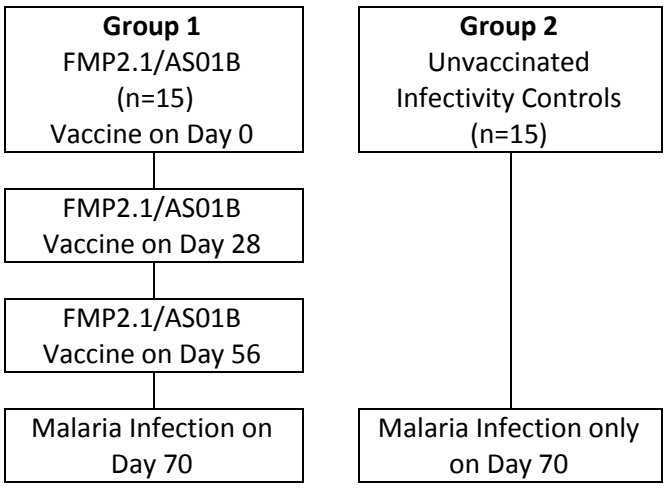
We will also recruit 15 individuals to be control subjects – these participants won't receive any vaccinations but will be challenged with malaria in the same way. It is extremely important to have control participants, as by developing malaria infection, they help us prove our method of giving participants malaria is effective. Otherwise we may think our vaccines have worked when actually the malaria parasites in the red blood cells weren't infective.

Do I have to take part?
No. It is up to you to decide whether or not to take part. Your decision will not result in any penalty, or loss of benefits to which you are otherwise entitled. If you do decide to take part, you will be asked to complete a questionnaire assessing your understanding of the study in order for us to be confident that you fully understand what taking part will involve. You need to answer all questions correctly in order to take part in the study. If you don't answer all the questions correctly the first time, you will be able to complete the questionnaire again after discussion with the Investigator. You will then be asked to sign a consent form. You are free to withdraw at any time without giving a reason, but you may be asked to return to the clinic for follow up for safety reasons.

The University of Oxford does not urge, influence, or encourage any employees/students of the institution to take part in this research study. Your decision to not participate in the study, or a decision on your part to withdraw from the study, will have no effect whatsoever on your employment/student status at the University.

What will happen if I decide to take part?
You will either;

- Receive three vaccinations according to the schedule below and then undergo challenge infection with malaria (**Group 1**)
  or
- Receive no vaccines and undergo challenge with malaria (**Group 2**)



You can choose whether you would like to receive the malaria vaccines or not, providing there is space available in the group.

Length of research
Group 1 participants will participate in the study for approximately 8 months from the time they have the first vaccination. Group 2 participants will take part in the study for approximately 3 months from the time they are enrolled (1 day before challenge).

Am I eligible to be involved in the trial?
In order to be involved in the study you must be:

- A healthy adult aged between 18 and 45 years.
- Able and willing (in the Investigators' opinion) to comply with all study requirements.
- Willing to allow the Investigators to discuss your medical history with your GP (General Practitioner).
- Willing to refrain from future blood donation in the UK.

You cannot participate in this study if:

- You have had malaria before.
- You have travelled to a malaria endemic region in the last 6 months or are intending to travel to a malaria endemic region during the study period.
- You have used antibiotics which could treat malaria (e.g. doxycycline) in the 30 days prior to malaria challenge.
- You have previously received an investigational malaria vaccine.
- You have sickle cell anaemia, sickle cell trait, thalassameia trait or thalassemia or any other haematological condition that might affect susceptibility to malaria infection.
- You have had immunoglobulins and/or any blood products in the three months preceding your involvement in this trial.
- You have problems with your immune system.
- You have an abnormal heart rhythm.
- You have a family history of congential QT prolongation or sudden death.
- Close family members have developed heart disease when aged less than 50 years.
- You are pregnant, breast feeding or intend to become pregnant during the study.
- You have a history of allergic disease or reactions likely to be exacerbated by any component of the vaccine, by malaria infection or by the medications used to treat malaria infection.
- You have had an anaphylaxis after vaccination.
- You have a history of cancer.
- You have a history of a serious psychiatric condition that may affect participation in the study.
- You have any other serious long-term illnesses requiring hospital follow-up.
- You drink on average more than 42 units of alcohol a week (a pint of beer is two units, a small glass of wine 1 unit and a shot of spirits one unit).
- You have injected drugs at any time in the last 5 years.
- You have hepatitis B, hepatitis C or HIV infection.
- You are unable to stay in Oxford from the day before challenge to up to 3 weeks following the malaria challenge.

Mild conditions, such as childhood asthma which is well controlled would not automatically exclude you from participating. If you are unclear whether you are eligible to be involved in the study you can contact the study team (details at the end of this information sheet) who will be able to advise you.

CONSIDERATIONS BEFORE TAKING PART IN THIS STUDY

Screening Visit: This takes place up to 3 months before the study starts and lasts up to two and a half hours. This visit will take place in your local trial site (in Oxford, London or Southampton). The purpose of the screening visit is for you to discuss the trial with us and decide if you still wish to enter the study. If you decide to participate, you will be asked to complete a questionnaire to assess your understanding of the study and to sign a consent form.

We then need to check that you are eligible to participate. You will be asked some medical questions and a doctor will examine you. Some **blood tests** will be taken to check your **red and white cells**, your **liver** and your **kidney function**. These tests need to be normal for you to be enrolled in the study. Your blood will also be tested for infection with **hepatitis B, hepatitis C** or **HIV**. These viruses are transmitted by infected blood or sexually and can affect the immune response to infection and vaccines. If you test positive to any of these infections, we will inform you of the result and offer referral for medical review and treatment with your permission. Positive results may be reported to the appropriate health authority. Your blood will also be tested for **cholesterol** to calculate your cardiovascular risk over the next 10 years. If your cardiovascular risk is > 5% you cannot participate in the trial. An electrocardiogram (ECG; to check the rhythm of the heart) will be done at your screening visit, to ensure that there are no rhythm problems with the heart.

All participants are asked for **urine samples** at screening to check for **glucose** (to exclude diabetes), **protein** and **blood** (which can indicate kidney disease). For women, a **urine pregnancy test** will also be performed. To avoid repeated testing, if you are not enrolled into this study and apply to enter another study conducted by the Jenner Clinical Trials Group based at the Centre for Clinical Vaccinology & Tropical Medicine (CCVTM) the screening blood results may be used in that study, where appropriate.

Blood Donation: Under current UK regulations, **you would not be permitted to donate blood** after taking part in this trial. This is because the malaria challenge involves the injection of red blood cells from another person, which is classified as a small blood transfusion.

Medications: You should not take any drugs other than vitamin pills, contraceptive pills or those medications assessed as appropriately safe during a malaria challenge by the doctor at screening. This also applies for drugs bought over the counter. Of course, your health and well-being is much more important than the conduct of this study and if at any time you need any medication then you should take it. However, it is very important that you let us know **before** you start on any treatment. For example, any antibiotics that you take within 4 weeks of the planned challenge day may affect the malaria parasite. If you ask the prescribing doctor to discuss with a study doctor (contact details at the end of this information sheet) before you start treatment then he or she may be able to advise on an appropriate antibiotic that will treat you but won't interfere with the study. If during the study any other treatment, especially with antifungal, antidepressant, antipsychotic or anti-arrhythmic drugs becomes necessary, it is important that you inform us immediately, since many of these drugs might interfere with the treatment against malaria you would receive.

Pregnancy and Contraception: The potential effect of the vaccine used in this study on a foetus is unknown. Also, malaria infection can be particularly dangerous during pregnancy to both the mother and the foetus. Women are therefore asked to use an effective method of contraception for the whole study period to avoid pregnancy during the study. Volunteers will take the antimalarial medication, Riamet, for three days, to treat malaria infection. As Riamet may temporarily reduce the effectiveness of hormonal contraceptives, women taking hormonal contraceptives will need to use an additional form of contraception while taking

Riamet and until the start of the next menstruation after Riamet treatment. A urinary pregnancy test will be carried out at screening, just prior to each study vaccination (as applicable), before malaria challenge and again before anti-malarial treatment is started.

If you are a woman using a hormonal contraceptive, you will need to use an alternative method of contraception while you are taking the medication for malaria, and until the start of the next menstrual period.

Private Insurance: If you have private medical or travel insurance you are advised to contact your insurance company before participating in this trial, because involvement in this study may affect the cover provided by private insurance.

VACCINATIONS

What is the vaccine being tested?
FMP2.1/AS01B is a 'protein in adjuvant' vaccination, using the protein **FMP2.1** and the adjuvant **AS01B**. FMP2.1 is a part of a malaria protein known as AMA1 which is expressed by the malaria parasite during infection. AS01B is an adjuvant – a substance that helps improve the body's immune response to a vaccine.

FMP2.1 has been given to over 340 people, including over 200 children, with no serious safety concerns. It has been given with a different adjuvant (AS02A) in the majority of cases, but has been given with AS01B in a previous trial to 20 healthy adults with no serious safety concerns. AS01B used with other antigens has been used in hundreds of adults with no emerging specific safety concerns. The same adjuvant at a paediatric dose, AS01E, has been used in thousands of children in the only malaria vaccine to reach Phase III clinical trials, RTS,S.

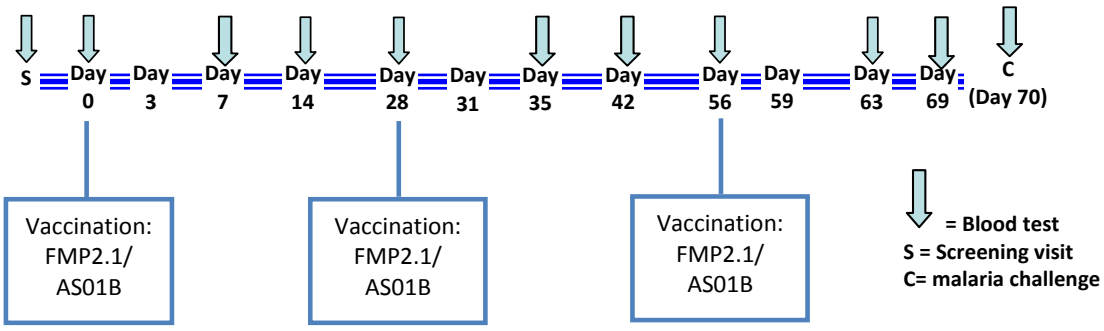
Vaccinations will be given into the muscle of the upper arm. Control volunteers (Group 2) will receive no vaccinations.

Vaccination Visits: Group 1
Vaccination visits will take place at your local trial site (in Oxford at the CCVTM, Churchill Hospital; in Southampton at the NIHR WTCRF; or in London at the Hammersmith Hospital). We will ask you to wait for 60 minutes after each vaccination to check there are no immediate problems. You will be given a diary card, thermometer and tape measure to take away. We will ask you to record your symptoms and the size of any redness or swelling at the injection site, every day for 7 days after each vaccination on the diary card.

We may ask to photograph your vaccination site(s) and you can choose whether or not to agree to this when you sign the consent form. You will not be identifiable in these photographs, as only the vaccination site and your unique trial number will be visible. These photographs may be shown to other professional staff, used for educational purposes or included in a scientific publication.

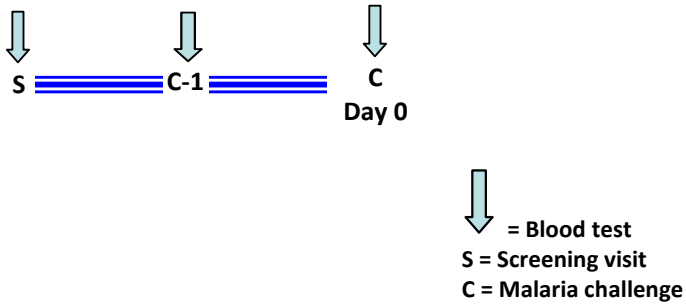
Number, timing and purpose of follow-up visits post vaccination:
The diagram below shows the timing of post vaccination follow-up visits. Visits include a medical assessment, temperature, pulse and blood pressure readings, examination by a doctor if needed and blood tests. Vaccinations and visits after vaccination will take place at your local clinic site (i.e. Southampton, London or Oxford).

Group 1 (vaccinees)



Group 1 visits up to malaria challenge. Screening visits (S), vaccination visits and pre-challenge follow-up visits, will take place at your local trial site; whereas the C-1 (the day before challenge) and challenge (Day 70) visits will take place at the CCVTM in Oxford.

Group 2 (controls)



Group 2 visits up to malaria challenge. Screening visits (S) will take place at your local trial site; whereas the C-1 (the day before challenge) and challenge (day 0) visits will take place at the CCVTM in Oxford.

THE MALARIA CHALLENGE

What happens during the challenge?

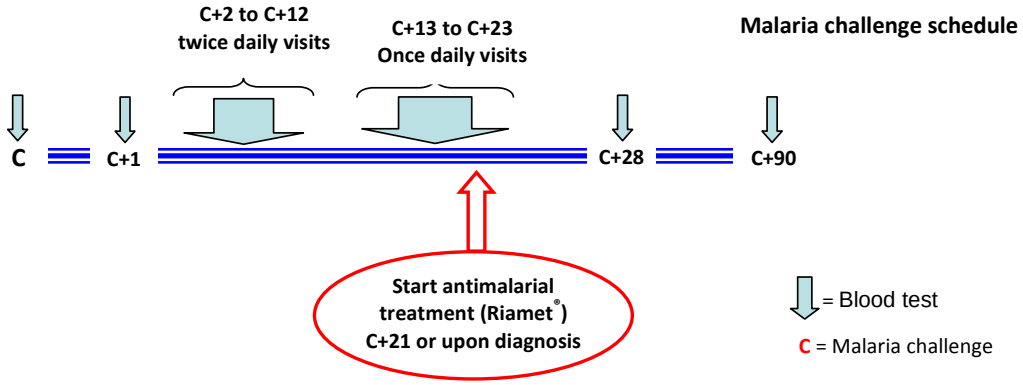
The best way of assessing how well new malaria vaccines work is to test whether they protect against malaria – as we plan to do in this trial. The malaria challenge is 70 days after the vaccination group (Group 1) receive their first vaccination, and the day after the C-1 visit for control volunteers (Group 2), and **will take place at the CCVTM in Oxford for all trial participants** (i.e. including those recruited in Southampton and London). All volunteers will need to attend the CCVTM the day before challenge (C-1) where blood tests will be taken and female volunteers will have a urinary pregnancy test. On the day of challenge an intravenous cannula ('drip') will be inserted into a vein in your arm. After this, a small amount (5 ml or 1 teaspoonful) of a solution containing red blood cells which are infected with malaria parasites will be injected into the vein. You will need to stay in CCVTM for 1 hour after being given the injection, in case you have an immediate reaction.

What happens at follow up after the Malaria Challenge?

The malaria challenge follow up visits are very important for your safety. We need to assess you once the day after the malaria challenge, (C+1, or day 71) then twice daily until C+12 (inclusive) and once daily until C+23 or until you have completed antimalarial treatment if you develop malaria (see diagram below). **All these clinic visits will take place at the Centre for Clinical Vaccinology & Tropical Medicine (CCVTM) at the Churchill Hospital in Oxford. It is**

essential that you reside in Oxford during this time for careful monitoring and regular review by the study team. If you do not normally live in or close to Oxford and if you joined the study at the Southampton or London sites, accommodation will be provided for this time as well as transportation to and from Oxford. You will be required to stay in Oxford until you have had two negative malaria films after starting antimalarial treatment, or completing 2 days of antimalarial treatment at day 21 if you do not develop malaria. We will give you a medication diary card on which you will be asked to record all medications that you take.

Each time we see you, we will assess your symptoms and a doctor will examine you, if necessary. A small amount of blood will be taken and examined under the microscope for malaria parasites. This is called a thick film and it is the standard test for diagnosing malaria infection. Your blood will also be tested for malaria parasite genetic material (DNA) using a technique called polymerase chain reaction (PCR). These visits will last approximately 10 minutes, although you may have to wait to be seen. The total number of visits post challenge will vary depending on when and if you get malaria. It is important you are able to attend all the visits. **If you plan to travel outside of Oxford at any time from the day before challenge to 21 days following the challenge, then you should discuss your plans with one of the study physicians before participating in this study.**



If you are diagnosed with malaria you will be immediately started on a course of anti-malarial tablets. Usually the blood test result is available after you have already left clinic, although if you wish to wait for the result after each test you are welcome to do so. If you have left and your blood test is positive for malaria we will contact you by telephone and ask you to return to the CCVTM as soon as possible to start treatment. It is therefore essential that we are able to contact you at all times on your telephone and that you are available to return to the CCVTM to start treatment at short notice any time between day 2 – 21 post challenge. The drug you will be treated with is called Riamet®. It is a licensed drug in the UK for the treatment of malaria caused by *Plasmodium falciparum*, the type of malaria you will be infected with. Each Riamet® tablet is a combination of 20mg artemether and 120mg lumefantrin. Each dose is 4 tablets and you will need 2 doses each day for 3 days. We will give you the first dose and ask you to take the second dose 8 hours later. The next day and the day after that, you will need to take 2 doses, 12 hours apart. We will watch you take one dose each day. Tablets should be taken with a meal.

When you start treatment, you may not feel better straight away, but most people start to feel better after about 24 hours. After starting treatment, you will need a blood test once every day until two blood tests in a row have been negative for malaria parasites.

If you are feeling unwell and your symptoms are like malaria, but no malaria parasites are seen in your blood, we may not treat you straight away. If you are feeling ill for one or two days, we may decide to start treatment even if no parasites are seen. If you have still not developed

malaria after 21 days, you will be given the malaria treatment regardless of whether or not we have seen malaria parasites in your blood.

If there any problems with Riamet® for any reason, we have different anti-malarial treatments available which are called Malarone or chloroquine.

Our experience tells us that the malaria parasites should disappear from your blood within 2 or 3 days of starting the treatment. If you do develop malaria then you will be seen each day during the treatment until there are no malaria parasites in your blood on two consecutive days. We will give you a diary card when you finish treatment on which to note when any on-going symptoms of malaria stop.

If you become unwell with malaria then you may be admitted to the John Warin ward (the Infectious Diseases and Tropical Medicine Unit at the Churchill Hospital in Oxford) as a precaution until you have recovered, but it is very unlikely that this will be necessary.
If the vaccines do not work or you are in the control group (group 2) you are most likely to develop malaria between day 7 and 9 following challenge. If the vaccines protect you against malaria you may develop malaria later than day 9 or not at all. If we do not find malaria parasites in your blood by day 21 post challenge then we will presume the vaccines have protected you against malaria and give you a course of antimalarial treatment anyway so that any parasites that we have not detected are killed.

Days 28 and 90 after malaria challenge
You will be seen in clinic on day 28 and day 90 post challenge. At these visits a blood sample will be taken. The appointments will last about 10 minutes and will take place at your local clinic site (i.e. London, Southampton or Oxford).

WHAT ARE THE RISKS OF TAKING PART IN THE STUDY?

The potential risks in the study can be divided into seven categories;

1. **Blood Tests**

The total volume of blood taken during the study depends on the group. The amount taken at each visit will vary between around 3mL (less than a teaspoon) to around 92 mL (about 6 tablespoons). The volume of blood being taken over the course of the trial should not cause any problems in healthy people. There may be some temporary mild discomfort, such as bruising and tenderness at the site where the blood tests are taken from. You may experience faintness as a result of collecting blood. We will give you a copy of your blood tests if you request them, will only send the results to your GP if you wish us to and will not report them to anyone without your permission.

If abnormal results or undiagnosed conditions are found in the course of the study these will be discussed with you and, if you agree, your GP will be informed. For example, a new diagnosis of anaemia might be made. Any newly diagnosed conditions will be looked after by your GP within the NHS.

Once malaria has been diagnosed and treated, with 2 consecutive negative thick blood films confirmed after treatment, the twice daily / daily blood tests after challenge will no longer be required.

Blood tests that will be taken during visits for Participants in <u>GROUP 1</u>:

	Screening	Vaccination Visits	Vaccination follow ups	Days Post - Challenge follow up Visits
Tests of Red and White Blood Cells*	X	X	X	Within 24 hrs of diagnosis & at Days 6, 28 & 90 post challenge
Test of Liver and Kidney Function*	X	X	X	Within 24 hrs of diagnosis & at Days 6, 28 & 90 post challenge
Hepatitis B, Hepatitis C & HIV infection	X			
EBV and CMV[$]			X	Day 90 post challenge
Cholesterol	X			
HLA typing		X[£]		
Tests of Immune Response to Vaccines		X	X	Day of diagnosis and days 28 & 90 post-challenge
Tests for malaria #				Days 1-23 post challenge

***We will test your red and white blood cells and liver and kidney function at various points throughout the study to check you remain well and whether the vaccines are safe.*

[$] EBV and CMV are viruses that cause glandular fever. We will check to see whether you have been exposed to these before. Whether you have or not will not affect your ability to take part in the trial.This will be checked once prior to malaria challenge (at C-1 visit) and once later in the trial.

***#** Tests for malaria use only small volumes of blood (3mL). We perform one prior to challenge to ensure we get accurate readings during the follow-up post challenge. These tests will be performed until you have had 2 negative blood films following starting antimalarial treatment or until day 23 post-challenge if you do not develop malaria.*

[£] This will only be performed once

Blood tests that will be taken during visits for Participants in <u>GROUP 2</u>:

	Screening Visit	Day before Malaria Challenge	Follow ups Post Challenge
Tests of Red & White Blood Cells*	X	X	Within 24 hrs of diagnosis & at Days 6, 28 & 90 post Challenge
Tests of Liver & Kidney Function*	X	X	Within 24 hrs of diagnosis & at Days 6, 28 & 90 post Challenge
Hepatitis B, Hepatitis C & HIV infection	X		
EBV and CMV[$]		X	Day 90 post challenge
Cholesterol	X		
HLA Typing		X	
Tests of Immune Response to Malaria		X	Alternate days from day 2 post-challenge until Day of diagnosis (inclusive) and days 28 & 90 post-challenge
Tests for malaria [#]		X	Days 1 – 23 post Challenge

** We will test your red and white cells and your liver and kidney function at various points throughout the study in order to check you remain well.*

[$] EBV and CMV are viruses that cause glandular fever. We will check to see whether you have been exposed to these before. Whether you have or not will not affect your ability to take part in the trial.This will be checked once at the beginning of the study and once at the end.

#Tests for malaria use only small volumes of blood (3mL). We perform one prior to challenge to ensure we get accurate readings during the follow-up post challenge.If you are in the control group you are likely to get malaria within 7-9 days after challenge, therefore it is very unlikely that you would continue to have blood tests to day 23. These tests will be performed until you have had 2 negative blood films following starting antimalarial treatment or until day 23 post-challenge if you do not develop malaria.

2. Vaccination Side Effects (Group 1 only)

Side effects of Vaccination
It is likely that you will experience some symptoms at the vaccination site as well as general symptoms due to vaccination. It is important to remember these are vaccines in the early stage of development therefore the amount of safety data available is limited.
For this reason there is a chance you could experience a side effect that is more severe than that described below, or that has not been seen before with these vaccines.

The vaccine being tested in this trial has been used before, in small numbers of healthy volunteers, so we can predict from past experience what the symptoms should be like. We don't expect any new symptoms and we expect that symptoms will be mild in strength most of the time, but symptoms may also be moderate or severe in how strong they are. All symptoms should resolve completely within a few days.

Local Reactions
You may experience some discomfort at the site as the injection is given. This usually gets better in 5 minutes. Later, you might experience pain resulting in some difficulty moving your arm but should resolve within a few days. In addition to pain, you may experience redness, swelling, or warmth at the injection site.

General reactions
You may experience flu-like symptoms such as muscle aches, joint aches, feverishness, chills, headache, nausea, tiredness and/or feeling generally unwell in the first 24 - 48 hours after the injection, which should generally resolve within a few days.

You are encouraged to take over the counter medications such as paracetamol or ibuprofen as soon as you experience these symptoms as this is likely to reduce the intensity of any symptoms you have.

Serious Reactions
With any vaccination there is a risk of rare serious adverse events, such as an allergic reaction, which may be related to the nervous system or the immune system. Severe allergic reactions to vaccines (anaphylaxis) are also rare, but can be fatal. Reactions in the nervous system are also extremely rare following vaccination and can cause an illness called Guillain-Barré syndrome. Guillain-Barré syndrome is an illness in which people can develop severe weakness and can also be fatal. These adverse events have not previously been seen following administration of this vaccine.

Autoimmune diseases are a class of diseases resulting from a disordered attack of the immune system on the body's own organs and tissues. Such diseases have been reported in individuals having received components similar to components present in FMP2.1/AS01B. The relationship of the product or any of its components to these events has not been established but cannot be excluded. Evaluation of these and similar events continues.

The Investigators are contactable any time if you are concerned about any possible vaccine side effects.

3. Blood Transfusion Reaction

The malaria challenge in this trial involves receiving a very small number of malaria-infected red blood cells. If blood is given from one person to another there is a risk of an allergic reaction. Normally, the blood groups of the blood donor and the individual receiving the blood must be the same to avoid allergic reactions. The donor of the blood we will be using was blood group O, rhesus negative. This means the donor's blood can be given to people of the same or any other blood group, without causing an allergic reaction.

4. Transmission of Blood-borne Infection

The blood transfused in this study has a smaller risk of infection than normal blood transfusions. Firstly, the volunteer who donated the malaria-infected blood was screened for a wide range of blood borne diseases both before and after the blood was collected. The blood was then kept frozen for over 1 year while the donor was observed and retested for any evidence of infection. During this time the donor remained healthy and repeat screenings did not reveal any infections that may have not been detected by initial tests. This procedure took place over 15 years ago and the donor has remained healthy since. Secondly, the volume of blood injected for this study (0.1mL) is thousands of times smaller than the volume in a transfused unit of blood (400mL). Thirdly, the blood cells have been washed and the white blood cells removed, both of which lower the risk of infection due to transfusion.

The donor was known to have had viral infections with Epstein-Barr virus and Cytomegalovirus in the past. These are viruses which, over a person's lifetime, they are very likely to be exposed to, and are the most common causes of 'glandular fever'. They remain within white blood cells after the initial infection so there is a theoretical risk of transmitting these infections from the donor to someone receiving the transfused blood. This risk is extremely small however, given that the white blood cells have been removed from the blood. The blood has also been tested since to look for the virus and these tests were negative. Furthermore, over 30 volunters who had not had these viruses before have received the inoculum and none of them have acquired the infection.

5. Malaria Infection

If untreated, the malaria infection that we propose to give you could result in death. Worldwide over 1300 people have been deliberately infected with malaria and all have made a complete recovery. In Oxford more than 400 people have been infected with malaria. The risks of taking part in this study are low provided that you return for follow-up as outlined above.

The early symptoms of malaria include a flu-like illness, fever, chills, headache, muscle aches, diarrhoea and vomiting. If you develop any of these then you **must let one of the study physicians know immediately.** Study doctors can be contacted 24 hours a day. We hope to diagnose and treat your infection before the onset of symptoms but in previous studies most participants did experience some of the above symptoms. About one-fifth of participants temporarily develop symptoms graded as severe (i.e. symptoms that prevent daily activities). It is possible that you might need to take one or two days off work due to symptoms of malaria. We will prescribe pain-killers such as paracetamol and anti-sickness tablets which you can take as required. Symptoms can start or persist after treatment has started but usually last no more than 1 to 3 days. If malaria is not treated appropriately, possible complications include jaundice, kidney failure, fluid on the lung, low blood sugar and collapse. Seizures, altered consciousness, coma and even death may occur. It is for this reason **it is crucial that you attend all the scheduled follow-up visits and contact us immediately if you have any**

symptoms at all.

In the unlikely event that it is necessary, you may be admitted to the Infectious Diseases ward (the John Warin ward) at the Churchill Hospital, Oxford for observation and treatment. In the last 10 years, only 4 participants out of more than 400 challenged with malaria in Oxford have required hospital admission. There have been no long term problems in participants challenged with malaria.

There have been two unexpected serious adverse events in persons infected in malaria challenge studies in The Netherlands. The first individual experienced an episode of chest pain diagnosed as acute coronary syndrome that occurred two days after completion of malaria treatment with a full recovery. It is uncertain whether this was a form of coronary artery spasm or blockage or cardiac inflammation. More recently, a second individual was found to have an abnormal blood test suggesting cardiac inflammation. This second individual subsequently suffered a very short episode of chest pain. They were also found to be suffering with a viral upper respiratory tract infection (common cold virus) at the time. Again, this individual made a full recovery. It is unclear at this stage whether these findings were related to the malaria vaccine the participants received, the malaria infection, malaria treatment or some other cause. As a result of these events we will exclude people at high risk of heart disease from involvement in this study. These individuals will be identified by medical history, family history, appropriate blood tests, and performing an ECG.

In 2010 in a malaria challenge study in Oxford, a participant failed to attend for a scheduled study visit after being infected with malaria. The police were immediately informed and began a nationwide search for the individual that involved the national media. The participant was found 17 days following challenge when he had mild malaria symptoms. He was admitted to a local hospital where he received treatment for malaria and made a full recovery. The reason for the participant's disappearance was unrelated to the malaria vaccine he received or the malaria challenge.

It is important that you understand that if you fail to attend a clinic appointment after challenge but before you have completed a full course of anti-malarial therapy, the police may be notified and your name may be released to the national media in order to find you.

For 6 months after the challenge if you develop any of the symptoms of malaria as detailed above please contact one of the study doctors or your General Practitioner and remind them that you have been involved in this study.

6. Treatment of Malaria

The drug you will be treated with is called Riamet. It is a licensed drug in the UK for treatment of acute uncomplicated malaria caused by *Plasmodium falciparum* (the type of malaria you will be infected with). Riamet is a combination drug consisting of 20mg artemether and 120mg lumefantrine per tablet.

A treatment course of Riamet consists of 6 doses of 4 tablets. The first 4 tablets will be given when diagnosis is made, followed by additional doses after 8, 24, 36, 48 and 60 hours. We will need to watch you take at least three of these doses. We will continue taking blood to look for parasites until 2 consecutive blood tests are negative for malaria parasites. Blood tests usually become negative for malaria parasites after 24 hours of treatment. Tablets should be taken with a meal or snack. We will provide a light snack with your doses of Riamet which we observe at the CCVTM. You should avoid taking grapefruit juice while taking Riamet.

Riamet is generally well tolerated, but may cause some side effects. Side effects can include

headache, dizziness, abdominal pain and loss of appetite, sleeping problems, palpitations, nausea, vomiting, diarrhoea, skin rash, cough, muscle or joint pain and fatigue. Side effects such as dizziness may impact on the performance of skilled tasks such as driving. Riamet can have an effect on the electrical conduction in the heart (increase in the QT interval) which could potentially increase the risk for a cardiac arrhythmia as an extremely rare side effect; as a precaution we will use a different malaria treatment if we find any reason that you would be at increased risk. Severe allergic reactions could potentially occur, but the exact frequency is unknown.

Signs of severe allergic reactions include rash and itching, sudden wheezing, tightness of the chest or throat, or difficulty breathing, swollen eyelids, face, lips, tongue or other part of the body. If you experience any of these symptoms you should contact the trial doctor immediately on the emergency contact number you will be provided with, or telephone 999 and ask for an ambulance if you are having difficulty breathing.

Taking some other medicines is not compatible with taking Riamet at the same time. If you cannot take Riamet or need to stop taking Riamet during the study, then there are other anti-malarial drugs that can be used effectively instead. If at screening the doctor thinks you may not be able to take Riamet they will discuss with you an alternative medication (Malarone or Chloroquine) and give you an information sheet from the manufacturer for this drug to take away.

7. Treatment of Symptoms Associated with Challenge

Provided there are no contraindications, all participants will be given some medications to help with symptoms associated with malaria challenge. These are licensed, commonly used, medications. If you wish you can see the sheets from the manufacturers, provided inside the packets of these medications, prior to taking part in the study. As with all medications, these drugs can cause a severe allergic reaction in a small number of people. If you develop any concerning symptoms you should contact the trial doctor on the emergency contact number you will be provided with immediately.

Cyclizine: This is a tablet that can be taken as and when needed to help reduce nausea and vomiting. Cyclizine is generally well tolerated however side effects include skin rashes or itching, drowsiness, headache, dry mouth, nose or throat, blurred vision, palpitations, difficulty passing water, constipation, anxiety, or difficulty sleeping. It should be noted that drowsiness may affect your performance of skilled tasks such as driving.

Paracetamol: Is a tablet that can be taken as and when needed to reduce feverishness, muscle and joint pain, back ache and headache. Paracetamol is generally well tolerated.

There may be risks, or side effects which are unknown at this time.

OTHER INFORMATION

Expenses and Payments

You will be compensated for:

- Travel expenses: £10 per visit to local clinic
- Time required for visit: £20 per hour
- Inconvenience of blood tests: £10 per blood donation
- Compensation for illness £480

If you choose to leave the study early or are withdrawn from the study you will be compensated according to the length of your participation based on these figures. You should

note that compensation payments received in this trial may have an impact on your entitlement to benefits.

GroupNo.	Time in Trial (approx.)	Maximum No. of Clinic Visits*	Maximum Volume of Blood Taken (ml)	Compensation Amount
1	8 months	51	787-823**	£2285
2	3 months	39	627-639**	£1540

*The exact number of visits depends on when/if you are diagnosed with malaria following challenge.

**The exact amount of blood taken will depend on when/if you are diagnosed with malaria and also where you are recruited- blood volumes for volunteers outside of Oxford may be slightly higher than those for Oxford volunteers.

In addition to the 15 participants to be included in group 2, we will also recruit a number of 'back-up participants' for this group. These participants will be asked to be available to take part in the study in group 2 at short notice if another participant is unavailable to take part at the last minute. 'Back-up participants' who are not enrolled in the study will be compensated £200 in addition to compensation for visits they may have attended.

What do I have to do?

- You **must** provide a name and 24 hour phone number for someone who lives near to you and who will know where you are for the duration of the study. If you fail to attend for review during the 21 days after challenge and are un-contactable we will contact this person. If you cannot be located we will take additional steps to locate you which may involve contacting the police and national media.
- You must attend all the visits that are outlined above.
- Women must use an effective method of contraception for the duration of the study. If you are using a hormonal contraceptive, you will need to use an alternative method of contraception while you are taking the medication for malaria, and until the start of the next menstrual period.
- You must not donate blood in the UK following participation in the study.

What alternatives are present?

Your alternative is not to participate in this study.

What are the possible benefits of taking part?

This study will not benefit you, but the information gained from the trial might help to prevent malaria infection and disease in those who live in areas where malaria is common and in travellers. At present, there is no malaria vaccine licensed anywhere in the world. There are other malaria vaccines in various stages of development.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

What happens when the research study stops?

If you have any queries or concerns once the study is over, please do not hesitate to get in touch with us.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available. If this happens, we will tell you about it and discuss whether you want to or should continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. On receiving new information, we might consider it to be in your best interests to withdraw you from the study. Your participation in this study may also be stopped at any time by the study doctor or the Sponsor without your consent for other reasons.

What will happen if I don't want to carry on with the study?
If at any time after agreeing to participate you change your mind about being involved with this study, you are free to withdraw without giving a reason. Your decision will not result in any penalty, or loss of benefits to which you are otherwise entitled. However, **if you wish to leave after malaria challenge then you must take the treatment course of Riamet (or an agreed alternative) because of the potentially very serious consequences of untreated malaria infection.** Your compensation would be paid as a proportion of the total compensation according to the length of your participation.

What if there is a problem?

Complaints:
If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should ask to speak with the researchers who will do their best to answer your concerns. You can contact the researchers via the contact details provided at the bottom of this information sheet. Alternatively, you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or the head of CTRG, email ctrg@admin.ox.ac.uk.

Harm:
The Investigators recognise the important contribution that participants make to medical research, and will make every effort to ensure your safety and well-being. If you are harmed as a result of taking part in this study, the study doctor can advise you of further action and refer you to a doctor within the NHS for treatment, if necessary. The University has a specialist insurance policy in place which would operate in the event of any participant suffering harm as a result of their involvement in the research (Newline Underwriting Management Ltd, at Lloyd's of London) as you may be entiltled to compensation. NHS indemnity operates in respect of the clinical treatment which may be provided if you needed to be admitted to hospital. In the event of harm being suffered, while the University will cooperate with any claim, you may wish to seek independent legal advice to ensure that you are properly represented in pursuing any complaint. At any time during the study you will be entirely free to change your mind about taking part, and to withdraw from the study. This will not affect your subsequent medical care in any way.

Will my taking part in this study be kept confidential?
All information that is collected about you during the course of the research will be coded with a study number and kept confidential. The information is available to the study team, the safety monitors, the ethical review committee(s), the Western Institutional Review Board (WIRB), the Sponsor (University of Oxford), government regulatory agencies, authorised

collaborators and external monitors who can ask to audit or monitor the study. Subject's research records may be independently reviewed by United States Agency for International Development (USAID) staff and consultants to ensure compliance with USAID regulations for protection of human research subjects. Any information about you that leaves the clinic will have your name and address removed so that you cannot be identified from it. Your information is stored on a secure server and on paper in a locked filing cabinet. Records are stored for at least 5 years, and in accordance with the applicable regulations.

Information generated from this study will be shared with GlaxoSmithKline (GSK), who are providing the AS01B adjuvant for this trial. GSK is a company that studies and makes vaccines, medicines and other health products. GSK will have access to the study data collected, however, the link between your name and the code number will not be shared. Only the code number and coded information will be sent to GSK.

Involvement of the General Practitioner (GP)/Family doctor
In order to enrol into this study you will be required to sign a form documenting that you consent for us to contact your GP. This is to inform him / her that you are interested in being involved in the study and to check there are no medical reasons that they are aware of why that would make your participation inappropriate. Your GP may be asked to share information about your medical history and give access to any other medical records as required. The final decision about your eligibility will be the responsibility of the study Investigators.

Prevention of 'Over Participating'
Subjects participating in this study must not be concurrently receiving medications or vaccines in another study. In order to check this, you will be asked to provide your National Insurance (NI) or Passport number (if you do not have a NI number). This will be entered on to a national database which helps prevent participants from taking part in too many clinical trials. More information can be found at *www.tops.org.uk*. Your national insurance or passport number is also required to allow processing of compensation payments.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

What will happen to any samples I give?
All samples will be stored in an anonymised form. The blood tests mentioned in part 1 will be analysed in your local hospital laboratory, Oxford University research laboratories, and our collaborating research laboratories. If you consent, some of your leftover blood samples will be stored and may be used for further studies of the human body's immune response to malaria and/or the vaccines used in this study, and/or your safety. Any such tests will have an appropriate ethical review. Upon your request at any time, your remaining blood samples will be destroyed. Your participation in this study will not be affected by your decision to allow or not allow storage and future use of your leftover blood samples.

Will any genetic tests be done?
Yes. Some blood will be used to look at the pattern of your genes that can affect the immune system (including the Human Leukocyte Antigen or HLA genes). The immune response to vaccines is in part genetically controlled, so knowing your pattern of genes that regulate immune responses (such as the HLA type) may help us to understand the responses to vaccination.

What will happen to the results of the research study?
The results of this research study may be published in a scientific medical journal or presented

at scientific conferences and meetings. Publication may not happen until 1 or 2 years after the study is completed. If you contact the researchers in the future you can obtain a copy of the results. You will not be identified in any report or publication.

The anonymised data from this study will be shared with the collaborating partners who are organising and funding this research work, including GSK.

Data from this study may be used to file patents, licence vaccines in the future or make profits in other ways. You will not be paid for any part of this.

Data from this study may be used as part of a student post-graduate degree, for example an MD.

Who is organising and funding the research?
The study is organised by the University of Oxford. The major collaborators are the PATH Malaria Vaccine Initiative, Walter Reed Army Institute of Research (WRAIR), US Agency for International Development (USAID) and a division of the pharmaceutical company GlaxoSmithKline (GSK Vaccines). The study is funded through financial support to Oxford University from several funders, primarily PATH (an international public health organisation) with support provided by the Infectious Disease Division, Bureau for Global Health, US Agency for International Development (USAID); the UK Medical Research Council (MRC); and the National Institutes of Health Research through the Oxford Biomedical Research Centre. Neither your GP nor the researchers are paid for recruiting you into this study.

Who has reviewed the study?
This study has been reviewed by NRES Committee South Central - Oxford A (Reference number: 13/SC/0596). Review is also provided by the Western Institutional Review Board, USA. The Medicines and Healthcare products Regulatory Agency (MHRA) which regulates the use of all medicines in the UK has reviewed the study design and has granted permission to use these unlicensed vaccines in this clinical study.

Thank you for reading this information sheet. If you are interested in being involved in the study please contact the study team at your local trial site below to arrange a screening appointment.

E-mail:vaccinetrials@ndm.ox.ac.uk

Telephone: 01865 857406

Centre of Clinical Vaccinology and Tropical Medicine,
Churchill Hospital, Old Road, Headington, Oxford, OX3 7LE

Contact Professor Adrian Hill, DM, FRCP, at +44 1865 857401 if you have questions, concerns or complaints about the research or if you feel you have been injured due to your participation in the study.

Professor Adrian Hill
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Tel: 01865 857401

NRES Committee South Central - Oxford A ref number: 14/SC/0120

Centre for Clinical Vaccinology and Tropical Medicine (CCVTM)
Churchill Hospital,
Oxford,
OX3 7LE

Recruitment Co-ordinator 01865 857406

PARTICIPANT INFORMATION SHEET: VAC057

A study to assess new malaria vaccines ChAd63 RH5 and MVA RH5

A Phase Ia clinical trial to assess the safety and immunogenicity of new *Plasmodium falciparum* malaria vaccine candidates ChAd63 RH5 alone and with MVA RH5

We would like to invite you to take part in a research study. Before you make a decision, it is important you take the time to understand why the research is being done and what it would involve. Please read the following information carefully and discuss it with friends, relatives and your General Practitioner (GP) if you wish. Please ask us if there is anything that is not clear or if you would like more information.

- Part 1 tells you the purpose of the study and what will happen to you if you take part.
- Part 2 tells you more information about the conduct of the study.

Part 1

What is the purpose of the study?

Malaria is a major global problem, affecting around 216 million people each year and causing around 655,000 deaths. There is a great need for a safe, effective malaria vaccine as the range of effective medicines for treating malaria is limited and resistance to commonly used medicines is increasing. Currently there is no approved vaccine available for malaria.

The purpose of this study is to assess two new malaria vaccines, **ChAd63 RH5** and **MVA RH5**, at different doses and alone or in combination. The study will enable us to assess the safety of the vaccines and the extent of the immune response in healthy volunteers. We will do this by giving volunteers one or two vaccinations, doing blood tests and collecting information about any symptoms that occur after vaccination. This is the first trial to use these vaccines in humans. We plan to recruit a total of 24 volunteers to be vaccinated.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep (or sent it electronically) and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason, but you may be asked to return to the clinic for follow up for safety reasons.

What will happen if I decide to take part?

This study involves having one or two vaccinations and then being followed up with blood tests. You will be asked to complete a diary, recording any symptoms you experience after the vaccination. You will be able to choose which group you are enrolled in, although as groups fill up there will be less choice.

Length of research

If you decide to take part in this study, you will be involved in the trial for approximately 6 to 8 months, depending on what group you are in.

Am I eligible to be involved in the trial?

In order to be involved in the study you **must**:

- Be a healthy adult aged between 18 and 50 years.
- Be able and willing (in the Investigator's opinion) to comply with all study requirements.
- Allow the Investigators to discuss your medical history with your GP.
- Practice continuous effective contraception for the duration of the study **(women only)**.
- Refrain from blood donation during the course of the study.

You cannot participate in this study if:

- You have had malaria before.
- You have travelled to a malaria endemic region in the last 6 months or are intending to travel to a malaria endemic region during the time you would be involved in the study.
- You have participated in another research study in the last 30 days.
- You are planning to participate in another study at the same time as this study.
- You have previously received an investigational malaria vaccine.
- You have had immunoglobulins and/or any blood products (such as a blood transfusion) in the 3 months preceding your involvement this trial.
- You have problems with your immune system.
- You are pregnant, breast feeding or intend to become pregnant during the study.
- You have a history of a severe allergic reaction to a vaccination.
- You have a history of cancer.
- You have a history of a serious psychiatric condition that may affect participation in the study.
- You have any other serious long-term illnesses requiring hospital follow-up.
- You drink on average more than 42 units of alcohol a week (a pint of beer is 2 - 3 units, a small glass of wine (125mL) 1 unit and a shot of spirits (25mL) one unit).
- You have injected drugs at any time in the last 5 years.
- You have hepatitis B, hepatitis C or HIV infection.

Mild conditions, such as childhood asthma, which are well-controlled, would not automatically exclude you from participating. If you are unclear whether you are eligible to be involved in the study you can contact the study team who will be able to advise you.

CONSIDERATIONS BEFORE TAKING PART IN THIS STUDY

Screening Visit: This takes place at your local study site and will last approximately one and a half hours. The purpose of the screening visit is for you to discuss the trial with us and decide if you wish to enter the study. If you decide to participate, you will be asked to sign a consent form and we will check that you are eligible to participate.

During the screening visit:

- You will be asked some medical questions
- A doctor will examine you
- Blood samples and a urine sample will be taken. These tests will need to be normal for you to be enrolled in the study
- All women will have a urinary pregnancy test

Please note – The screening blood tests will look at your blood counts (e.g. to check if you are anaemic), your liver function and your kidney function. We will also test your blood to see if you are infected with hepatitis B, hepatitis C or HIV, as these conditions can affect your body's response to the vaccines we are assessing. If you test positive to any of these, we will let you know and offer to refer you for treatment. We may also report positive results to the appropriate health authority.

Blood Donation: Under current UK regulations, volunteers will not be able to donate blood during the course of the study.

Private Medical Insurance: If you have private medical insurance you are advised to contact your insurance company before participating in this trial, as involvement may affect the cover provided.

Malaria Prophylaxis: If in future you travel to an area where malaria is common, you should not assume that the experimental vaccines you received in this study will give you any protection against malaria. Make sure you visit your GP or a travel clinic before travelling to a malaria endemic region and follow their advice on prevention measures.

Contraception: It is currently unknown whether the vaccines being tested are safe during pregnancy. For this reason, it is important that all women use adequate contraception throughout the trial. If you were to become pregnant during the trial you must tell us immediately and you will be withdrawn from the study, although we will ask to follow you up for safety reasons.

VACCINATIONS

What are the vaccines that are being tested?

We are testing two vaccines; ChAd63 RH5 and MVA RH5. These vaccines will be injected into the muscle of your upper arm(s).

ChAd63 RH5

ChAd63 RH5 is based on a virus (ChAd63) that has been genetically altered so that it is impossible for it to grow in humans. To this virus we have added a gene containing a protein from the malaria parasite called RH5. The malaria parasite needs this protein in order to get into red blood cells, which is when malaria makes people sick. We are hoping to make the body develop an immune response to this protein, in order to stop the malaria parasite from getting into blood cells. RH5 has not been given to humans before, but side effects from these types of vaccines are usually due to the viruses used rather than the proteins. We have given the virus with other genes for malaria proteins to over 1000 volunteers and it has been safe and well-tolerated. It can, however, cause some short-lived side effects as described below.

MVA RH5

MVA RH5 is based on a different virus (MVA) but contains the same malaria gene as the ChAd63 RH5 vaccine. We have given MVA carrying genes for other malaria proteins to over 1000 people with no serious side effects. It appears safe and well tolerated but can cause short-lived side-effects.

What are the expected side effects from these vaccines?

Once the vaccinations have been given they cannot be undone, so it is important you are clear of the potential risks of the vaccines before you agree to be involved in the study.

These particular vaccines have not been used in humans before but we do not expect the side effects of these vaccines to be significantly different from previous trials where these viruses have been used with different malaria proteins.

- Injection site pain (most likely mild; however there is a chance this could be moderate or severe in intensity). MVA vaccines have tended to cause more reaction than ChAd63 vaccines in previous studies.
- Redness, swelling, itching and warmth at the vaccine site (symptoms are likely to be mild if present).
- A 'flu-like illness within 24 hours of vaccination which usually resolves within 48 hours. (This can include headache, muscle aches, joint aches, feverishness, tiredness and feeling generally unwell). The majority of general symptoms are likely to be mild but there is a possibility of moderate or severe symptoms occurring.

It is important to remember these are vaccines in the early stage of development; therefore the amount of safety data available is limited. The malaria protein (RH5) has not previously been given to humans before, so there is a chance you could experience a side effect more severe than or different to those described.

Severe Reactions

With any vaccination there is a rare risk of serious reactions, which may be related to the nervous system or the immune system. Severe allergic reactions to vaccines (anaphylaxis) are very rare but can be fatal. Doctors qualified in the management of anaphylaxis will be present at each vaccination. Reactions in the nervous system are also extremely rare following vaccination, but can cause an illness called Guillain-Barré syndrome. Guillain-Barré syndrome is an illness in which people can develop severe weakness and may be fatal. However, these reactions have not previously been seen with the types of vaccines used in this study. If you experience unexpected symptoms, or become in any way concerned you should contact one of the Investigators (who are available 24 hours a day) using the emergency contact details that you will be given once you have been vaccinated.

Vaccination days

All women will have a urinary pregnancy test before each vaccination. We will ask you to wait for 1 hour after each vaccination to check there are no immediate problems. You will be assessed again before leaving and we will ask you to record your symptoms and measure any redness or swelling every day for 7 days after each vaccination. After these 7 days we will ask you to record if you feel unwell or take any medications over the next 3 weeks.

We may ask to photograph your vaccination site. You will not be identifiable in these photographs and you can choose whether or not to agree to this when you sign the consent form. Photographs may be shown to other professional staff, used for educational purposes or included in a scientific or academic publication.

Number, timing and purpose of visits

You will receive either 1 or 2 vaccinations and attend between 8 and 12 visits in total, plus a final telephone call. Visits may include a medical assessment, temperature, pulse and blood pressure readings, examination by a doctor if needed and blood tests. All visits will take place at your local trial site – either the CCVTM in Oxford on the NIHR WTCRF in Southampton. During the course of the trial you may be asked to attend for an extra visit, for example, if a blood test needs to be repeated. You will be compensated for the time and inconvenience of any extra visits.

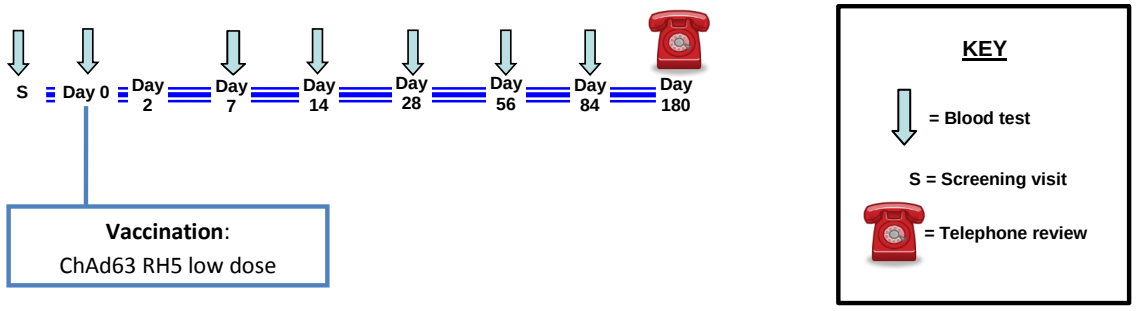
You will be able to choose which group you are enrolled in, although as groups fill up there will be less choice.

The vaccination groups are summarised in the following table:

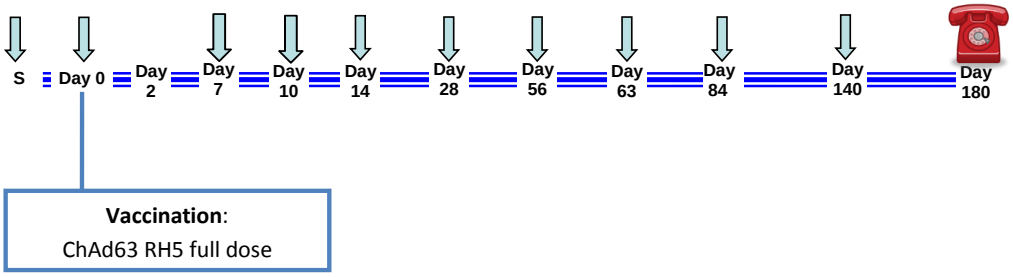
Group Number		Number of volunteers	Dose ChAd63 RH5 Day 0	Dose MVA RH5 Day 56
1		4	Low dose	--
2	A	4	Full dose	--
	B	8	Full dose	Low dose
	C	8	Full dose	Full dose

Timeline for vaccinations

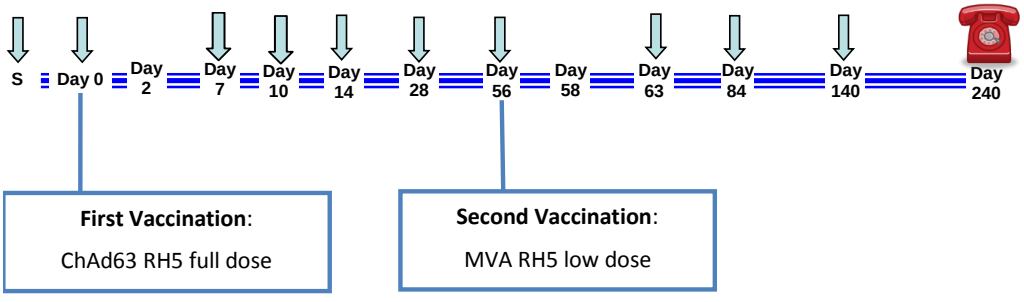
Group 1



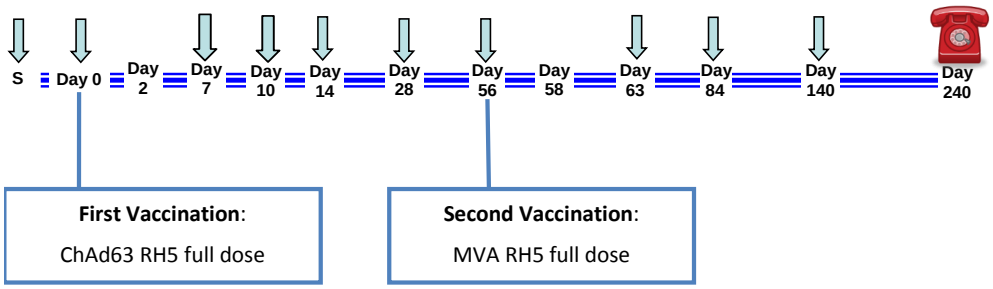
Group 2A



Group 2B



Group 2C



OTHER INFORMATION

Blood Tests

We take blood tests as part of the screening visit and at the study visits in order for us to assess your general health, immune response to the vaccine and for safety reasons. The volume of blood taken at each visit ranges from 10 to 75 mL. If you would like them, we can give you the results of your blood tests. Anonymised blood samples will be stored after testing, and may be used in future malaria research. You will be asked to consent specifically for blood to be stored and shared with other researchers.

Group 2 will have an additional blood test at day 10. Some cells from this blood may be used to produce specific antibodies with activity against malaria (known as 'monoclonal antibodies') in the laboratory. We will also use blood from other time-points for this work (e.g. 7 days after the boost vaccination). These antibodies will help us investigate the response to vaccination in greater detail and could be useful commercially in providing antibodies or products for preventing or treating malaria in the future. If this occurs there will be no financial or other benefit to participants who have provided the blood samples from which products are developed.

To avoid repeated testing, if you are not enrolled into this study and apply to enter another study conducted by the Jenner Clinical Trials Group based at the CCVTM in Oxford, the screening blood results may be used in that study, where appropriate.

Abnormal Results

If abnormal results or undiagnosed conditions are found in the course of the study these will be discussed with you and, if you agree, your GP (or a hospital specialist, if more appropriate) will be informed. Any newly diagnosed conditions will be looked after within the NHS.

Expenses and Payments

You will be compensated for:

- Travel expenses: £10 per visit
- Time required for visit: £20 per hour
- Inconvenience of blood tests: £10 per blood donation

Group No.	Time in Trial (approx.)	Number of Visits	Number. of Blood Tests	Approximate Volume of Blood Taken
1	6 months	8 (+ telephone review)	7	400mL
2A	6 months	10 (+ telephone review)	10	530mL
2B and 2C	8 months	11 (+ telephone review)	10	570mL

The total compensation for taking part in this study is between £360 and £540, depending on which group you are in. If you choose to leave the study early or are withdrawn from the study, you will be compensated according to the length of your participation based on these figures. Please note that if you do leave the study early it can take several weeks for your final payment to be made. You should note that compensation payments received in this trial may have an impact on your entitlement to benefits.

What alternatives are present?

At present, there is no malaria vaccine approved anywhere in the world. There are other malaria vaccines in various stages of development. This study may help develop an effective malaria vaccine.

What are the possible benefits of taking part?

This study will not benefit you, but the information gained from the study might help to prevent malaria infection and disease in those living in areas where malaria is common and in travellers to those areas.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

This completes Part 1 of the Information Sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?

Sometimes during the course of a trial, new information becomes available about the vaccine being studied. If this happens, we will tell you about it and discuss whether you want to or should continue in the study. If you decide to continue to take part you will be asked to sign an updated consent form. On receiving new information, we may consider it to be in your best interests to withdraw you from the study.

What will happen if I don't want to carry on with the study?

If, at any time after agreeing to participate you change your mind about being involved with this study, you are free to withdraw without giving a reason. Unless you state otherwise, any blood taken whilst you have been in the study will continue to be stored and used for research as detailed above. You are free to request that your blood samples are destroyed at any time during or after the study.

What if there is a problem?

The University of Oxford, as Sponsor, has appropriate insurance in place in the unlikely event that you suffer any harm as a direct consequence of your participation in this trial.

The investigators recognise the important contribution that volunteers make to medical research, and make every effort to ensure your safety and well-being. In the unlikely event of harm being suffered, while the University will cooperate with any claim, you may wish to seek independent legal advice to ensure that you are properly represented in pursuing any complaint. At any time during the study you will be entirely free to change your mind and withdraw from the study. This will not affect your subsequent medical care in any way.

Complaints statement

If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact your local trial team (contact details at the end of this document) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224 or the head of CTRG, email ctrg@admin.ox.ac.uk.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be coded with a study number and kept confidential. The information is available to the study team, safety monitors, ethical review committee, Sponsors, government regulatory agencies and external monitors who can ask to audit or monitor the study. Any information about you that leaves the hospital or clinic will have your name and address removed so that you cannot be identified from it. Your information is stored electronically on a secure server and any paper notes are kept in a locked filing cabinet.

Involvement of the General Practitioner/Family doctor (GP)

In order to enrol into this study, you will be required to sign a form, documenting that you consent for us to contact your GP. This is to inform them that you are interested in being involved in the study and to check there are no medical reasons that they are aware of why your participation would be unadvisable. The researchers will not enrol you in the trial if they have any concerns about your eligibility or safety. We will write to your GP to let them know whether or not you are finally enrolled in the study, and whether or not you completed the study, so they can update your medical records accordingly.

Prevention of 'Over Volunteering'

Volunteers participating in this study must not be involved in another study at the same time. In order to check this, you will be asked to provide your National Insurance or Passport number. This will be entered on to a national database which helps prevent volunteers from taking part in too many clinical trials. More information can be found at *www.tops.org.uk*. Your national insurance or passport number is also required to allow processing of compensation payments.

What will happen to any samples I give?

If you consent, some of your leftover blood samples will be stored and may be used for further studies of the human body's immune response. Any such tests will have an appropriate ethical review. Upon your request at any time, your remaining blood samples will be destroyed. Your participation in this study will not be affected by your decision whether to allow storage and future use of your leftover samples.

Your study visit blood tests will be analysed in the hospital laboratories and Oxford University research laboratories. Other blood tests to look at the response of your body to the vaccine may be done with collaborating laboratories in other countries. Any samples or data sent to them would be anonymous.

Will any genetic tests be done?

Yes. Some blood may be used to look at the pattern of your genes that can affect the immune system (for example 'human leukocyte antigen [HLA] type). The immune response to vaccines is in part genetically controlled, so knowing your pattern of genes that regulate immune responses may help us to understand the responses to vaccination. You can opt out of 'genetic tests' if you wish, without any effect on your participation in the trial.

What will happen to the results of the research study?

The results of this research study may be presented at scientific meetings or conferences and published in a scientific medical journal. This may not happen until 1 or 2 years after the study is completed. If you contact the researchers in the future you can obtain a copy of the results. You will not be identified in any report or publication.

The anonymised data from this study will be shared with the collaborating partners who are organising and funding this research work, including the pharmaceutical company GlaxoSmithKline (GSK). Data from this study may be used to file patents, licence vaccines in the future or make profits in other ways. You will not be paid for any part of this. Data from this study may be used as part of a student post-graduate degree, for example a MD or PhD.

Who is sponsoring, organising and funding the research?

The study is sponsored by the University of Oxford and funded by European Commission funding.

The study is organised by a research team at The Jenner Institute at the University of Oxford, headed by Dr Simon Draper. Neither your GP, nor the researchers are paid for recruiting you into this study.

Who has reviewed the study?

This study has been reviewed by Oxford Research Ethics Committee A and has been given a favourable ethical opinion. The Medicines and Healthcare products Regulatory Agency (MHRA) which regulates the use of all medicines in the UK has reviewed the study design and has granted permission to use these unlicensed vaccines in this clinical study.

Thank you for reading this information sheet. If you are interested in taking part in the study please contact the study team at your local study site to arrange a screening appointment.

Contact details for further information:

Recruitment Co-ordinator
Tel: 01865 857406
Email: vaccinetrials@ndm.ox.ac.uk

Appendix 2: Participant consent forms

VAC051 Consent form v2.0

Malaria Vaccine Trials
Centre for Clinical Vaccinology and Tropical Medicine
University of Oxford
Churchill Hospital
Old Road, Headington
Oxford OX3 7LE

A phase Ia study to assess the safety and immunogenicity of new *Plasmodium vivax* malaria vaccine candidate ChAd63 PvDBP alone and with MVA PvDBP

Chief Investigator: Prof AVS Hill **Study Code:** VAC051

NRES Committee South Central - Oxford A ref number: 13/SC/0001

		Please initial
1	I confirm that I have read and understand the Volunteer Information Sheet, Version_____, dated _____ for the above study. I have I have spoken to_____ and had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	1……
2	I agree to have blood tests for this study, including testing for HIV and Hepatitis B and C.	2……..
3	I agree that my blood samples are a gift to the University of Oxford and I understand I will not gain any direct personal benefit from these.	3……..
4	I agree that blood tests looking at the immune response to vaccination, including some genetic tests, can be carried out. (*Participation in this study will not be affected by your decision to allow or not allow genetic tests to be carried out*)	4……..
5	I agree that some of my leftover blood samples will be stored indefinitely and that my stored blood may be used for further ethically approved studies of the body's immune response to malaria vaccination and malaria. I understand that I can ask for these to be destroyed at any time. (*Participation in this study will not be affected by your decision to allow or not allow storage and future of blood samples*)	5……..
6	I agree that samples may be passed in an anonymised form to research collaborators, including collaborators in other countries.	6……..
7	I agree to abstain from donating blood for the duration of the study	7……..
8	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	8……..
9	I give permission for relevant sections of any of my medical notes and data collected during the study to be looked at by responsible individuals from regulatory authorities and the University of Oxford, and Clinical Trials Research Governance (CTRG) for the purposes of audit.	9……..
10	I understand that I will be given live (attenuated) viral vector vaccine(s) during the course of this study.	10……
11	I agree to my GP being contacted and being asked to share information about my medical history and give access to any other medical records as required.	11……

12	I agree to my details being registered with and checked against a confidential national database (TOPS) to prevent me taking part in more than one trial at a time.	12……
13	I have received enough information about the vaccine and follow up schedule.	13……
14	**Women only:** I agree to use effective contraception for the whole study and understand that I will be required to have pregnancy tests at regular intervals during the trial.	14……
15	I agree to allow photographs of the injection site(s) to be taken for clinical comparison. I understand I will not be identifiable in these photographs other than by my unique study number.	15……
16	I agree to take part in this study.	16……

Signature of Volunteer: **Date:**

Name of Volunteer:
(in block letters)

Signature of Investigator: **Date:**

Name of Investigator:

(in block letters) ***When completed 1 for volunteer, 1 for research file, (both original signatures)***

A Phase I/IIa Study of the Safety, Immunogenicity and Efficacy of FMP2.1/AS01B, an Asexual Blood-Stage Vaccine for *Plasmodium falciparum* Malaria

APPROVED
Jan 30, 2014
WIRB®

Chief Investigator: Prof AVS Hill
E-mail: vaccinetrials@ndm.ox.ac.uk
Tel: 01865 857401

Study Code: VAC054
REC REF : 13/SC/0596
Subject ID:_____

Study Procedures	**Please initial**	
1. I confirm that I have read and understand the Information Sheet pertaining to **VAC054 Version_____, Dated_____** and have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	**1.....**	
2. I have spoken to (name of investigator): _____.	**2.....**	
3. I agree to have blood tests as part of this trial including testing for HIV, Hepatitis B & C, cytomegalovirus (CMV) and Epstein-Barr Virus (EBV).	**3.....**	
4. Group 1 only: I understand that I will be given three injections of an investigational protein-in-adjuvant malaria vaccine.	**4.....**	**N/A** ☐
5. I understand that I may develop local and/or general [13]symptoms after vaccination.	**5.....**	
6. I understand I will be given blood containing malaria parasites during this trial with the intention of giving me malaria.	**6.....**	
7. I understand that there is potentially a very small risk of acquiring a blood-borne infection, including EBV or CMV (the viruses that most commonly cause glandular fever).	**7.....**	
8. I understand that I may develop symptoms of malaria which may be severe.	**8.....**	
9. I understand that I will not be able to donate blood in the UK again.	**9.....**	
10. I have received enough information about the challenge procedure, the follow-up schedule and the proposed anti malarial therapies.	**10.....**	
Personal Information		
11. I agree to my GP being contacted and being asked to share information about my medical history and give access to any other medical records as required.	**11.....**	
12. I agree to my details being registered with and checked against a confidential national database (**TOPS**) to prevent me taking part in more than one trial at a time.	**12.....**	
13. I give permission for relevant sections of any of my medical notes and data collected during the study to be looked at by responsible individuals from the study group, regulatory authorities, collaborators, the University of Oxford and the external monitor for the purposes of audit.	**13.....**	

14. I agree that investigators may speak to my nominated contact, next of kin, the police and national media if I fail to attend for follow up after being infected with malaria. **14.....**

Withdrawing from the study

15. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. **15.....**

16. If I should wish to withdraw from the trial after I have been infected with malaria, I understand that I must take a course of anti-malarial medication, and that I will be asked to attend for review on at least 2 further days for safety reasons. **16.....**

17. I understand that should I fail to return for review as outlined in the volunteer Information sheet that I may become seriously ill and die. **17.....**

18. I understand that if I withdraw my consent, the blood samples collected before my withdrawal will be used unless I specifically request otherwise. **18.....**

Contraception

19. Women: I understand the crucial need to use an effective method of birth control for the whole study and that I will be required to have pregnancy tests at regular intervals during this trial. I understand that Riamet may temporarily reduce the effectiveness of hormonal contraceptives, and the need to use an additional form of contraception while taking Riamet and until the start of the next menstruation after Riamet treatment. **19.....** **N/A** ☐

Consent

20. I agree to take part in this study. **20.....**

The following are optional, answering "No" to any or all will not affect your ability to participate in the study.

21. To avoid repeated testing, I agree that if I am not enrolled into this study and apply to enter another study conducted by the Jenner Clinical Vaccine Trials Group based at the CCVTM, my screening blood results may be used in that study, where appropriate. **21.....** **No** ☐

22. I agree that my leftover blood samples will be a gift to the University of Oxford, where they may be stored in accordance with the Human Tissue Act 2004 indefinitely**.** **22.....** ☐

23. I understand that my samples will be identifiable only by a unique ID number and I will not be identifiable to researchers. **23.....** ☐

24. I agree that my stored blood samples may be used for further studies of the body's immune response to malaria in future ethically approved research. **24.....** ☐

25. I agree that my leftover blood samples may also be shared with other collaborating study teams based within the United Kingdom. **25.....** ☐

26. I agree that my leftover blood samples may also be shared with other collaborating study teams based in the European Union, United States of America and with responsible institutions around the world. **26.....** ☐

27. I understand I am able to withdraw consent for the storage and use of samples at any time. **27.....** ☐

28. I agree to allow photographs to be taken for clinical comparison, and I understand I will not be identifiable in these photographs other than by the unique study number. ☐ **28.....**

Signature of Volunteer:.....Name:.....Date: .....

Signature of Investigator:.....Name:.....Date: .....

When completed 1 for volunteer, 1 for research file, (both original signatures)

VAC057 Consent form v3.0 (Oxford)

E-mail: vaccinetrials@ndm.ox.ac.uk
Tel: 01865 857401

UNIVERSITY OF OXFORD

A Phase Ia clinical trial to assess the safety and immunogenicity of new *Plasmodium falciparum* malaria vaccine candidates ChAd63 RH5 alone and with MVA RH5

Chief Investigator: Prof AVS Hill **Study Code:** VAC057
NRES Committee South Central - Oxford A ref number: 14/SC/0120

		Please initial/tick
1	I confirm that I have read and understand the Participant Information Sheet, Version_____, dated _____ for the above study. I have spoken to_____ and had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	1……
2	I agree to have blood tests for this study, including testing for HIV and Hepatitis B and C.	2……
3	I agree that my blood samples are a gift to the University of Oxford, to be used for looking at the immune response to vaccination, and I understand I will not gain any direct personal benefit from these.	3……
4	I agree that blood tests looking at the immune response can include some genetic tests. (*Participation in this study will not be affected by your decision to allow or not allow genetic tests to be carried out*)	4 Yes ☐ No ☐
5	I agree that some of my leftover blood samples will be stored indefinitely and that my stored blood may be used for further ethically approved studies of the body's immune response to malaria vaccination and malaria. I understand that I can ask for these to be destroyed at any time. (*Participation in this study will not be affected by your decision to allow or not allow storage and future of blood samples*.)	5……
6	I agree that cells from my blood may be used to produce specific antibodies ('monoclonal antibodies') which could be used in commercial activity in the future. I understand that I will not gain any direct personal benefit from this.	6……
7	I agree that samples may be passed in a coded form to research collaborators, including collaborators in other countries.	7……
8	I agree that coded data may be passed on to other organisations, which may include commercial organisations.	8……
9	I agree to abstain from donating blood for the duration of the study.	9……
10	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	10……
11	I give permission for relevant sections of any of my medical notes and research data collected during the study to be looked at by responsible individuals from regulatory authorities, the University of Oxford, and University Hospital Southampton NHS Foundation Trust for the purposes of audit and monitoring.	11……
12	I understand that I will be given live (attenuated) viral vector vaccine(s) during the course of this study.	12……
13	I agree to my GP being contacted and being asked to share information about my medical history and give access to any other medical records as required.	13……
14	I agree to my details being registered with and checked against a confidential national database (TOPS) to prevent me taking part in more than one trial at a time.	14……

15	Women only: I agree to use effective contraception for the whole study and understand that I will be required to have pregnancy tests at regular intervals during the trial.	15……
16	I agree to allow photographs of the injection site(s) to be taken for clinical comparison. I understand I will not be identifiable in these photographs other than by my unique study number.	16 Yes ☐ No ☐
17	I agree to take part in this study.	17……

Signature of Volunteer: **Date:**

Name of Volunteer:
(in block letters)

Signature of Investigator: **Date:**

Name of Investigator:
(in block letters)
When completed 1 for volunteer, 1 for research file, (both original signatures)

Appendix 3: VAC054 Informed Consent Questionnaire v1.0

Professor Adrian V.S. Hill (DM FRCP)

E-mail: vaccinetrials@ndm.ox.ac.uk

Tel: 01865 857401

REC REF : 13/SC/0596

A study to assess the safety and effectiveness of an experimental malaria vaccine by infecting vaccinated volunteers with malaria parasites using malaria-infected red blood cells

A Phase I/IIa Study of the Safety, Immunogenicity and Efficacy of FMP2.1/AS01B, an Asexual Blood-Stage Vaccine for *Plasmodium falciparum* Malaria (VAC054)

INFORMED CONSENT QUESTIONNAIRE

This questionnaire is designed to test your understanding of the study in order for us to be confident that you fully understand what taking part will involve. Please make sure you have read the information sheet in full and asked the Investigator any questions you have. You need to answer all questions correctly in order to take part in the study. If you don't answer all the questions correctly the first time, you will be able to complete the questionnaire again after discussion with the Investigator.

Volunteer Name:……

Volunteer Trial Number:…..……..

Date……..……Time……..

Attempt Number……. of ……

Please clearly circle one answer for each question;

1. By participating in this study you may develop which of the following:
A. Tuberculosis
B. Malaria
C. Typhoid

2. The study involves volunteers being given malaria by:
A. Intramuscular injection
B. Mosquito bite
C. Transfusion of infected red blood cells

3. Is it likely that a single treatment course will be effective to treat malaria in this study?
A. Yes
B. No

4. Medical screening for this study will include which of the following?
A. Laboratory tests (including an HIV test)
B. Physical examination
C. Review of medical history
D. All of the above
E. None of the above

5. If you wish to withdraw from the study you may:
A. Withdraw voluntarily at any time provided you complete a course of anti-malarial therapy (if needed)
B. Withdraw from the study only if the investigators say it is ok
C. Never withdraw from the study

6. Which of the following are true regarding pregnancy and participation in this study?
A. Pregnant women may participate in this study
B. Women should not get pregnant for 12 months after getting malaria
C. An effective method of birth control is required for women while participating in this study

7. What are common symptoms associated with malaria infection?
A. Fever
B. Chills
C. Headache
D. All of the above
E. None of the above

8. If you develop any concerning symptoms in between clinic visits after being given malaria, what should you do?
A. Wait until your next clinic appointment
B. Call the 24hr emergency phone number and talk to the trial doctor
C. Ask a friend for advice

9. What will happen if you fail to attend a follow-up visit after being given malaria?
A. The police may be informed
B. Your next of kin may be contacted
C. Your identity may be given to the press
D. All of the above

10. How is malaria diagnosed in the study?
A. Looking at a sample of blood under a microscope
B. Chest X-ray
C. Having you walk on a treadmill

11. If you develop malaria, we will:
A. Treat you immediately with effective medications
B. See how sick you can get without treating you

12. If you take part in this study, for how long will you be unable to donate blood?
A. 1 year
B. 2 years
C. Never able to donate blood again

13. In the follow-up period when you may be diagnosed with malaria, which of the following is true?

A. You must remain in Oxford and the surrounding area
B. You must be contactable by the study team at all times
C. You must be able to attend clinic at short notice
D. All of the above

_____
Volunteer signature / date

Score: ___/13 Reviewer signature / date_____

Appendix 4: Schedules of attendance

VAC051 Schedules of attendance

	S	ChAd63 PvDBP					
Attendance number	1	2	3	4	5	6	7
Timeline (days)		0	2	14	28	56	84
Window (days)			±1	±2	±7	±7	±7
Inclusion / Exclusion criteria	X						
Informed consent	X						
Medical History	X	(x)	(x)	(x)	(x)	(x)	(x)
Physical Examination^	X	(x)	(x)	(x)	(x)	(x)	(x)
Urinalysis	X						
B-HCG urine test (♀)	X	X					
Review contraindications	X	X					
Vaccination		X					
Physical observations	X	X	X	X	X	(x)	(x)
AEs reviewed		X	X	X	X	X	X
Diary cards provided		X					
Diary cards collected				X			
HLA typing (mL)		4					
HBV,HCV,HIV (mL)	5						
Haematology (mL)	2			2	2	2	2
Biochemistry (mL)*	3			3	3	3	3
Exploratory immunology(/ serology)[$]		60		60	60	60	60
Blood volume per visit (mL)	10	64		65	65	65	65
Cumulative blood volume (mL)	10	74	74	139	204	269	334

VAC051: Schedule of attendances for Group 1

(Windows refer to time since last visit rather than time since scheduled attendance). S = screening visit; (x) = If considered necessary; * *Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, Liver Function Tests; & ^ Physical observations includes blood pressure, pulse and temperature.* [$]*Exploratory immunology will include PvDBP IFN-γ T cell ELISPOT, B cell ELISPOT, and PvDBP antibody ELISA; anti-adenovirus antibodies may be measured from serum at a later date.*

	S	ChAd63 PvDBP							
Attendance number	1	2	3	4	5	6	7	8	9
Timeline (days)	≥-90	0	2	14	28	56	63	84	140
Window (days)		0	±1	±2	±7	±7	±7	±7	±14
Inclusion / Exclusion criteria	X	X							
Informed consent	X	(X)							
Medical History	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical Examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Urinalysis	X								
B-HCG urine test (♀)	X	X							
Review contraindications	X	X							
Vaccination		X							
Physical observations	X	X	X	X	X	(X)	(X)	(X)	(X)
AEs reviewed		X	X	X	X	X	X	X	X
Diary cards provided		X							
Diary cards collected				X					
HLA typing (mL)		4							
HBV,HCV,HIV (mL)	5								
Haematology (mL)	2			2	2	2	2	2	2
Biochemistry (mL)	3			3	3	3	3	3	3
Exploratory immunology(/serology)[$]		60		60	60	60	60	60	60
Blood volume per visit (mL)	10	64		65	65	65	65	65	65
Cumulative blood volume (mL)	10	74		139	204	269	334	399	464

VAC051 Schedule of attendances for Group 2A

(Windows refer to time since last visit rather than time since scheduled attendance). S = screening visit; (x) = If considered necessary; * *Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, Liver Function Tests; & ^ Physical observations includes blood pressure, pulse and temperature.* [$]*Exploratory immunology will include PvDBP IFN-γ T cell ELISPOT, B cell ELISPOT, and PvDBP antibody ELISA; anti-adenovirus antibodies may be measured from serum at a later date.*

	S	ChAd63 PvDBP				MVA PvDBP				
Attendance number	1	2	3	4	5	6	7	8	9	10
Timeline (days)		0	2	14	28	56	58	63	84	140
Window (days)			±1	±2	±7	±7	±1	±2	±7	±14
Inclusion / Exclusion criteria	X	X				X				
Informed consent	X	(X)				(X)				
Medical History	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Physical Examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
Urinalysis	X									
B-HCG urine test (♀)	X	X				X				
Review contraindications	X	X				X				
Vaccination		X				X				
Physical observations	X	X	X	X	X	X	X	X	X	(X)
AEs reviewed		X	X	X	X	X	X	X	X	X
Diary cards provided		X				X				
Diary cards collected				X				X		
HLA typing (mL)		4								
HBV,HCV,HIV (mL)	5									
Haematology (mL)	2			2	2	2		2	2	2
Biochemistry (mL)	3			3	3	3		3	3	3
Exploratory immunology(/serology)[$]		60		60	60	60		70	60	60
Blood volume per visit (mL)	10	64		65	65	65		75	65	65
Cumulative blood volume (mL)	10	74		139	204	269		344	409	474

VAC051 Schedule of attendances for Groups 2B and 2C

*(Windows refer to time since last visit rather than time since scheduled attendance). S = screening visit; (x) = If considered necessary; * Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, Liver Function Tests; & ^ Physical observations includes blood pressure, pulse and temperature. [$]Exploratory immunology will include PvDBP IFN-γ T cell ELISPOT, B cell ELISPOT, and PvDBP antibody ELISA; anti-adenovirus antibodies may be measured from serum at a later date.*

VAC054 Schedules of attendance

	S	FMP2.1/ AS01B (1)				FMP2.1/ AS01B (2)				FMP2.1/ AS01B (3)			C-1	C	C+1	C+2-12	(Day of diagnosis)	C+13-23	C+28	C+90	C+170
Attendance number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16-37		38-48	49	50	51
Timeline (days)		0	3	7	14	28	31	35	42	56	59	63	69	70	71	72-82 (AM+PM)		83-93	98	160	240
Window (days)	(-90)		±1	±2	±2		±1	±2	±2		±1	±2			0	0		0	±3	±7	±14
Inclusion / Exclusion criteria	X	X				X				X				X							
Informed Consent Questionnaire	X																				
Informed consent	X																				
Medical History	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)							
Physical Examination	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)							
Urinalysis	X																				
Electrocardiogram	X	(X)																			
β-HCG urine (♀)	X	X				X				X			X				X				
Review contraindications	X	X				X				X			X	X							
Vaccination		X				X				X											
Physical Observations^	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
AEs reviewed		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	(x)[$]
Diary card provided		X				X				X				X							
Diary card collected				X				X				X							X		

	S	FMP2.1/ AS01B (1)				FMP2.1/ AS01B (2)				FMP2.1/ AS01B (3)			C-1	C	C+1	C+2-12	(Day of diagnosis)	C+13-23	C+28	C+90	C+170
Medic Alert Card Given to Volunteers														X							
Treatment for Malaria																	X	(X)			
HLA typing (mL)		4																			
HBV,HCV,HIV (mL)	5																				
EBV,CMV (mL)													5							5	
Serum for storage													5							5	
Haematology (mL)	2	2		2	2	2		2	2	2		2	2			2[£]	2		2	2	
Biochemistry (mL)**	3	3		3	3	3		3	3	3		3	3			3[£]	3		3	3	
Immunology		60			60	60		10	60	60		10	70				70		60	60	
Blood Film / PCR													3		3	3 x 22	3	3 x 11			
Blood volume per visit (mL)	10	69	0	5	65	65	0	15	65	65	0	15	88	0	3	71	78	33	65	75	0
Cumulative blood volume (mL)	**10**	**79**	**79**	**84**	**149**	**214**	**214**	**229**	**294**	**359**	**359**	**374**	**462**	**462**	**465**	**536**	**614**	**647**	**712**	**787**	**787***

S = screening visit, **(x)** = If considered necessary, emphasising any acute complaints.

^ Physical observations includes blood pressure, pulse and temperature, height and weight, however height and weight will only be measured at screening and dC-1.

[$] The visit on day 240 may be conducted by telephone and will involve collection of information about any SAEs that have occurred since the C+90 visit.

*** Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, Liver Function Tests, Magnesium & Cholesterol, however Magnesium and cholesterol will only be measured at screening.*

[£] Biochemistry and haematology bloods will be checked on day 6 post malaria challenge

**Cumulative blood volume for Oxford volunteers if blood taken as per schedule, and excluding any repeat safety blood test that may be necessary. Southampton and Hammersmith volunteers may have a slightly higher cumulative volume due to use of higher volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures. The maximum cumulative volume for these volunteers would be up to 823mL.*

VAC054 Schedule of attendances for Group 1 (Windows refer to time since last visit, but windows between vaccinations must be a minimum of 21 days and a maximum of 35 days. The window between final vaccination and challenge must be a minimum of 14 days and maximum of 16 days).

	S	C-1	C	C+1	C+2-12	(Day of diagnosis)	C+13-23	C+28	C+90
Attendance number	1	2	3	4	5-26		27-37	38	39
Timeline (days)		-1	0	1	2-12 (AM+PM)		13-23	28	90
Window (days)	(-90)	-2	0	0	0		0	±3	±7
Inclusion / Exclusion criteria	X	X	X						
Informed Consent Questionnaire	X								
Informed consent	X								
Medical History	X	(x)	(x)						
Physical Examination	X	(x)	(x)						
Urinalysis	X								
Electrocardiogram	X	(X)							
β-HCG urine (♀)	X	X				X			
Review contraindications	X	X	X						
Vaccination									
Physical Observations^	X	X	X	X	X	X	X	X	X
AEs reviewed		X	X	X	X	X	X	X	X
Diary card provided			X						
Diary card collected								X	
Medic Alert Card Given to Volunteers			X						
Treatment for Malaria						X	(X)		
HLA typing (mL)		4							

	S	C-1	C	C+1	C+2-12	(Day of diagnosis)	C+13-23	C+28	C+90
HBV,HCV,HIV (mL)	5								
EBV,CMV (mL)		5							5
Serum for storage		5							5
Haematology (mL)	2	2			2[£]	2		2	2
Biochemistry (mL)**	3	3			3[£]	3		3	3
Immunology		70			20 x 6[#]	70	20 x 4[#]	60	60
Blood Film / PCR		3		3	3 x 22	3	3 x 11		
Blood volume per visit(s) (mL)	10	92	0	3	191	78	113	65	75
Cumulative blood volume (mL)	**10**	**102**	**102**	**105**	**296**	**374**	**487**	**552**	**627***

S = screening visit, **(x)** = If considered necessary, emphasising any acute complaints.

^ Physical observations includes blood pressure, pulse and temperature, height and weight, however height and weight will only be measured at screening and C-1.

*** Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, Liver Function Tests, Magnesium & Cholesterol, however Magnesium and cholesterol will only be measured at screening.*

[£] Biochemistry and haematology bloods will be checked on day 6 post malaria challenge

[#] Immunology blood to be taken at AM visits on days 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 (but only until day of diagnosis)

** Cumulative blood volume for Oxford volunteers if blood taken as per schedule, and excluding any repeat safety blood test that may be necessary. Southampton and Hammersmith volunteers may have a slightly higher cumulative volume due to use of higher volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures. The maximum cumulative volume for these volunteers would be up to 639mL*

VAC054 Schedule of attendances for Group 2 (Windows refer to time since last visit).

VAC057 Schedules of attendance

	S	ChAd63 RH5							Telephone review
Attendance number	1	2	3	4	5	6	7	8	9
Timeline (days)	≤90	0	2	7	14	28	56	84	180
Window (days; since last visit rather than since scheduled attendance)			±1	±1	±2	±7	±7	±7	±14
Inclusion / Exclusion criteria	X								
Informed consent	X								
Medical History	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	
Physical Examination^	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	
Urinalysis	X								
B-HCG urine test (♀)	X	X							
Review contraindications	X	X							
Vaccination		X							
Physical observations	X	X	X	X	X	X	(x)	(x)	
AEs reviewed		X	X	X	X	X	(x)	(x)	
SAEs reviewed		X	X	X	X	X	X	X	X
Diary cards provided		X		(x)					
Diary cards collected				(x)		X			
HLA typing (mL)		4							
HBV,HCV,HIV (mL)	5								
Haematology (mL)	2	2		2		2			
Biochemistry (mL)*	3	3		3		3			
Exploratory immunology[$]		60	0	60	60	60	60	60	
Blood volume per visit (mL)	10	69	0	65	60	65	60	60	
Cumulative blood volume (mL)	10	79	79	144	204	269	329	389**	

VAC057 Schedule of attendances for Group 1.

S = screening visit; (x) = If considered necessary;

**Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, and Liver Function Tests.*

^ Physical observations include blood pressure, pulse and temperature.

[$]Exploratory immunology will include PfRH5 IFN-γ T cell ELISPOT, B cell assays, PfRH5 antibody ELISA, and functional antibody assays; anti-adenovirus antibodies may be measured from serum at a later date.

***Cumulative blood volume for Oxford volunteers if blood taken as per schedule, and excluding any repeat safety blood test that may be necessary. Southampton volunteers may have a slightly higher cumulative volume due to use of higher volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures.*

	S	ChAd 63 RH5										Telephone review
Attendance number	1	2	3	4	5	6	7	8	9	10	11	12
Timeline (days)		0	2	7	10	14	28	56	63	84	140	180
Window (days; since last visit rather than since scheduled attendance)			±1	±1	±1	±2	±7	±7	±2	±7	±14	±14
Inclusion / Exclusion criteria	X											
Informed consent	X	(x)										
Medical History	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	
Physical Examination^	X	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)	
Urinalysis	X											
B-HCG urine test (♀)	X	X										
Review contraindications	X	X										
Vaccination		X										
Physical observations	X	X	X	X	X	X	X	(x)	(x)	(x)	(x)	
AEs reviewed		X	X	X	X	X	X	(x)	(x)	(x)	(x)	
SAEs reviewed		X	X	X	X	X	X	X	X	X	X	X
Diary cards provided		X		(x)								
Diary cards collected				(x)			X					
HLA typing (mL)		4										
HBV,HCV,HIV (mL)	5											
Haematology (mL)	2	2		2			2					
Biochemistry (mL)*	3	3		3			3					
Exploratory immunology$		60		60	20	60	60	60	60	60	60	
Blood volume per visit (mL)	10	69	0	65	20	60	65	60	60	60	60	
Cumulative blood volume (mL)	10	79	79	144	164	224	289	349	409	469	529**	

VAC057 Schedule of attendances for Group 2A

S = screening visit; (x) = If considered necessary;

**Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, and Liver Function Tests.*

^ Physical observations include blood pressure, pulse and temperature.

$ Exploratory immunology will include PfRH5 IFN-γ T cell ELISPOT, B cell assays, PfRH5 antibody ELISA, and functional antibody assays; anti-adenovirus antibodies may be measured from serum at a later date.

***Cumulative blood volume for Oxford volunteers if blood taken as per schedule, and excluding any repeat safety blood test that may be necessary. Southampton volunteers may have a slightly higher cumulative volume due to use of higher volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures.*

	S	ChAd63 RH5						MVA RH5					Telephone review
Attendance number	1	2	3	4	5	6	7	8	9	10	11	12	13
Timeline (days)		0	2	7	10	14	28	56	58	63	84	140	240
Window (days; since last visit rather than since scheduled attendance)			±1	±1	±1	±2	±7	±7		±1	±7	±14	±14
Inclusion / Exclusion criteria	X	X						X					
Informed consent	X	(X)						(X)					
Medical History	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	
Physical Examination	X	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	
Urinalysis	X												
B-HCG urine test (♀)	X	X						X					
Review contraindications	X	X						X					
Vaccination		X						X					
Physical observations	X	X	X	X	X	X	X	X	X	X	X	(X)	
AEs reviewed		X	X	X	X	X	X	(x)	X	X	X	(x)	
SAEs reviewed		X	X	X	X	X	X	X	X	X	X	X	X
Diary cards provided		X		(X)				X		(X)			
Diary cards collected				(X)			X			(X)	X		
HLA typing (mL)		4											
HBV,HCV,HIV (mL)	5												
Haematology (mL)	2	2		2			2	2		2	2		
Biochemistry (mL)	3	3		3			3	3		3	3		
Exploratory immunology[$]		60		60	20	60	60	60		70	70	60	
Blood volume per visit (mL)	10	69	0	65	20	60	65	65	0	75	75	60	
Cumulative blood volume (mL)	10	79	79	144	164	224	289	354	354	429	504	564**	

VAC057 Schedule of attendances for Groups 2B and 2C

S = screening visit; (x) = If considered necessary;

**Biochemistry will include Sodium, Potassium, Urea, Creatinine, Albumin, and Liver Function Tests.*

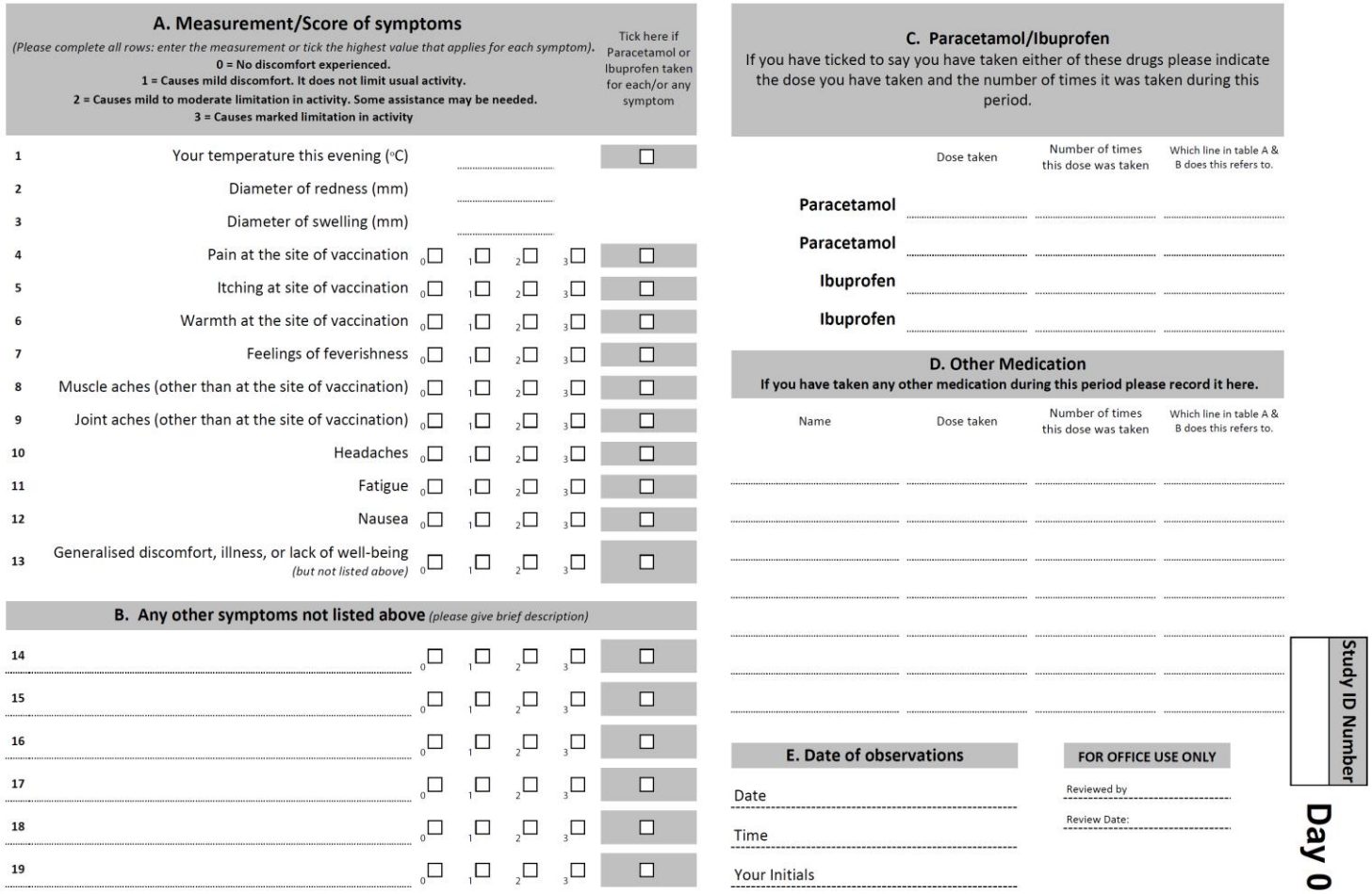
^ Physical observations include blood pressure, pulse and temperature.

[$] Exploratory immunology will include PfRH5 IFN-γ T cell ELISPOT, B cell assays, PfRH5 antibody ELISA, and functional antibody assays; anti-adenovirus antibodies may be measured from serum at a later date.

***Cumulative blood volume for Oxford volunteers if blood taken as per schedule, and excluding any repeat safety blood test that may be necessary. Southampton volunteers may have a slightly higher cumulative volume due to use of higher volume vacutainers for biochemistry, haematology and serology samples as per local Trust standard procedures.*

Appendix 5: Participant diary cards

Generic paper diary card (example page)



A. Measurement/Score of symptoms

(Please complete all rows: enter the measurement or tick the highest value that applies for each symptom).
0 = No discomfort experienced.
1 = Causes mild discomfort. It does not limit usual activity.
2 = Causes mild to moderate limitation in activity. Some assistance may be needed.
3 = Causes marked limitation in activity

						Tick here if Paracetamol or Ibuprofen taken for each/or any symptom
1	Your temperature this evening (°C)	.....				☐
2	Diameter of redness (mm)	.....				
3	Diameter of swelling (mm)	.....				
4	Pain at the site of vaccination	0☐	1☐	2☐	3☐	☐
5	Itching at site of vaccination	0☐	1☐	2☐	3☐	☐
6	Warmth at the site of vaccination	0☐	1☐	2☐	3☐	☐
7	Feelings of feverishness	0☐	1☐	2☐	3☐	☐
8	Muscle aches (other than at the site of vaccination)	0☐	1☐	2☐	3☐	☐
9	Joint aches (other than at the site of vaccination)	0☐	1☐	2☐	3☐	☐
10	Headaches	0☐	1☐	2☐	3☐	☐
11	Fatigue	0☐	1☐	2☐	3☐	☐
12	Nausea	0☐	1☐	2☐	3☐	☐
13	Generalised discomfort, illness, or lack of well-being *(but not listed above)*	0☐	1☐	2☐	3☐	☐

B. Any other symptoms not listed above *(please give brief description)*

14	.....	0☐	1☐	2☐	3☐	☐
15	.....	0☐	1☐	2☐	3☐	☐
16	.....	0☐	1☐	2☐	3☐	☐
17	.....	0☐	1☐	2☐	3☐	☐
18	.....	0☐	1☐	2☐	3☐	☐
19	.....	0☐	1☐	2☐	3☐	☐

C. Paracetamol/Ibuprofen

If you have ticked to say you have taken either of these drugs please indicate the dose you have taken and the number of times it was taken during this period.

	Dose taken	Number of times this dose was taken	Which line in table A & B does this refers to.
Paracetamol	.....	.....	.....
Paracetamol	.....	.....	.....
Ibuprofen	.....	.....	.....
Ibuprofen	.....	.....	.....

D. Other Medication

If you have taken any other medication during this period please record it here.

Name	Dose taken	Number of times this dose was taken	Which line in table A & B does this refers to.
.....	.....	.....	.....
.....	.....	.....	.....
.....	.....	.....	.....
.....	.....	.....	.....
.....	.....	.....	.....
.....	.....	.....	.....
.....	.....	.....	.....

E. Date of observations

Date .....

Time .....

Your Initials .....

FOR OFFICE USE ONLY

Reviewed by .....

Review Date: .....

Study ID Number

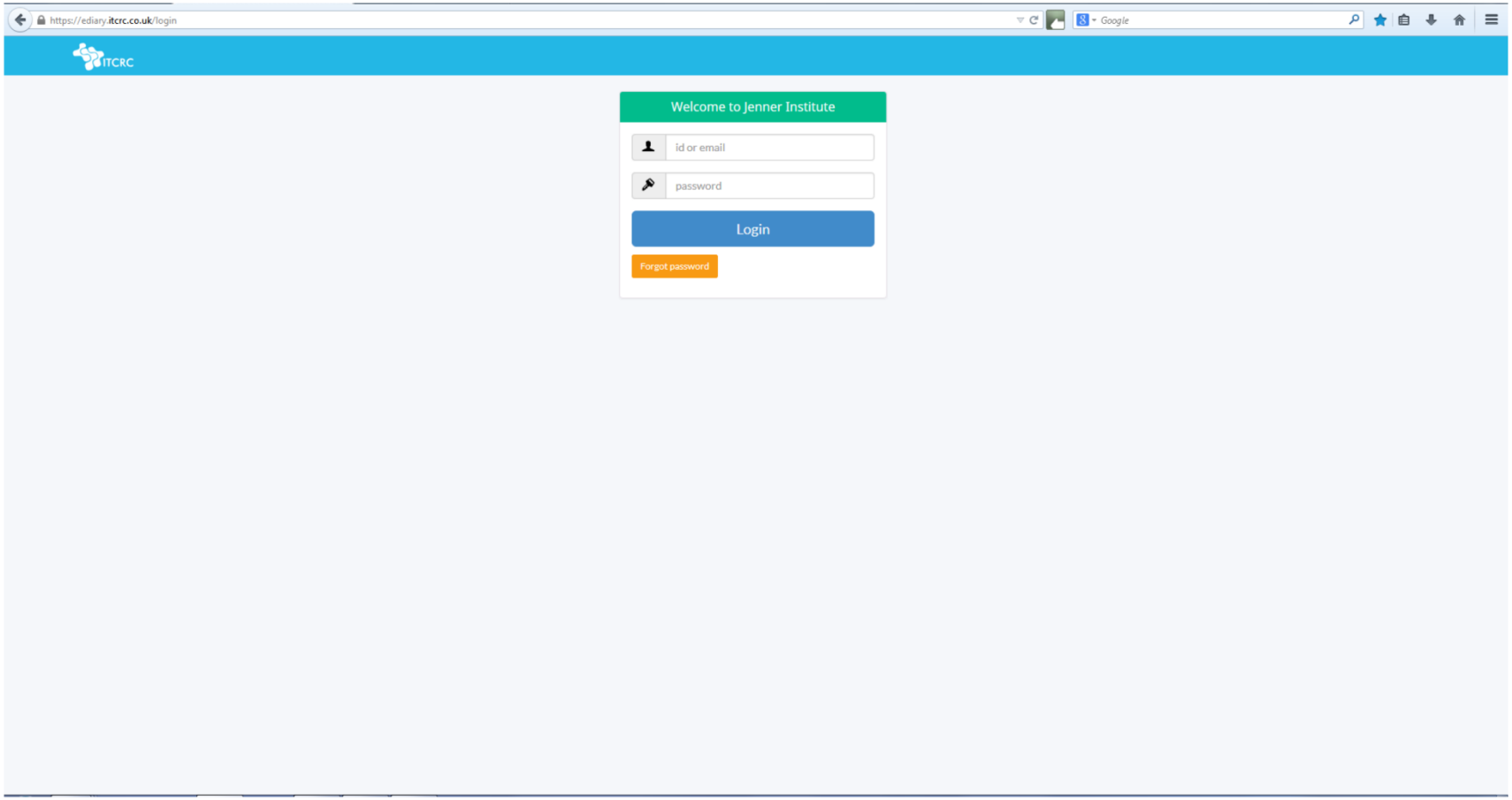
Day 0

Jenner Institute Vaccine Diary Card (IM-7), v1.1 (11 April 2013)

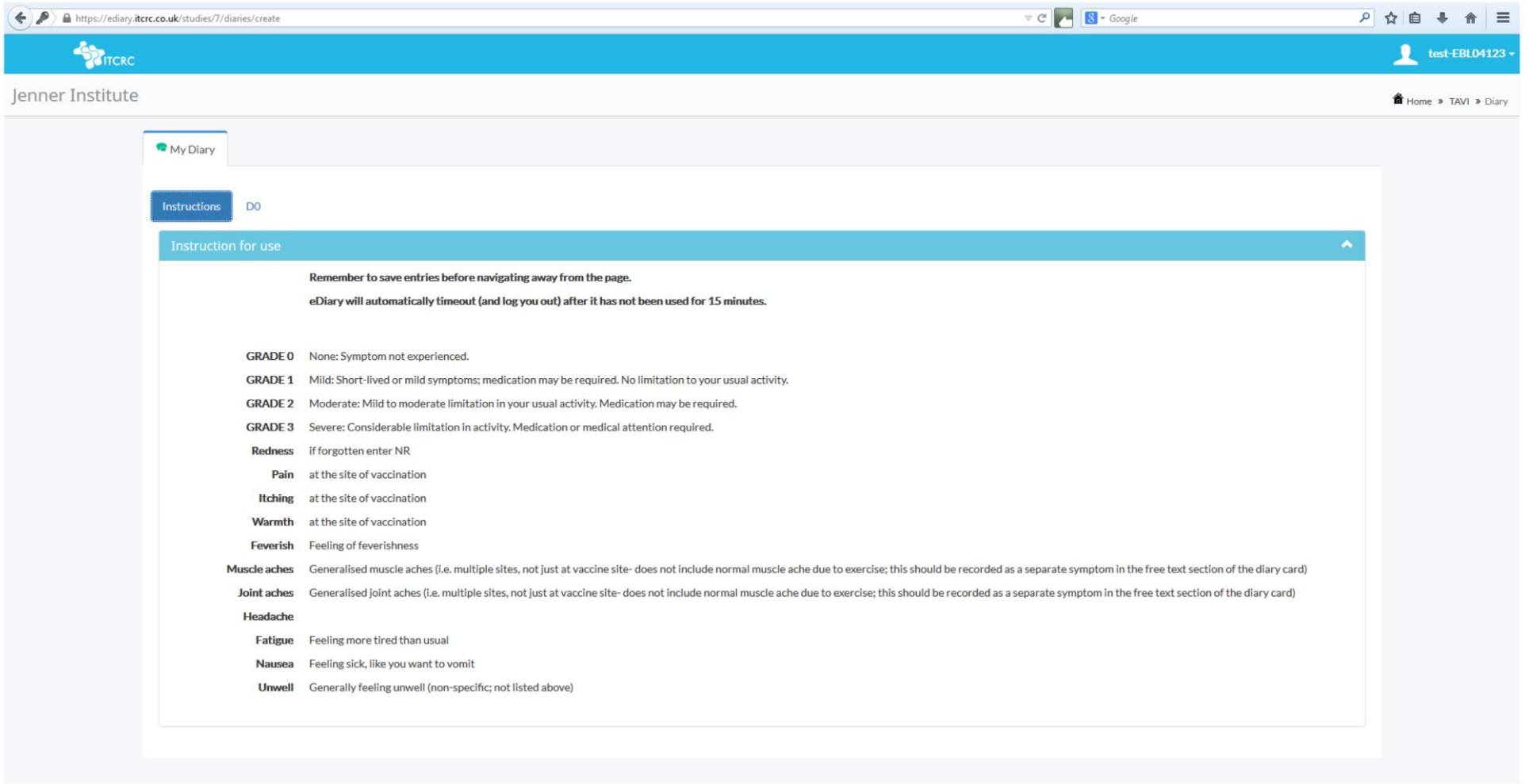
Page 3 of 12

Generic eDiary screenshots

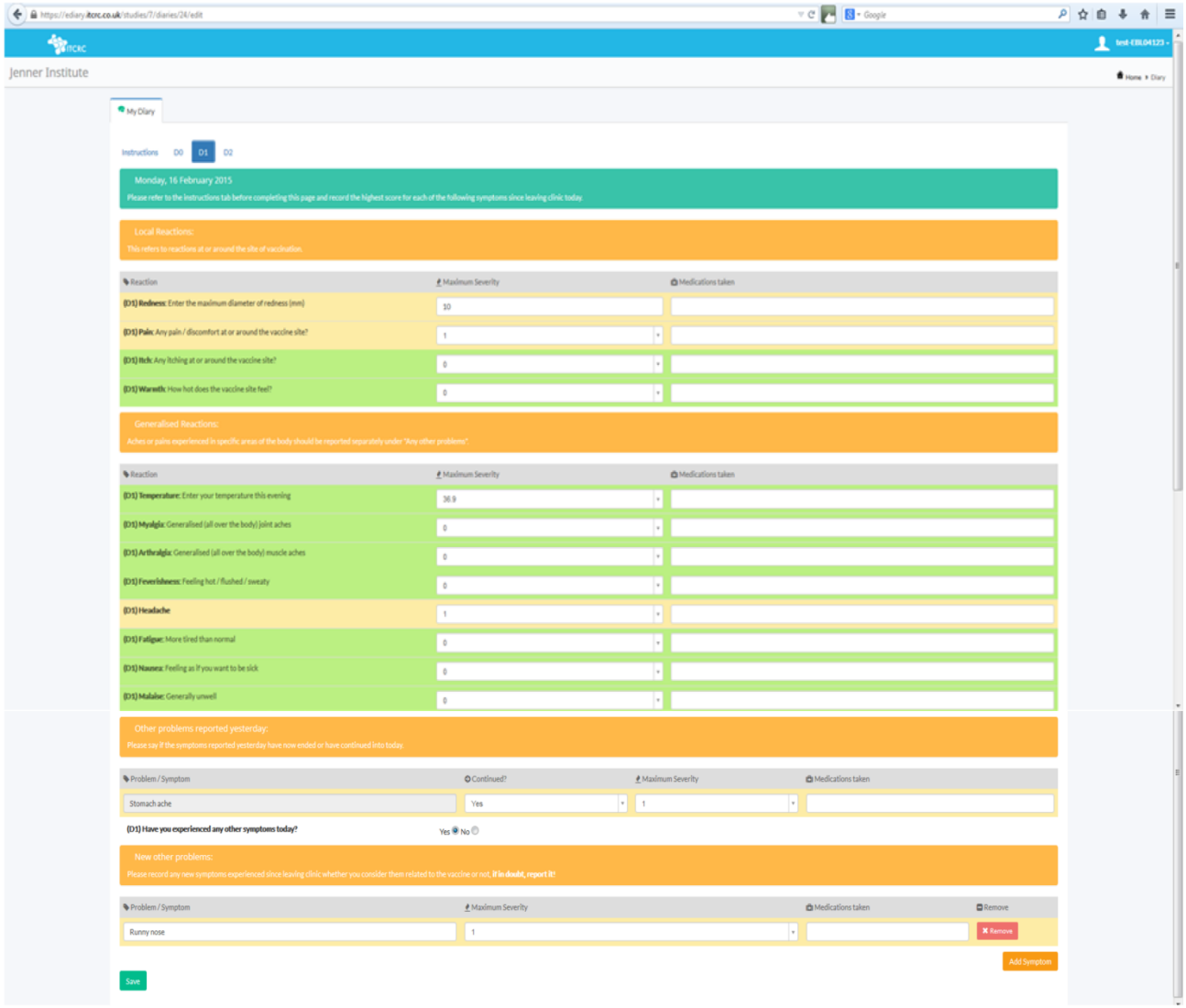
Login page



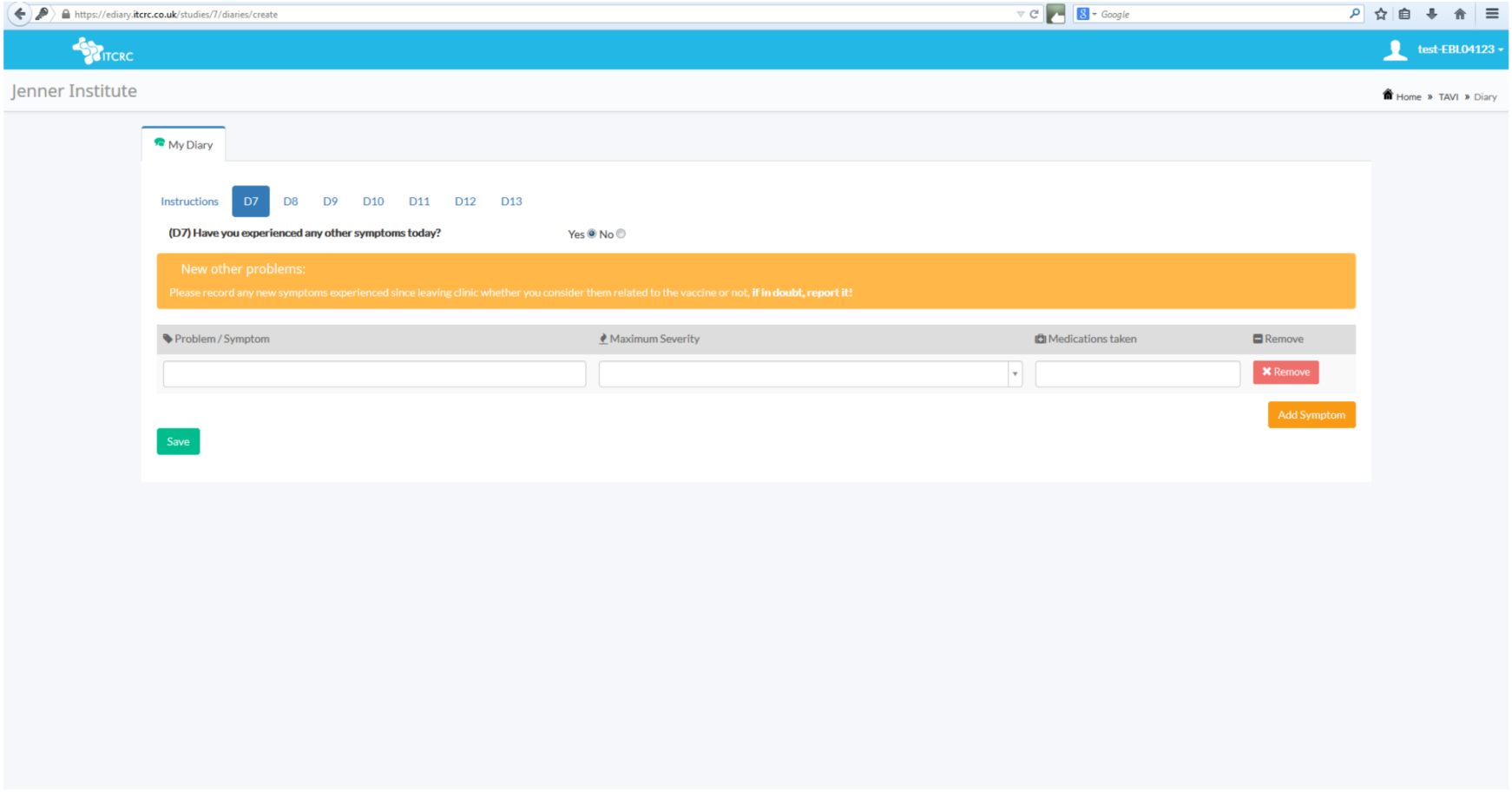
Instruction page



Example page days 0 – 6



Example page days 7 - 27



Appendix 6: Site-specific severity grading tables for laboratory adverse events

GRADING OF LABORATORY ADVERSE EVENTS- OXFORD v1.0

Severity grading criteria for clinically significant laboratory abnormalities; adapted from FDA guidelines (1) using Oxford University Hospitals NHS Trust laboratory reference ranges.

Laboratory Test	Grade 1	Grade 2	Grade 3
Hgb (female) – gm/dL Ref range 12.0 – 15.0	10.5 – 11.5	9.0 – 10.4	<9.0
Hgb (male) – gm/dL Ref range 13.0 – 17.0	11.5 – 12.5	10.0 – 11.4	<10.0
WBC- elevated (x10*9/L) Ref range 4.0-11.0	11.50 – 15.00	15.01 – 20.00	>20.0
WBC- low (x10*9/L) Ref range 4.0-11.0	2.50 – 3.50	1.50 – 2.49	<1.50
Neutrophils decrease (x10*9/L) Ref range 2.0-7.0	1.00 – 1.49	0.50 – 0.99	<0.50
Lymphocytes decrease (x10*9/L) Ref range 1.0-4.0	0.75 – 1.00	0.50 – 0.74	<0.50
Eosinophils (x10*9/L) Ref range 0.0-0.5	0.65 – 1.50	1.51 – 5.00	>5.00
Platelets (x10*9/L) Ref range 150-400	125 – 135	100 – 124	<100
Bilirubin – when accompanied by any increase in Liver Function Test increase by factor Ref range 3-17 (umol/L)	1.1 – 1.25 x ULN	>1.25 – 1.5 x ULN	>1.5 – 1.75 x ULN
Bilirubin- when LFTs normal; increase by factor Ref range 3-17 (umol/L)	1.3 – 1.5 x ULN	1.6 – 2.0 x ULN	>2.0 x ULN
ALT, AST; increase by factor Ref range 10-45 (IU/L)	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 x ULN
Alkaline phosphate- increase by factor Ref range 95-280 (IU/L)	1.1 – 2.0 x ULN	2.1 – 3.0 x ULN	>3.0 x ULN
Albumin- low (g/L) Ref range 35-50	28 – 31	25 – 27	<25
Creatinine Ref range 54-145 (umol/L)	1.1–1.5 x ULN	>1.6–3.0 x ULN	>3.0 x ULN
Urea (mmol/L) Ref range 2.5-6.7	8.2 – 8.9	9.0 – 11.0	>11.0
Sodium- elevated (mmol/L) Ref range 135-145	146 – 147	148 – 149	≥150
Sodium- low (mmol/L) Ref range 135-145	132 – 134	130 – 131	≤129
Potassium- elevated (mmol/L) Ref range 3.5-5.0	5.1 – 5.2	5.3 – 5.4	≥5.5
Potassium- low (mmol/L) Ref range 3.5-5.0	3.2 –3.3	3.0 – 3.1	≤2.9

1. FDA. Toxicity Grading Scale for Healthy Adult & Adolescent Volunteers Enrolled in Preventative Vaccine Clinical Trials

GRADING OF LABORATORY ADVERSE EVENTS- SOUTHAMPTON v1.0

Severity grading criteria for clinically significant laboratory abnormalities; adapted from FDA guidelines (1) using University Hospital Southampton NHS Foundation Trust laboratory reference ranges.

Laboratory Test	Grade 1	Grade 2	Grade 3
Hgb (female) – gm/L Ref range 120 – 150	105 – 115	90 – 104	<90
Hgb (male) – gm/L Ref range 130 – 170	115 – 125	100 – 114	<100
WBC- elevated (x10*9/L) Ref range 4.0-11.0	11.50 – 15.00	15.01 – 20.00	>20.0
WBC- low (x10*9/L) Ref range 4.0-11.0	2.50 – 3.50	1.50 – 2.49	<1.50
Neutrophils decrease (x10*9/L) Ref range 2.0-7.5	1.00 – 1.49	0.50 – 0.99	<0.50
Lymphocytes decrease (x10*9/L) Ref range 1.5-4.0	0.75 – 1.00	0.50 – 0.74	<0.50
Eosinophils (x10*9/L) Ref range 0.0-0.5	0.65 – 1.50	1.51 – 5.00	>5.00
Platelets (x10*9/L) Ref range 150-400	125 – 135	100 – 124	<100
Bilirubin – when accompanied by any increase in Liver Function Test increase by factor Ref range 0-20 (umol/L)	1.1 – 1.25 x ULN	>1.25 – 1.5 x ULN	>1.5 – 1.75 x ULN
Bilirubin- when LFTs normal; increase by factor Ref range 0-20 (umol/L)	1.2 – 1.5 x ULN	1.6 – 2.0 x ULN	>2.0 x ULN
ALT, AST; increase by factor Ref range 7-40 (IU/L)	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 x ULN
Alkaline phosphate- increase by factor Ref range 30-130 (IU/L)	1.1 – 2.0 x ULN	2.1 – 3.0 x ULN	>3.0 x ULN
Albumin- low (g/L) Ref range 35-50	28 – 31	25 – 27	<25
Creatinine Ref range 53-97 (umol/L)	1.1–1.5 x ULN	>1.6–3.0 x ULN	>3.0 x ULN
Urea (mmol/L) Ref range 2.5-7.8	8.2 – 8.9	9.0 – 11.0	>11.0
Sodium- elevated (mmol/L) Ref range 133-146	147 – 148	149 – 150	>150
Sodium- low (mmol/L) Ref range 133-146	131 – 132	129 – 130	<129
Potassium- elevated (mmol/L) Ref range 3.5-5.3	5.4 – 5.5	5.6 – 5.7	>5.7
Potassium- low (mmol/L) Ref range 3.5-5.3	3.2 –3.3	3.0 – 3.1	≤2.9

1. FDA. Toxicity Grading Scale for Healthy Adult & Adolescent Volunteers Enrolled in Preventative Vaccine Clinical Trials

GRADING OF LABORATORY ADVERSE EVENTS FOR HAMMERSMITH HOSPITALS v1.1

Severity grading criteria for clinically significant laboratory abnormalities; adapted from FDA guidelines (1) using Hammersmith Hospitals NHS Trust laboratory reference ranges.

Laboratory Test	Grade 1	Grade 2	Grade 3
Hgb (female) – gm/L Ref range 130 – 168	105 – 115	90 – 104	<90
Hgb (male) – gm/L Ref range 130 – 168	115 – 125	100 – 114	<100
WBC- elevated (x10*9/L) Ref range 4.2-10.6	11.50 – 15.00	15.01 – 20.00	>20.0
WBC- low (x10*9/L) Ref range 4.2-10.6	2.50 – 3.50	1.50 – 2.49	<1.50
Neutrophils (x10*9/L) Ref range 2.0-7.1	1.00 – 1.49	0.50 – 0.99	<0.50
Lymphocytes Decrease (x10*9/L) Ref range 1.1-3.6	0.75 – 1.00	0.50 – 0.74	<0.50
Eosinophils (x10*9/L) Ref range 0.0-0.5	0.65 – 1.50	1.51 – 5.00	>5.00
Platelets (x10*9/L) Ref range 130-370	110 – 120	95 – 109	<95
Bilirubin – when accompanied by any increase in Liver Function Test increase by factor Ref range 0-21 (umol/L)	1.1 – 1.25 x ULN	>1.25 – 1.5 x ULN	>1.5 – 1.75 x ULN
Bilirubin- when LFTs normal; increase by factor Ref range 0-21 (umol/L)	1.2 – 1.5 x ULN	1.6 – 2.0 x ULN	>2.0 x ULN
ALT, AST; increase by factor Ref range 0-40 (IU/L)	1.25 – 2.5 x ULN	>2.5 – 5.0 x ULN	>5.0 x ULN
Alkaline phosphate- increase by factor Ref range 30-130 (IU/L)	1.1 – 2.0 x ULN	2.1 – 3.0 x ULN	>3.0 x ULN
Albumin- low (g/L) Ref range 35-50	28 – 31	25 – 27	<25
Creatinine Ref range 60-125 (umol/L)	1.1–1.5 x ULN	>1.6–3.0 x ULN	>3.0 x ULN
Urea (mmol/L) Ref range 2.5-7.8	8.2 – 8.9	9.0 – 11.0	>11.0
Sodium- elevated (mmol/L) Ref range 133-146	146 – 147	148 – 149	≥150
Sodium- low (mmol/L) Ref range 133-146	132 – 134	130 – 131	≤129
Potassium- elevated (mmol/L) Ref range 3.5-5.3	5.4 – 5.5	5.6 – 5.7	≥5.8
Potassium- low (mmol/L) Ref range 3.5-5.3	3.2 –3.3	3.0 – 3.1	≤2.9

1. FDA. Toxicity Grading Scale for Healthy Adult & Adolescent Volunteers Enrolled in Preventative Vaccine Clinical Trials

Appendix 7: Standard Operating Procedures

ML002: Malaria PBMC Separation and Freezing

Document Category:	Standard Operating Procedure
Code:	ML002
Title:	Malaria PBMC Separation and Freezing
Version:	4.0
Authors:	Katie Ewer
Authorised By:	Adrian Hill
Section:	Jenner Institute Malaria Trials Laboratory
Number Of Copies:	1
Location Of Copy:	Malaria SOPs ORCRB

Table of Contents

1 PURPOSE

During malaria vaccine trials, blood samples are taken from volunteers for exploratory immunology. This SOP contains the detailed protocol for separating and storing PBMC, plasma and serum.

2 INTRODUCTION

The aim of our clinical vaccine trials is to induce protection against malaria in human volunteers and characterise the cells involved in inducing protective immune responses. The primary endpoint in Phase I and Phase II trials is immunogenicity as measured by the ex vivo IFNγ ELISpot assay, which quantifies the frequency of antigen-specific effector T cells producing IFNγ after overnight culture. However, other immunological assays may be performed at different time points to the ex vivo IFNγ ELISpot assay, requiring the preservation and storage of additional samples.

3 SCOPE

This SOP covers all blood samples taken for exploratory immunology from volunteers in malaria vaccine trials in Oxford for which ex vivo ELISpot is not the primary readout. It does not cover booking in of samples to the laboratory, which is described in ML001 Malaria Sample Handling.

4 DEFINITIONS/ABBREVIATIONS

PBMC	Peripheral Blood Mononuclear Cell
IFN	Interferon
ELISPOT	Enzyme Linked Immunosorbent Spot

5 RESPONSIBILITIES

All staff employed by University of Oxford who work on the malaria vaccine, trial including clinicians or visiting scientists working in the lab, must follow this procedure.
Senior Immunologist ensures staff are competent to perform these procedures.

6 PROCEDURE

6.1 Reagents

Reagent	Company	Cat No
RPMI	Sigma	R0883
Penicillin/Streptomycin	Gibco BRL/ Invitrogen	15140-122
L-Glutamine	Gibco	25030-24
FCS (heat-inactivated, filtered and batch tested)	Biosera	S1810
Leucosep tubes	Greiner	227209
0.22 µm filters for serum PVDF membrane	Fisher Scientific	FDR-120-060Y
Casyton counting buffer	Sedna Scientific	43003
Casyton tubes	Sedna Scientific	43001
DMSO	Sigma	D2650
Cryobabies labels	Jencons	
Mr Frosty Cryocontainers	Fisher	CRY-120-010T
CoolCell	Biocision	BCS-405
Propan-2-ol	BDH	296946H
Lymphoprep	Axis Shield	1114545
Cryovials	Nunc	CRY-960-130J
RBC Lysis Solution	Qiagen	158902/4

FCS: Is heat-inactivated by the supplier. Filter with 0.22µm filter, make 50mL aliquots and store at -20°C.

Pen/Strep and L-Gln: prepare aliquots (typically 5mL) of each reagent as required. Store at -20°C.

6.2 Day 0 day before sample arrives in lab (or further in advance if applicable).

1. Prepare Leucosep tubes in MSC. Pipette 15mL Lymphoprep into a 50mL Leucosep tube. Centrifuge at 1000 x g for 1 min to get the Lymphoprep below the below the porous filter disc. Label tubes and lids with subject number and store in the dark.
2. Label serum, plasma, and cryovials. Print labels onto cryobabies sheets. Wear gloves when attaching labels to tubes to maximize adhesiveness.
3. Place Mr Frostys in the fridge, (1 per 3 volunteers). Alternatively use CoolCell which does not require previous chilling.
4. Make up R0 (500mL RPMI + 5mL Pen/strep + 5mL L-glutamine) and R10 (500mL RPMI + 5mL Pen/strep + 5mL L-glutamine + 50mL filtered FCS).

6.3 Day 1 samples must be processed in a microbiological safety cabinet.

1. Record samples in the MVT lab book (see *ML001* Malaria Sample Handling.

Processing blood

2. Store serum tubes in the fridge (if not immediately processed), otherwise spin at 1800 rpm for 5 min, collect serum with a transfer pipette and store in aliquots as required by the trial specific Analytical Plan. Store at -80ºC and record location in record folder.

3. Pour 15-30mL heparinised blood into a Leucosep tube (typically each 40-50mL blood sample is divided into two Leucosep tubes, although for bleed volumes refer to the trial specific Analytical Plan) and centrifuge at 1000 x g for 13 min at RT without brake (programme 4). Record the time on the sample record sheet. Once centrifuge begins to slow, brake may be applied once it reaches below 500 x g.

4. Take samples of the plasma fraction (as required in the trial specific Analytical Plan) and place in labelled cryovials. Store at -80°C and record location in record folder.

5. Pour excess plasma containing the PBMC from each tube into a new, labelled 50mL falcon tube. This should amount to approximately 10-20mL of cells.

6. Top the tubes up to 40mL with R0, and spin at 1800rpm for 5min at room temperature (RT) (programme 1).

7. Pour off the supernatant, and flick the cell pellet to resuspend it, and pool the 2 tubes of cells from one donor together; add 30mL of R0 to cells in one of the falcon tubes, transfer this volume to the other tube. Spin at 1800rpm for 5 min at RT (programme 1).

8. If the pellet shows significant contamination with red blood cells, then pour off the supernatant and resuspend the pellet in 5mL RBC Lysis Solution. Rest the cells for maximum 5 min before topping up to 30mL with R0. Spin at 1800 rpm for 5 min at RT (programme 1).

9. Pour off the supernatant, flick the pellet, and resuspend in 10 ml R10 for counting.

10. If required to store the cells prior to plating out, do so with the lids loosely attached and in the 37°C incubator.

Counting cells

11. Prepare the CASY counter by running CASY Clean twice through the chamber, then running CASYTON through the chamber twice.

12. Casy counter Program 1 (CELL DIAMETER 5.75 µM – 15µM, count 2 samples, each of volume 400µL, 150µM capillary).

13. Place 10µL of each cell suspension into exactly 10mL CASYTON solution. Record the number of cells counted (cnt) and the time they are counted on the sample record sheet.

14. If the counts don't agree within 15%, then the above step should be repeated. If the counter reports the counts are too high, then the above step should be repeated except that 5µL of each cell suspension should be pipetted into exactly 10mL CASYTON solution and the count doubled.

15. Enter the counts into the trial-specific Excel file on the network (V:\1.Malaria\1. Master Files\VAC Studies\VACxxx\xx. Immunology\Cell counts). Print and attach to the Standard record sheet.

16. Resuspend cells according to calculations on the cell count spreadsheet. This will vary between trials as described in the trial specific Analytical Plan.

17. Proceed to assay.

Freezing cells

18. Resuspend the remaining cells up to a maximum volume of 5 mL FCS (i.e. 0.5 mL of FCS per vial to be frozen, always have at least 5 million cells per vial, normally only freeze a maximum of 10 vials). Record the label on the FCS to the Standard record sheet.

19. Place the cells in the fridge for 20-30 min.

20. Add an equal volume of ice-cold 20% DMSO in FCS (5 mL for 10 vials) to the cells and mix by pipetting gently up and down. Record the time of adding DMSO-FCS to the Standard record sheet.

21. Aliquot the cell suspensions to labelled cryovials (1mL per vial) and immediately place them in the Mr Frosty or CoolCell containers. As soon as one container is full, place it in the –80°C freezer. If using CoolCell and samples to not fill container, add pre-made freeze media containing 'blank' vials to fill spaces before placing at –80°C.

22. After an overnight (or over weekend if frozen on a Friday) incubation in the –80°C, transfer the frozen cells to liquid nitrogen and record location in record folder.

7 ASSOCIATED DOCUMENTS

Vaccine trials record sheet
ML001 Malaria Sample Handling

8 REFERENCES

None

9 REVIEW HISTORY

Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
12/04/12	Simon Draper Katie Ewer	3.0	Update to reflect current practice. Reformatted. Update or references and removal of sample sample tracking form.	2.1
25/01/16	Sean Elias	4.0	Updated to include CoolCell freezing procedure.	3.0

ML006: Ex Vivo ELISPOT

Document Category:	Standard Operating Procedure
Code:	ML006
Title:	Ex Vivo ELISPOT
Version:	5.0
Authors:	Katie Ewer
Authorised By:	Adrian Hill
Section:	Jenner Institute Malaria Trials Laboratory
Number Of Copies:	1
Location Of Copy:	Malaria SOPs ORCRB

Table of Contents

1 PURPOSE

During vaccine trials, the *ex vivo* ELISPOT is either the primary or secondary assay for monitoring the immunological response to vaccination in human volunteers. This SOP describes the procedure for reading and counting ELISPOT plates and the criteria for assessing and interpreting spot counts.

2 INTRODUCTION

The aim of our clinical vaccine trials is to induce protection against disease in human volunteers and characterise the cells involved in inducing protective immune responses. The primary/secondary endpoint (depending on antigen/trial) in Phase I and Phase II trials is immunogenicity as measured by the *ex vivo* IFNγ ELISpot assay, which quantifies the frequency of antigen-specific effector T cells producing IFNγ after overnight culture. In this case, the antigens are pools of peptides from the vaccine as well as positive controls. Different types of positive controls are included; PHA-L is used to induce a mitogenic response and demonstrate viability of lymphocytes; SEB and PMA may also be included as positive controls; CTL (also known as FEC or CEF) is a pool of peptides from influenza, Epstein Barr virus and cytomegalovirus known to induce CD8+ T cell responses. Typically, a mixture of PHA-L and SEB is used as positive control. A negative (cells and medium) control is also included.

ELISPOT plates are coated with a monoclonal antibody against IFNγ overnight The assay is performed by separating PBMC from fresh blood and incubating a known number of cells per well with antigen in duplicate or triplicate for each stimulation. After overnight incubation, cells are discarded, the plate is washed and a second, biotinylated monoclonal antibody against IFNγ is added. After another wash step, streptavidin alkaline phosphatase is added and then finally the alkaline phosphatase substrate is added after a third wash step. The substrate is left to develop until spots are clearly visible when the plate is washed. After drying overnight, the spots in each well are counted by an automated plate counter. The result is obtained by subtracting any background response and then taking an average of the duplicate pairs or triplicate set of wells, depending on antigen/trial.

In order for the results of an ELISPOT assay to be considered valid, certain criteria must be met to ensure the reliability and reproducibility of the assay. It is important that the procedures involved in determining final ELISPOT results for clinical vaccine trial samples are as consistent as possible across trials to allow valid comparison of results. This relies on following the same procedures and minimising subjective intervention, either in reading or interpreting ELISPOT data.

3 SCOPE

This SOP covers all blood samples taken from volunteers in malaria vaccine trials in Oxford for which *ex vivo* ELISpot is either the primary or secondary readout. It does not cover booking in of samples to the laboratory, which is described in ***ML001*** Malaria Sample Handling, nor does it cover counting and interpretation of ELISpot results which is contained in ***VL001*** ELISPOT Calibration Procedure and Settings and ***VL002*** ELISPOT Counting and Pass/Fail Criteria.

4 DEFINITIONS/ABBREVIATIONS

Nil

5 RESPONSIBILITIES

All staff employed by University of Oxford who work on the malaria vaccine, trial including clinicians or visiting scientists working in the lab, must follow these protocols.

Senior Immunologist ensures staff are competent to perform these procedures.

6 PROCEDURE

6.1 Reagents

Reagent	Company	Cat No
RPMI	Sigma	R0883
Penicillin/Streptomycin	GibcoBRL/ Invitrogen	15140-122
L-Glutamine	Gibco	25030-24
FCS (heat-inactivated, filtered and batch tested)	Biosera	S1810
PBS	Gibco	21600-069
TWEEN-20	Sigma	9005-64-5
Leucosep tubes	Greiner	227209
0.22 µm filters for serum PVDF membrane	Fisher Scientific	FDR-120-060Y
Casyton counting buffer	Sedna Scientific	43003
Casyton tubes	Sedna Scientific	43001
DMSO	Sigma	D2650
Cryobabies labels	Jencons	
Mr Frosty Cryocontainers	Fisher	CRY-120-010T
Cool Cell Cryocontainers	Biocision	FTS30 BTS-170
Propan-2-ol	BDH	296946H
Lymphoprep	Axis Shield	1114545
Cryovials	Nunc	CRY-960-130J
PHA-L	Sigma	L4144
SEB	Sigma	S4881
PBS	Sigma	3813
Plate Sealers – TiterTops	Scientific Laboratory Supplies	PJB-720-030M
U-bottom 96 well plates	VWR	402030716
ELISPOT plates (MultiScreen-IP)	Millipore	MAIPS4510
Carbonate Buffer Capsules	Sigma	C-3041
RBC Lysis Solution	Qiagen	158902/4
IFN-γ Antibody – Catcher (1-DIK)	Mabtech	3420-2A
IFN-γ Antibody – Detector (7-1B6-Biotin)	Mabtech	3420-2A
Streptavidin-ALP	Mabtech	3420-2A
BCIP/ NBT plus	Moss Inc / Europa Bioproducts	NBTH-1000
Peptides	Variable	

Peptide Pools: See ***ML007*** Peptide Pool Preparation. Peptide pool calculations should be done so that there are enough stimulant plates for the whole trial. Plates should be pre-aliquotted into 96 well plates, sealed with plate sealers and stored at -80°C until needed. Final peptide concentration in the well is dependent on the trial (typically 5-10µg/mL). See trial specific Analytical Plan for peptide specific requirements as well as positive control information.

FCS: Is heat-inactivated by the supplier. Filter with 0.22µm filter, make 50mL aliquots and store at -20℃.

Pen/Strep and L-Gln: prepare aliquots (typically 5mL) of each reagent as required. Store at -20℃.

Coating Buffer: One carbonate buffer capsule should be added per 100mL filtered water. Typically prepare bottles of 500mL. These should be autoclaved and labeled with name and date of preparation.

Positive Controls: These should be prepared in single use aliquots as described in the trial specific Laboratory Plan.

SEB: If required for use in the positive control (see trial specific Analytical Plan), storage of SEB aliquots must be recorded with the Jenner Institute Laboratory Manager or named person responsible.

6.2 Day 0 Preparation - day before sample arrives in lab (or further in advance if applicable).

- **Coat MAIP plates in Class II microbiological safety cabinet (MSC)**, typically 2-4 volunteers per plate (see trial specific Analytical Plan). Make the coating solution by adding 10 µl of catcher antibody (1-D1K) per mL of ELISPOT coating buffer resulting in a concentration of 10µg/mL. Add 50µL per well of the coating solution to the ELISPOT plate. Keep at RT for 3-8 h or at +4℃ for 8-72 h, plates must be wrapped in cling film to prevent evaporation of antibody solution. Note time of coating on plate, which must be transferred to the sample record sheet the following day.

- **Prepare Leucosep tubes in MSC.** Pipette 15mL Lymphoprep into a 50mL Leucosep tube. Centrifuge at 1000 x g for 1 min to get the Lymphoprep below the below the porous filter disc. Label tubes and lids with subject number and store in the dark.

- **Label serum, plasma, and cryovials.** Print labels onto cryobabies sheets. Wear gloves when attaching labels to tubes to maximize adhesiveness.

- **Place Mr Frostys in the fridge**, (1 per 3 volunteers).

- **Make up R0** (500mL RPMI + 5mL Pen/strep + 5mL L-glutamine) **and R10** (500mL RPMI + 5mL Pen/strep + 5mL L-glutamine + 50mL filtered FCS).

6.3 Day 1 Sample Processing

Samples must be processed in a microbiological safety cabinet.

Record samples, plate number(s) and experiment number in the MVT lab book (see ***ML001*** Malaria Sample Handling.)

6.3.1 Blocking plates

- Flick off the coating solution, and wash the plates 3 times with 100µL per well of sterile PBS using a multichannel pipette.

- Block the wells with 100µL per well of R10. Keep the plate at RT for 1-8 h or at +4°C for 8-48 h. Record the time of blocking on the sample record sheet.

- Thaw peptide plates in the hood.

6.3.2 Processing Blood

- Store serum tubes in the fridge (if not immediately processed), otherwise spin at 1800 rpm for 5 min, collect serum with a transfer pipette and store in aliquots as required by the trial specific analytical Plan. Store at -80ºC and record location in record folder.

- Pour 15-30mL heparinised blood into a Leucosep tube (typically each 40-50mL blood sample is divided into two Leucosep tubes, although for bleed volumes refer to the trial specific analytical Plan) and centrifuge at 1000 x g for 13 min at RT without brake (programme 4). Record the time on the sample record sheet.

- Take samples of the plasma fraction (as required in the trial specific analytical Plan) and place in labelled cryovials. Store at -80℃ and record location in record folder.

- Pour excess plasma containing the PBMC from each tube into a new, labelled 50mL falcon tube. This should amount to approximately 10-20mL of cells.

- Top the tubes up to 40mL with R0, and spin at 1800rpm for 5min at room temperature (RT) (programme 1).

- Pour off the supernatant, and flick the cell pellet to resuspend it, and pool the 2 tubes of cells from one donor together; add 30mL of R0 to cells in one of the falcon tubes, transfer this volume to the other tube. Spin at 1800rpm for 5 min at RT (programme 1).

- If the pellet shows significant contamination with red blood cells, then pour off the supernatant and resuspend the pellet in 5mL RBC Lysis Buffer. Rest the cells for maximum 5 min before topping up to 30mL with R0. Spin at 1800 rpm for 5 min at RT (programme 1).

- Pour off the supernatant, flick the pellet, and resuspend in 10 ml R10 for counting.

- If required to store the cells prior to plating out, do so with the lids loosely attached and in the 37℃ incubator.

6.3.3 Counting cells

- Prepare the CASY counter by running CASY Clean twice on the clean cycle. Then run the CASY clean once on the start cycle before running CASYTON twice on the start cycle.

- Casy counter Program 1 (CELL DIAMETER 5.75 µM – 15µM, count 2 samples, each of volume 400µL, 150µM capillary.)

- Place 10µL of each cell suspension into exactly 10mL CASYTON solution and invert at least 20 times to mix thoroughly. Record the number of cells counted (cnt) and the time they are counted on the sample record sheet. (Note: count immediately as CASYTON is toxic to cells.)

- If the counts don't agree within 15%, then the above step should be repeated. If the counter reports the counts are too high, then the above step should be repeated except that 5µL of each cell suspension should be pipetted into exactly 10mL CASYTON solution and the count doubled.

- Enter the counts into the trial-specific Excel file on the network (V:\1.Malaria\1. Master Files\VAC Studies\VACxxx\xx. Immunology\Cell counts). Print and attach to the Standard record sheet.

- Suspend cells according to calculations on the cell count spreadsheet. This will vary between trials as described in the trial specific analytical Plan.

6.3.4 Plating out

- Flick off the blocking solution from plate, and add peptides and other stimulants to the wells by transferring 50µL of solution from the stimulant plate to the corresponding wells on the ELISPOT plate with a multichannel pipette. Add 50µL of cell suspension the appropriate wells on the plate. Record the time of addition of cells to the plate on the sample record sheet.

- Incubate the plate for 18-20 h at +37℃ 5%CO_2 in the TC incubator.

- Spin remaining cells in the 50mL falcon at 1800 rpm 5 min at RT.

6.3.5 Freezing cells

- Resuspend the remaining cells up to a maximum volume of 5 mL FCS (i.e. 0.5 mL of FCS per vial to be frozen, always have at least 5 million cells per vial, normally only freeze a maximum of 10 vials). Record the label on the FCS to the Standard record sheet.
- Place the cells in the fridge for 30 min.
- Add an equal volume of ice-cold 20% DMSO in FCS (5 mL for 10 vials) to the cells and mix by pipetting gently up and down. Record the time of adding DMSO-FCS to the Standard record sheet.
- Aliquot the cell suspensions to labelled cryovials (1mL per vial) and immediately place them in the Mr Frosty or Cool Cell containers. As soon as one container is full, place it in the –80℃ freezer.
- After an overnight (or over weekend if frozen on a Friday) incubation in the –80℃, transfer the frozen cells to liquid nitrogen and record location in record folder.

6.4 Day 2 Developing

- Wearing safety goggles, flick off the cell suspension into a container of Microsol or other suitable disinfectant, and, using the immunowash, wash 6 times with PBS-Tween. Flick off the washing solution and blot onto absorbent paper. At this stage, the plate may be left with 100µL of PBS in each well and left in the fridge for up to 48 h before developing.
- Dilute the detector antibody (7-B6-1-Biotin) 1:1000 in PBS resulting in a concentration of 1µg/mL, and add 50µL to each well. Record the time of addition of the detector antibody and the batch number of the antibody on the Standard record sheet.
- Incubate the plate for 2-4 h at RT.
- Flick off the detector antibody and, using the immunowash, wash 6 times with PBS-Tween. Flick off the washing solution and blot onto absorbent paper.
- Dilute the SA-ALP 1:1000 in PBS, and add 50µL to each well. Record the time of addition of the SA-ALP and the batch number of the SA-ALP to the Standard record sheet. Incubate for 1-2 h at RT.
- Flick off the SA-ALP and, using the plate washer, wash 6 times with PBS-Tween. Flick off the washing solution and blot onto absorbent paper.
- Pour 5mL of developer per plate into a Falcon tube. Add 50µL per well and develop for approximately 3-15 min. Record time of developer addition on the standard record sheet and length of developer incubation.
- Wearing safety goggles, flick off the developing reagent into the sink. Wash plates in tap water, remove and wash backing and dry overnight on paper towel. Record time of washing on the standard record sheet.

7 ASSOCIATED DOCUMENTS

None

8 REFERENCES

None

9 REVIEW HISTORY

The review history allows a record to be kept of when and who reviews the document to ensure it remains fit for purpose. Significant changes should be recorded in the 'Detail' section.

Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
19/May/09	Katie Ewer	3.0	Re-format.	2.0
29/March/12	Katie Ewer & Simon Draper	4.0	Minor changes	3.0
01/April/14	Kathryn Milne & Sean Elias	5.0	Minor changes and reformatting	4.0

ML026: DBP ELISA

1 Purpose

During malaria vaccine trials, the Enzyme-Linked ImmunoSorbent Assay (ELISA) is the primary assay for monitoring the antibody responses induced by vaccination in the serum of human volunteers. This SOP contains the detailed experimental protocol for performing this assay for the antigen *Plasmodium vivax* Duffy-Binding Protein (PvDBP). There are multiple alleles of the PvDBP antigen, but only one is tested in this ELISA assay: PvDBP_RII (Salvador I allele). This SOP details the experimental protocol for performing this assay for this allele of PvDBP.

2 Introduction

The aim of our clinical vaccine trials is to induce protection against malaria in human volunteers and characterize the cells and/or antibodies involved in inducing protective immune responses. One of the primary endpoints in Phase I and Phase II trials of blood-stage malaria vaccines is immunogenicity as measured by ELISA, which quantifies the level of antigen-specific antibodies in the serum of immunized volunteers. In this case, the PvDBP antigen was provided by Jing Jin. The ELISA protocol has been standardised according to published methods (see reference in section 8). This protocol uses a reference serum on each ELISA plate to detect antibodies induced by experimental malaria vaccines.

Different types of controls are included; reference serum from a high responding volunteer is included on each ELISA plate to produce a standard curve which is used to quantify and assign ELISA units to each unknown sample on the plate; also an internal positive control sample and negative control / no serum (“blank wells”) are used to perform QC analysis on each ELISA plate.

ELISA plates are coated over-night with PvDBP_RII. The assay is performed by preparing a standard curve and internal controls from the reference serum and adding these samples to the plate. Unknown test serum samples from immunized volunteers are diluted and added in triplicate to the ELISA plate. After a two hour incubation period, the diluted sera are discarded, the plate is washed and a secondary polyclonal antibody against the γ–chain of human IgG is added. This secondary antibody is conjugated to the enzyme alkaline phosphatase. After another hour of incubation, followed by a wash step, the alkaline phosphatase substrate is added. The substrate is left to develop for 15-20 min and the absorbance at 405nm is read using a plate reader. The result is obtained by taking an average of the triplicate wells for each test sample, and using the standard curve to assign DBP_RII ELISA arbitrary units (AU).

3 Scope

This SOP covers all serum samples taken from volunteers in PvDBP_RII blood-stage malaria vaccine trials in Oxford for which ELISA is one of the primary readouts. It does not cover booking in of samples to the laboratory, which is described in ***ML001*** Malaria lab sample handling.

4 Definitions

ELISA = Enzyme-Linked ImmunoSorbent Assay.

5 Responsibilities

All staff employed by the University of Oxford who work on the blood-stage malaria vaccine trials, including clinicians or visiting scientists working in the lab, must follow these protocols.

The **Senior Immunologist** ensures that staff are competent to perform these procedures.

6 Procedure

6.1 Equipment & Reagents

Where appropriate, equipment and reagents used in the processing of samples from clinical trials is dedicated to study work. Equipment used forms part of the laboratory maintenance and monitoring plan.

Equipment:
Fridge at +4^{o}C
Freezer at -20^{o}C and -80^{o}C
Vortex
Eppendorf Racks
Pipettes including 8- or 12-well multi-channel and automatic multi-channel
Pipetteboy
Bio-tek ELx800 Microplate Reader with Gen5 ELISA software v1.10
Timer
Sufficient tips for pipettes. 0.1-10µl, 2-20µl, 20-200µl, 100-1000µl
Safety Glasses
PBS-Tween Hand Washer
Timer

Consumables & Reagents:

Reagent	Company	Cat #
NUNC Immuno Plates (442404)	Fisher	DIS-971-030J
Blocker Starting Block T20 in PBS	Fisher	10270404
Goat anti-human IgG (γ-chain)-alk phos	Sigma	A3187
Dulbecco's PBS (DPBS)	Sigma	D8537
5x Diethanolamine Buffer	Fisher	34064
4-Nitrophenyl Phosphate Tablets (20mg)	Sigma	N2765
Tween-20	Sigma	P7949
Aluminium Foil	Fisher	AKL-300-040J
1.5mL Eppendorf tubes	Fisher	FB74031
Reagent Reservoirs (Costar 4870)	Fisher	PMP-331-010C
10L PBS powder	Invitrogen	21600-069

Recombinant Protein Antigen:

The recombinant protein antigen required for this SOP is: PvDBP (Salvador I allele)

The PvDBP protein is provided by Jing Jin. It is stored in small aliquots (typically enough to coat two ELISA plates) at -20ºC in the human immunology freezer until needed. Coating antigen concentration is standard at 2µg/ml.

Reference Serum:

VAC051 Volunteer 028 day 84 serum is stored in the Equipment Bay MVT -80^{o}C freezer.

Buffers and Solutions:

Make up buffer and solutions as follows:

PBS/T (PBS with 0.05% Tween) for washing plates. Dissolve 10L PBS tub in 10L deionised water (15.0 MΩ setting). Add 5mL Tween-20. Shake and return to the ELISA plate wash station.

6.2 Day 1: Coating ELISA plates on the bench.

1. Print off a new MVT ELISA record sheet for each experiment. Number the experiment with the next experiment number and fill this in with the required information throughout the experiment. The next available experiment number can be found in the Excel spreadsheet contained in the same folder. Sign off the experiment number in the spreadsheet and re-save the file. Sheets can be found at:

   V:\1.Malaria\1. Master Files\5. Lab general trial info\Blood-Stage MVT\ Templates & Record Sheets

2. Calculate the number of Nunc Immuno ELISA plates required (max number of 22 test samples per plate). Thaw an aliquot of the recombinant antigen required. Make the coating solution by adding recombinant antigen to DPBS at a final concentration of 2µg/mL. Working on the bench, add 50 µl per well of the coating solution to the ELISA plate using a multi-channel pipette. Store the plates at 4°C (≥16 h), wrapped in cling film. Note time of coating and record on the experiment layout sheet.

6.3 Day 2: Blocking plates.

1. Bring Starting Block T20 to RT for >30mins and flick off the coating solution into the sink (wear eye protection).

2. Wash the plates 6x in PBS/T using the handheld washer.

3. Block the wells with 200 µL per well of Starting block T20 blocking buffer.

4. Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 1h (max 1h 30min) at RT. Note time of blocking and record on the experiment sheet.

5. During blocking prepare test samples and reference standard dilutions as below.

6.4 Sample Preparation

i) Standard Curve

1. Prepare dilutions of Volunteer 028 day 84 reference serum in Starting block T20 Block buffer:

   Take the serum and prepare one 1:100 dilution in an eppendorf tube: add 9µL to 891µL of blocking buffer. Label 1. Vortex to mix.

Prepare a dilution curve in eppendorf tubes. To make a 1:3 dilution set (enough for 4 ELISA plates):

1. Add 600µL of Starting block T20 block to nine eppendorfs labelled 2-10.
2. Add 300µL of the first dilution 1 to tube 2. Vortex to mix.
3. Add 300µL of 2 to tube 3. Vortex to mix. And so on, repeat this through to tube 10.
4. Each tube should now contain 600µL of liquid (except 10 which contains 900µL), with a 3-fold dilution series running from tube 1 to 10.

ii) Positive Control Sample

Prepare the positive control serum sample in Starting block T20 Block buffer and vortex to mix:

For DBP, make a 1:81 dilution of the reference serum by adding 3µL to 240µL of blocking buffer. Then make a 1:8100 dilution by adding 3µl of the 1:81 stock to 297µl blocking buffer.

Repeat this 2 more times to make 3 independent 1:8100 dilutions.

iii) Test Serum Samples

Prepare the test serum sample in Starting block T20 Block buffer. A dilution is required that will give an OD 405nm reading that is in the linear part of the standard curve (0.26 ≤ OD 405nm ≤ 2.1). Test samples can be tested at a single or multiple dilutions. The dilutions must be recorded on the ELISA record sheet.

Typical dilutions for serum from the vaccine trials include 1:300 for samples taken from Adenovirus only immunised volunteers, or for most samples from volunteers receiving Adeno-MVA regimes taken between d0 – d56. Do not test samples at a dilution lower than 1:300. Serum samples taken on or after d63 should be typically tested at higher dilutions in the range e.g. 1:1000-1:5000 – the necessary dilution will depend on the strength of the response and may range from 1:600-1:40,000.

- To prepare 1:300 dilutions: dilute 3µl serum in 897µl of Starting block T20 block.
- To prepare higher dilutions: dilute the 1:300 dilution appropriately in Starting block T20 block.
- Record all dilutions for each sample on the experimental record sheet.
- Vortex all samples to mix.

6.5 Plating Serum

1. After blocking is complete, wash the plates 6x in PBS/T. Tap them dry on blue roll.
2. Plate serum out using plate layout below. Each well should contain 50µl of sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
G	1	2	3	4	5	6	7	8	9	10	Blank	
H	1	2	3	4	5	6	7	8	9	10		

S1 to S22 (blue) = test sera (added in triplicate).
Standard Curve (pink) = dilution 1 (column 1) to dilution 10 (column 10). Transfer 50µl from tubes 1-10 (see step 6.4.2) to the appropriate wells of rows G and H of the ELISA plate. Repeat for subsequent ELISA plates, etc.
Blank = 50µl of Starting block T20 block solution.

Internal control = 1:8100 V028 day 84 reference serum (see step 6.4.3).

3. Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 2h (max 2h 30min) at RT. Note time of plating and record on the experiment sheet.

6.6 Secondary Antibody

1. After this time, wash the plates 6x in PBS/T. Tap them dry on blue roll.

2. Dilute the secondary antibody 1:1000 in Starting block T20 block solution. 6mL is required per plate, i.e. 6µl secondary antibody in 6mL Starting block T20 block. Secondary antibody is goat anti-human IgG (γ-chain) Sigma A3187 (stored at +4˚C). Vortex to mix.

3. Add 50µl secondary antibody per well.

4. Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 1h (max 1h 30min) at RT. Note time and record on the experiment sheet.

5. Prepare development buffer – for each plate 10ml is required. Buffer must be made up in units of 20ml. Dilute 5x diethanolamine buffer (stored at +4˚C) in deionised water (18.2 MΩ setting). For each unit of 20ml, add one 20mg 4-nitrophenylphosphate tablet (stored at -20˚C) to give a final concentration of 1mg/ml. Prepare the buffer in a suitable tube wrapped in foil, to prevent exposure to light. Leave to stand at RT until required, and shake to mix before use.

6.7 Development

1. Wash the plates 6x in PBS/T. Tap them dry on blue roll.

2. **Make sure the computer and plate reader are available and turned on, before developing.**

3. Using a multi-channel automatic pipette, add 100µl development buffer to each well of plate one. Using the timer, wait 60-90s and then add development buffer to plate 2. Continue adding development buffer to each plate in turn at the same 60-90s interval. Cover the plates in foil and leave on the bench. Make sure there are no bubbles in any of the wells, as this can aberrantly increase the absorbance readings. If bubbles are present, pop these with a clean yellow pipette tip (use a separate tip for each bubble to avoid cross-contamination of development buffer between wells).

6.8 Reading Plates and Analysis

1. During the development period, log on to ELISA station computer.
2. Load Gen5 ELISA software.
3. Create a new experiment using an existing protocol.
4. Select DBP ELISA protocol stored in X:\S Draper\Clinical Trials\ELISA Protocols\150622 MVT PvDBP_RII SS and click OK.
5. Click file menu and save as.
6. Save experiment files as “MVT E xx followed by initials and date (yymmdd) in V:\1.Malaria\1.Master files\2.VAC Studies\VAC051\11.Immunology\ELISA data\Experiments.
7. If you have more than one ELISA plate, right click on Plate 1 and select add plates. Add the number of extra plates required.
8. Click on the + icon next to plate 1 to expand the menu, and select Sample IDs.
9. Enter IDs for samples 1-22 (typically numbered 1-22) and then click OK.

10. Right click on Plate 1 and choose custom plate layout and select Yes.
11. Double click on custom layout in the menu for plate 1.
12. For Sample 1, select 3 vertical replicates and enter the dilution used. Click on Square A1.
13. Enter the dilution used for sample 2 and then click on square A2. Repeat for Sample 3 and click on A3 and so on, until all 22 samples have been accounted for. For Samples 13-22 click on squares D1-D10. Click OK at the end.
14. Repeat these steps for any extra plates in the experiment.
15. After approximately 15 min, read the Abs at 405nm of Plate 1 using the Bio-tek ELx800 microplate reader. Right click on Plate 1 and then “read plate 1” or click green play button in toolbar, click READ and then OK.
16. The Abs 405nm of the six wells of the internal control serum should have an average OD = 1.0 The typical total development time for DBP_II is ~ 20 min hence initial plate reading (in step 6.8.15) begins ~5min prior to this.
17. Repeat step 6.8.15 until the mean OD of the internal controls wells on plate 1 = ~ 1.0.
18. Wait for the plate to read and then select Plate 2. Click read plate after the 60-90s interval has expired..
19. Repeat for subsequent plates, reading each in order with the appropriate 60-90s time interval between reading each plate.
20. When you have read all the plates, save the experiment again (File menu, then save).
21. Now right click on Plate 1 and select Export.
22. Now right click on Plate 2 and select Export. Repeat this process for all plates in turn.
23. Once finished, exit Gen5 software.
24. Save the Excel worksheet on the V;\ drive in the correct experiment folder with the same name as the Gen5 Experiment.
25. Each plate is displayed on a separate worksheet. For each plate:
26. Check the R^2 value for the standard curve is >0.994.
27. Carefully review the Abs405 data for each well. Check for no aberrant readings in the triplicate values for each sample CV should be <20%. Check the Abs405 of the blank wells is <0.15.
28. Check the reference value is within 20% of 8100.
29. The worksheet will analyse the data and provide a readout in the bottom table for the antibody units of each sample (AU).
30. Any readings that are below the Abs405 threshold of 0.26 should be regarded as negative.
31. Any readings that are above the Abs405 threshold of 2.1 should be repeated in another ELISA assay at higher dilution.
32. Fill in the volunteer number and timepoint for each sample.
33. Copy the final data set to the DBP MVT ELISA database found at: V:\1.Malaria\1. Master Files\VAC Studies\ VAC051\ Immunology\ ELISA data. ELISA plates can be discarded once read.
34. MVT ELISA record sheets should be stored in the relevant trial ring-binder folder.

7 Associated documents

ELISA Experiment Record Sheets and Spreadsheet of MVT Exx numbers. Found at:

V:\1.Malaria\1. Master Files\Lab general trial info\Blood-stage MVT\ Templates & Record Sheets

MVT ELISA database stored at:

V:\1.Malaria\1. Master Files\VAC Studies\ VAC051\ Immunology \ ELISA data

8 References

Miura, K., A. C. Orcutt, O. V. Muratova, L. H. Miller, A. Saul, and C. A. Long. 2008. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine* 26:193-200.

9 Review History

The review history allows a record to be kept of when and who reviews the document to ensure it remains fit for purpose. Significant changes should be recorded in the 'Detail' section.

Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
27/Nov/13	Simon Draper & Kathryn Milne	1.0	New SOP based on ML023. The antigen has changed from AMA1/MSP1 to P.vivax DBP_RII. The source of this antigen and the reference serum used in setting up the standard curve. Specifies this SOP is for use in just blood-stage trials as opposed to both blood-stage and liver stage. Dilutions and volumes used to make the standard curve, as well as positive control. Range of expected O.D values in the positive control has changed as well as the	N/A
13/Nov/15	Sarah Silk	2.0	Updated version of SOP for new batch of DBP protein, new reference serum and change of blocking agent.	1.0

ML023 *P. falciparum* MSP1 and AMA1 ELISA

1 Purpose

During malaria vaccine trials, the Enzyme-Linked ImmunoSorbent Assay (ELISA) is the primary assay for monitoring the antibody responses induced by vaccination in the serum of human volunteers. This SOP contains the detailed experimental protocol for performing this assay for the antigens *Plasmodium falciparum* Merozoite Surface Protein 1 19kDa fragment ($PfMSP1_{19}$) and Apical Membrane Antigen 1 (PfAMA1). There are two alleles of the $PfMSP1_{19}$ antigen: i) 3D7/Mad20/ETSR – referred to here as ETSR, and ii) Wellcome/K1/FVO/QKNG – referred to here as QKNG. There are multiple alleles of the PfAMA1 antigen, but only two are tested in this ELISA assay: i) 3D7 AMA1, and ii) FVO AMA1. This SOP details the experimental protocol for performing this assay for both these alleles of $PfMSP1_{19}$ and PfAMA1.

2 Introduction

The aim of our clinical vaccine trials is to induce protection against malaria in human volunteers and characterise the cells and/or antibodies involved in inducing protective immune responses. One of the primary endpoints in Phase I and Phase II trials of blood-stage malaria vaccines is immunogenicity as measured by ELISA, which quantifies the level of antigen-specific antibodies in the serum of immunized volunteers. In this case, the $PfMSP1_{19}$ antigens are recombinant GST-$PfMSP1_{19}$ fusion proteins produced in *E. coli* (Jenner Protocol J136) and the PfAMA1 antigens are provided by external collaborators (3D7 AMA1 (1) was provided by Dr Chetan Chitnis (ICGEB, New Delhi, India) and FVO AMA1 (2) was provided by Dr Mike Blackman (NIMR, London, UK)). The ELISA protocol has been standardized according to published methods (3). This protocol uses a reference serum on each ELISA plate to detect antibodies induced by experimental malaria vaccines.

Different types of controls are included; a high-titre reference serum from naturally-immune African adults is included on each ELISA plate to produce a standard curve which is used to quantify and assign ELISA units to each unknown sample on the plate; also an internal positive control sample and negative control / no serum (“blank wells”) are used to perform QC analysis on each ELISA plate. A separate ELISA against GST only is also included for selected samples as a negative control for the $PfMSP1_{19}$ antigens.

ELISA plates are coated over-night with the relevant recombinant GST or GST-$PfMSP1_{19}$ fusion proteins or PfAMA1. The assay is performed by preparing a standard curve and internal controls from the reference serum and adding these samples to the plate. Unknown test serum samples from immunized volunteers are diluted and added in triplicate to the ELISA plate. After a two hour incubation period, the diluted sera are discarded, the plate is washed and a secondary polyclonal antibody against the γ–chain of human IgG is added. This secondary antibody is conjugated to the enzyme alkaline phosphatase. After

another one hour incubation, followed by a wash step, the alkaline phosphatase substrate is added. The substrate is left to develop for 10-25 mins (antigen dependent) and the absorbance at 405nm is read using a plate reader. The result is obtained by taking an average of the triplicate wells for each test sample, and using the standard curve to assign MSP1 or AMA1 ELISA arbitrary units (AU).

3 Scope

This SOP covers all serum samples taken from volunteers in MSP1 or AMA1 malaria vaccine trials in Oxford for which ELISA is one of the primary readouts. It does not cover booking in of samples to the laboratory, which is described in ***ML001*** Malaria lab sample handling.

4 Definitions

ELISA = Enzyme-Linked ImmunoSorbent Assay.

5 Responsibilities

All staff employed by the University of Oxford who work on the malaria vaccine trials, including clinicians or visiting scientists working in the lab, must follow these protocols.

The **Senior Immunologist** ensures that staff are competent to perform these procedures.

6 Procedure

6.1 Equipment & Reagents

Where appropriate, equipment and reagents used in the processing of samples from clinical trials is dedicated to study work. Equipment used forms part of the laboratory maintenance and monitoring plan.

Equipment:
Fridge at +4^{o}C
Freezer at -20^{o}C and -80^{o}C
Vortex
Eppendorf Racks
Pipettes including 8- or 12-well multi-channel and automatic multi-channel Pipetteboy
Bio-tek ELx800 Microplate Reader with Gen5 ELISA software
Timer
Sufficient tips for pipettes. 0.1-10µl, 2-20µl, 20-200µl, 100-1000µl
Safety Glasses
PBS-Tween Hand Washer
Timer

Consumables & Reagents:

Reagent	Company	Cat #
NUNC Immuno Plates (442404)	Fisher	DIS-971-030J
Blocker Casein in PBS	Pierce	37528
Goat anti-human IgG (γ-chain)-alk phos	Sigma	A3187

Dulbecco's PBS (DPBS)	Sigma	D8537
5x Diethanolamine Buffer	Fisher	34064
4-Nitrophenyl Phosphate Tablets (20mg)	Sigma	N2765
Tween-20	Sigma	P7949
Aluminium Foil	Fisher	AKL-300-040J
1.5mL Eppendorf tubes	Fisher	FB74031
Reagent Reservoirs (Costar 4870)	Fisher	PMP-331-010C
10L PBS powder	Invitrogen	21600-069

Recombinant Protein Antigens:

Recombinant protein antigens required for this SOP include:

i) GST-PfMSP1$_{19}$ ETSR ["ETSR"]
ii) GST-PfMSP1$_{19}$ QKNG ["QKNG"]
iii) GST control ["GST"]
iv) PfAMA1 (3D7)
v) PfAMA1 (FVO)

These GST (fusion) proteins are produced in *E. coli* and purified by affinity chromatography according to Jenner Protocol J136. The PfAMA1 proteins are provided by collaborators. These are stored in small aliquots (typically enough to coat five ELISA plates) at -20ºC in the human immunology freezer until needed. Coating antigen concentration is standard at 2µg/ml.

Reference Serum:

Human hyperimmune serum (sample 5H) from Kilifi Kenya. Diluted 1:100 and stored in aliquots in the MVT -20°C freezer.

Buffers and Solutions:

Make up buffer and solutions as follows:

i) PBS/T (PBS with 0.05% Tween) for washing plates. Dissolve 10L PBS tub in 10L deionised water (15.0 MΩ setting). Add 5mL Tween-20. Shake and return to the ELISA plate wash station.

Day 1.

6.2 Coating ELISA plates on the bench.

1 Print off a new MVT ELISA record sheet for each experiment. Number the experiment with the next experiment number and fill this in with the required information throughout the experiment. The next available experiment number can be found in the Excel spreadsheet contained in the same folder. Sign off the experiment number in the spreadsheet and re-save the file. Sheets can be found at:

V:\1.Malaria\1. Master Files\VAC Studies\VAC037\12. Immunology\ELISAs\ Templates & Record Sheets

2 Calculate the number of Nunc Immuno ELISA plates required (max number of 22 test samples per plate). Thaw an aliquot of the recombinant antigen required. Make the coating solution by adding recombinant antigen to DPBS at a final concentration of 2µg/mL. Working on the bench, add 50 µl per well of the coating solution to the ELISA plate using a multi-channel pipette. Store the plates at RT over-night (≥16 h), covered in foil (do not stack the plates, but

leave flat on the bench). Note time of coating and record on the experiment layout sheet.

3 Store any spare recombinant protein antigen at 4°C for maximum 1 week.

Day 2.

6.3 Blocking plates.

1 Flick off the coating solution into the sink (wear eye protection).

2 Wash the plates 6x in PBS/T using the handheld washer.

3 Block the wells with 200 µL per well of Casein blocking buffer.

4 Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 1h (max 1h 30min) at RT. Note time of blocking and record on the experiment sheet.

5 During blocking prepare test samples and reference standard dilutions as below.

6.4 Sample Preparation

iv) Standard Curve

1 Standard curve dilution: Thaw an aliquot of the 1:100 reference serum (stored at -20°C in the human immunology freezer).

2 Prepare dilutions of this reference serum in duplicate in Casein Block buffer:

- For MSP1: Take the aliquot and prepare one 1:900 dilution in an eppendorf tube: add 100µL 1:100 to 800µL of blocking buffer. Label 1. Vortex to mix.
- For AMA1: Take the aliquot and prepare one 1:1000 dilution in an eppendorf tube: add 100µL 1:100 to 900µL of blocking buffer. Label 1. Vortex to mix.

Prepare a dilution curve in eppendorf tubes. To make a 1:2 dilution set (enough for 4 ELISA plates):

- Add 450µL of Casein block to nine eppendorfs labelled 2-10.
- Add 450µL of the first dilution 1 to tube 2. Vortex to mix.
- Add 450µL of 2 to tube 3. Vortex to mix. And so on - repeat this through to tube 10.
- Each tube should now contain 450µL of liquid (except 10 which contains 900µL), with a 2-fold dilution series running from tube 1 to 10.

v) Positive Control Sample

3 Prepare the positive control serum sample in Casein Block buffer and vortex to mix:

For MSP1, make a 1:3600 dilution of the reference serum by adding 50µL 1:100 to 1750µL of blocking buffer.

For AMA1, make a 1:8000 dilution of the reference serum by adding 25µL 1:100 to 1975µL of blocking buffer.

vi) Test Serum Samples

4 Prepare the test serum sample in Casein Block buffer. A dilution is required that will give an OD 405nm reading that is in the linear part of the standard curve (0.15 ≤ OD 405nm ≤ 1.6). Test samples can be tested at a single or multiple dilutions. The dilutions must be recorded on the ELISA record sheet.

Typical dilutions for serum from the vaccine trials include 1:300 for samples taken from Adenovirus only immunised volunteers, or for most samples from volunteers receiving Adeno-MVA regimes taken between d0 – d56. Do not test samples at a dilution lower than 1:300. Serum samples taken on or after d63 should be typically tested at higher dilutions in the range e.g. 1:3000-1:6000 – the necessary dilution will depend on the strength of the response and can range from 1:600-1:40,000.

To prepare 1:300 dilutions: dilute 3µl serum in 897µl of casein block.

To prepare higher dilutions: dilute the 1:300 dilution appropriately in casein block.

Record all dilutions for each sample on the experimental record sheet.

Vortex all samples to mix.

6.5 Plating Serum

1 After blocking is complete, wash the plates 6x in PBS/T. Tap them dry on blue roll.

2 Plate serum out using plate layout below. Each well should contain 50µl of sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
G	1	2	3	4	5	6	7	8	9	10	Blank	
H	1	2	3	4	5	6	7	8	9	10		

S1 to S22 (blue) = test sera (added in triplicate).

Standard Curve (pink) = dilution 1 (column 1) to dilution 10 (column 10). Transfer 50µl from tubes 1-10 (see step 6.4.2) to the appropriate wells of rows G and H of the ELISA plate. Repeat for subsequent ELISA plates, etc.
Blank = 50µl of casein block solution.
Internal control = 1:3600 MSP1 or 1:8000 AMA1 reference serum (see step 6.4.3).

3 Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 2h (max 2h 30min) at RT. Note time of plating and record on the experiment sheet.

6.6 Secondary Antibody

1 After this time, wash the plates 6x in PBS/T. Tap them dry on blue roll.

2 Dilute the secondary antibody 1:1000 in casein block solution. 5mL is required per plate, i.e. 5µl secondary antibody in 5mL casein block. Secondary antibody is goat anti-human IgG (γ-chain) Sigma A3187 (stored at +4˚C). Vortex to mix.

3 Add 50µl secondary antibody per well.

4 Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 1h (max 1h 30min) at RT. Note time and record on the experiment sheet.

5 Prepare development buffer – for each plate 10ml is required. Buffer must be made up in units of 20ml. Dilute 5x diethanolamine buffer (stored at +4˚C) in deionised water (18.2 MΩ setting). For each unit of 20ml, add one 20mg 4-nitrophenylphosphate tablet (stored at -20˚C) to give a final concentration of 1mg/ml. Prepare the buffer in a suitable tube wrapped in foil, to prevent exposure to light. Leave to stand at RT until required, and shake to mix before use.

6.7 Development

1 Wash the plates 6x in PBS/T. Tap them dry on blue roll.

2 Make sure the computer and plate reader are available and turned on, before developing.

3 Using a multi-channel automatic pipette, add 100µl development buffer to each well of plate one. Using the timer, wait 60-90s and then add development buffer to plate 2. Continue adding development buffer to each plate in turn at the same 60-90s interval. Cover the plates in foil and leave on the bench. Make sure there are no bubbles in any of the wells, as this can aberrantly increase the absorbance readings. If bubbles are present, pop these with a clean yellow pipette tip (use a separate tip for each bubble to avoid cross-contamination of development buffer between wells).

6.8 Reading Plates and Analysis

1. During the development period, log on to ELISA station computer.
2. Load Gen5 ELISA software.
3. Create a new experiment.
4. Select either the MSP1 or AMA1 MVT Clinical Trail ELISA Protocol v1 (links below if required) and click OK.

   V:\1.Malaria\1. Master Files\VAC Studies\VAC037\12. Immunology\ELISAs\ MSP1 Protocol

   V:\1.Malaria\1. Master Files\VAC Studies\VAC036\13. Immunology\ELISAs\ AMA1 Protocol
5. Click file menu and save as.

6. Save experiment files as “MVT E xx followed by the date (yymmdd). It should save automatically to C:\Program Files\BioTek\Gen5 1.10\Experiments.
7. If you have more than one ELISA plate, right click on Plate 1 and select add plates. Add the number of extra plates required.
8. Click on the + icon next to plate 1 to expand the menu, and select Sample IDs.
9. Enter IDs for samples 1-22 (typically numbered 1-22) and then click OK.
10. Right click on Plate 1 and choose custom plate layout and select Yes.
11. Double click on custom layout in the menu for plate 1.
12. For Sample 1, select 3 vertical replicates and enter the dilution used. Click on Square A1.
13. Enter the dilution used for sample 2 and then click on square A2. Repeat for Sample 3 and click on A3 and so on, until all 22 samples have been accounted for. For Samples 13-22 click on squares D1-D10. Click OK at the end.
14. Repeat these steps for any extra plates in the experiment.
15. After approximately 20mins (for MSP1) and 10mins (for AMA1), read the Abs at 405nm of Plate 1 using the Bio-tek ELx800 microplate reader. Select Plate 1 and then “read plate” in the toolbar menu (grey box with green arrow pointing left) and then click READ and then OK.
16. The Abs 405nm of the six wells of the internal control serum should have an average OD = 1.0 (approx range 0.8 – 1.2). This value does not need to be exact, as the analysis takes this into account and antibody units are calculated in relation to the standard curve on each plate. The typical total development time for MSP1 is ~25mins and for AMA1 ~15mins, hence initial plate reading (in step 6.8.15) begins ~5mins prior to this.
17. Repeat step 6.8.15 until the mean OD of the internal controls wells on plate 1 = ~1.0.
18. Wait for the plate to read and then select Plate 2. Click read plate after the 60-90s interval has expired (from step 6.7.2).
19. Repeat for subsequent plates, reading each in order with the appropriate 60-90s time interval between reading each plate.
20. When you have read all the plates, save the experiment again (File menu, then save).
21. Now right click on Plate 1 and select power export.
22. If the Excel format box appears on the screen, click Continue. Excel will remain open after power export, but no worksheet will be visible.
23. Now right click on Plate 2 and select power export. Repeat this process for all plates in turn.
24. Once finished, exit Gen5 software.
25. An Excel worksheet should be saved in C:\Program Files\BioTek\Gen5 1.10\Experiments with the same name as the Gen5 Experiment.
26. Open the worksheet.
27. Each plate is displayed on a separate worksheet. For each plate:

28. Check the R^2 value for the standard curve is >0.994.
29. Carefully review the Abs405 data for each well. Check for no aberrant readings in the triplicate values for each sample. Check the Abs405 of the blank wells is <0.15.
30. The worksheet will analyse the data and provide a readout in the bottom table for the antibody units of each sample (AU). These are multipled by a conversion

factor if the internal reference (highlighted in green) is > ±10% of the expected value.

31. Any readings that are below the Abs405 threshold of 0.15 will be highlighted in bright blue. These should be regarded as negative.
32. Any readings that are above the Abs405 threshold of 1.6 will be highlighted in bright yellow. These should be repeated in another ELISA assay at higher dilution.
33. If you have tested two dilutions (e.g. sample positions 1&13, 2&14 etc), these are averaged in the final table and % difference calculated. This table can be ignored if only one dilution was tested for each sample.
34. Fill in the volunteer number and timepoint for each sample.
35. Copy the final data set to the MSP1 or AMA1 MVT ELISA database found at: V:\1.Malaria\1. Master Files\VAC Studies\VAC037\12. Immunology\ELISAs\ MSP1 ELISA Data. Note the data for each antigen are stored on separate worksheets (see tabs at the bottom).
36. Copy the Gen5 Experiment and Excel sheet into a folder labelled with the MVT Exx number in the appropriate Immunology / ELISA data folder for the Vac study on the V:// drive.
37. ELISA plates can be discarded once read.
38. MVT ELISA record sheets should be stored in the ring-binder folder in the MVT office.

7 Associated documents

ELISA Experiment Record Sheets and Spreadsheet of MVT Exx numbers. Found at:

V:\1.Malaria\1. Master Files\VAC Studies\VAC037\12. Immunology\ELISAs\ Templates & Record Sheets

MVT ELISA database stored at:

V:\1.Malaria\1. Master Files\VAC Studies\VAC037\12. Immunology\ELISAs\ MSP1 ELISA Data

8 References

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2. Pizarro, J. C., B. Vulliez-Le Normand, M. L. Chesne-Seck, C. R. Collins, C. Withers-Martinez, F. Hackett, M. J. Blackman, B. W. Faber, E. J. Remarque, C. H. Kocken, A. W. Thomas, and G. A. Bentley. 2005. Crystal structure of the malaria vaccine candidate apical membrane antigen 1. *Science* 308:408-411.

3. Miura, K., A. C. Orcutt, O. V. Muratova, L. H. Miller, A. Saul, and C. A. Long. 2008. Development and characterization of a standardized ELISA including a

reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine* 26:193-200.

9 Review History

The review history allows a record to be kept of when and who reviews the document to ensure it remains fit for purpose. Significant changes should be recorded in the 'Detail' section.

Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
26/Jul/10	Simon Draper & Sumi Biswas	1.0	New document	N/A

ML011: RH5 ELISA

1 Purpose

During malaria vaccine trials, the Enzyme-Linked ImmunoSorbent Assay (ELISA) is the primary assay for monitoring the antibody responses induced by vaccination in the serum of human volunteers. This SOP contains the detailed experimental protocol for performing this assay for the antigen *Plasmodium falciparum* reticulocyte-binding protein homologue 5 (PfRH5).

2 Introduction

The aim of our clinical vaccine trials is to induce protection against malaria in human volunteers and characterize the cells and/or antibodies involved in inducing protective immune responses. One of the primary endpoints in Phase I and Phase II trials of blood-stage malaria vaccines is immunogenicity as measured by ELISA, which quantifies the level of antigen-specific antibodies in the serum of immunized volunteers. In this case, the PfRH5 protein is cultured in S2 cells by Jing Jin. This assay will use RH5 v2 CTAG (batch: P0184).

The ELISA protocol has been standardized according to published methods (1). This protocol uses a reference serum on each ELISA plate to detect antibodies induced by experimental malaria vaccines.

Different types of controls are included; a reference serum from a high responding volunteer is included on each ELISA plate to produce a standard curve which is used to quantify and assign ELISA units to each unknown sample on the plate; also an internal positive control sample and negative control / no serum (“blank wells”) are used to perform QC analysis on each ELISA plate.

ELISA plates are coated over-night with the RH5 protein. The assay is performed by preparing a standard curve and internal controls from the reference serum and adding these samples to the plate. Unknown test serum samples from immunized volunteers are diluted and added in triplicate to the ELISA plate. After a two hour incubation period, the diluted sera are discarded, the plate is washed and a secondary polyclonal antibody against the γ–chain of human IgG is added. This secondary antibody is conjugated to the enzyme alkaline phosphatase. After another one hour incubation, followed by a wash step, the alkaline phosphatase substrate is added. The substrate is left to develop for 25 minutes and the absorbance at 405nm is read using a plate reader. The result is obtained by taking an average of the triplicate wells for each test sample, and using the standard curve to assign RH5 ELISA arbitrary units (AU).

3 Scope

This SOP covers all serum samples taken from volunteers in RH5 malaria vaccine trials in Oxford for which ELISA is one of the primary readouts. It does not cover booking in of samples to the laboratory, which is described in ***ML001*** Malaria lab sample handling.

4 Definitions

ELISA = Enzyme-Linked ImmunoSorbent Assay.

5 Responsibilities

All staff employed by the University of Oxford who work on the malaria vaccine trials, including clinicians or visiting scientists working in the lab, must follow these protocols.

The **Senior Immunologist** ensures that staff are competent to perform these procedures.

6 Procedure

6.1 Equipment & Reagents

Where appropriate, equipment and reagents used in the processing of samples from clinical trials is dedicated to study work. Equipment used forms part of the laboratory maintenance and monitoring plan.

Equipment:
Fridge at +4^{o}C
Freezer at -20^{o}C and -80^{o}C
Vortex
Eppendorf Racks
Pipettes including 8- or 12-well multi-channel and automatic multi-channel
Pipetteboy
Bio-tek ELx800 Microplate Reader with Gen5 ELISA software
Timer
Sufficient tips for pipettes. 0.1-10µl, 2-20µl, 20-200µl, 100-1000µl
Safety Glasses
PBS-Tween Hand Washer
Timer

Consumables & Reagents:

Reagent	**Company**	**Cat #**
NUNC Immuno Plates (442404)	Fisher	DIS-971-030J
Blocker Casein in PBS	Pierce	37528
Goat anti-human IgG (γ-chain)-alk phos	Sigma	A3187
Dulbecco's PBS (DPBS)	Sigma	D8537
5x Diethanolamine Buffer	Fisher	34064
4-Nitrophenyl Phosphate Tablets (20mg)	Sigma	N2765
Tween-20	Sigma	P7949
Aluminium Foil	Fisher	AKL-300-040J
1.5mL Eppendorf tubes	Fisher	FB74031
Reagent Reservoirs (Costar 4870)	Fisher	PMP-331-010C
10L PBS powder	Invitrogen	21600-069

Recombinant Protein Antigens:

Recombinant protein antigens required for this SOP include:

i) RH5 v2 Ctag

RH5 protein is cultured in S2 cells and purified by affinity chromatography by Jing Jin. The protein is stored in 28µl aliquots (enough to coat 2 ELISA plates) at -80ºC in the blood stage trial freezer until needed. Coating antigen concentration is standard at 2µg/ml.

Reference Serum:

VAC057 Volunteer 1020 G2B d84 serum is stored in the blood stage trial -80°C freezer.

Buffers and Solutions:

Make up buffer and solutions as follows:

ii) PBS/T (PBS with 0.05% Tween) for washing plates. Dissolve 10L PBS tub in 10L deionised water (15.0 MΩ setting). Add 5mL Tween-20. Shake and return to the ELISA plate wash station.

Day 1.

6.2 Coating ELISA plates on the bench.

6.2.1 Print off a new MVT ELISA record sheet for each experiment. Number the experiment with the next experiment number and fill this in with the required information throughout the experiment. The next available experiment number can be found in the Excel spreadsheet contained in the same folder. Sign off the experiment number in the spreadsheet and re-save the file. Sheets can be found at:

V:\1.Malaria\1. Master Files\5. Lab general info/blood stage mvt \Templates & Record sheets \ MVT ELISA record sheet

6.2.2 Calculate the number of Nunc Immuno ELISA plates required (max number of 22 test samples per plate). Thaw an aliquot of RH5 for >15minutes. Make the coating solution by adding RH5 to DPBS at a final concentration of 2µg/mL (DO NOT VORTEX). Working on the bench, add 50 µl per well of the coating solution to the ELISA plate using a multi-channel pipette. Store the plates at 4°C over-night (≥16 h), wrapped in cling film. Note time of coating and record on the experiment layout sheet.

Day 2.

6.3 Blocking plates.

6.3.1 Bring casein to RT for >30mins and flick off the coating solution into the sink (wear eye protection).

6.3.2 Wash the plates 6x in PBS/T using the handheld washer.

6.3.3 Block the wells with 200 µL per well of Casein blocking buffer.

6.3.4 Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 1h (max 1h 30min) at RT. Note time of blocking and record on the experiment sheet.

6.3.5 During blocking prepare test samples and reference standard dilutions as below.

6.4 Sample Preparation

vii) Standard Curve

6.4.1 Prepare dilutions of VAC057 Volunteer 1020 day 84 reference serum in duplicate in Casein Block buffer:

- For RH5: Take the serum and prepare one 1:100 dilution in an eppendorf tube: add 6µL to 594µL of blocking buffer. Label 1. Vortex to mix.

Prepare a dilution curve in eppendorf tubes. To make a 1:2 dilution set (enough for 2 ELISA plates):

- Add 300µL of Casein block to nine eppendorfs labelled 2-10.
- Add 300µL of the first dilution 1 to tube 2. Vortex to mix.
- Add 300µL of 2 to tube 3. Vortex to mix. And so on - repeat this through to tube 10.
- Each tube should now contain 300µL of liquid (except 10 which contains 600µL), with a 2-fold dilution series running from tube 1 to 10.

viii) Positive Control Sample

6.4.3 Prepare the positive control serum sample in Casein Block buffer and vortex to mix:

For RH5, make a 1:100 dilution of the reference serum by adding 3µL to 297µL of blocking buffer. Then make a 1:3200 dilution by adding 28µL of the 1:100 stock to 868µL blocking buffer.

Repeat this 2 more times to make 3 independent 1:3200 dilutions.

ix) Test Serum Samples

6.4.4 Prepare the test serum sample in Casein Block buffer. A dilution is required that will give an OD 405nm reading that is in the linear part of the standard curve ($0.15 \leq$ OD 405nm ≤ 2.5). Test samples can be tested at a single or multiple dilutions. The dilutions must be recorded on the ELISA record sheet.

Typical dilutions for serum from the vaccine trials include 1:100 for samples taken from Adenovirus only immunised volunteers, or for most samples from volunteers receiving Adeno-MVA regimes taken between d0 – d56. Do not test samples at a dilution lower than 1:100. Serum samples taken on or after d63 should be typically tested at higher dilutions in the range e.g. 1:100-1:3000 –

the necessary dilution will depend on the strength of the response and can range from 1:100-1:40,000.

To prepare 1:100 dilutions: dilute 3µl serum in 297µl of casein block.

To prepare higher dilutions: dilute the 1:00 dilution appropriately in casein block.

Record all dilutions for each sample on the experimental record sheet.

Vortex all samples to mix.

6.5 Plating Serum

6.5.1 After blocking is complete, wash the plates 6x in PBS/T. Tap them dry on blue roll.

6.5.2 Plate serum out using plate layout below. Each well should contain 50µl of sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
G	1	2	3	4	5	6	7	8	9	10	Blank	
H	1	2	3	4	5	6	7	8	9	10		

S1 to S22 (blue) = test sera (added in triplicate).

Standard Curve (pink) = dilution 1 (column 1) to dilution 10 (column 10). Transfer 50µl from tubes 1-10 (see step 6.4.2) to the appropriate wells of rows G and H of the ELISA plate. Repeat for subsequent ELISA plates, etc.

Blank = 50µl of casein block solution.

Internal control = 1:3200 RH5 reference serum (see step 6.4.3).

6.5.3 Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 2h (max 2h 30min) at RT. Note time of plating and record on the experiment sheet.

6.6 Secondary Antibody

6.6.1 After this time, wash the plates 6x in PBS/T. Tap them dry on blue roll.

6.6.2 Dilute the secondary antibody 1:1000 in casein block solution. 6mL is required per plate, i.e. 6µl secondary antibody in 6mL casein block. Secondary antibody is goat anti-human IgG (γ-chain) Sigma A3187 (stored at +4˚C). Vortex to mix.

6.6.3 Add 50µl secondary antibody per well.

6.6.4 Stack the plates (with an empty blank plate on top) and cover in foil. Leave for 1h (max 1h 30min) at RT. Note time and record on the experiment sheet.

6.6.5 Prepare development buffer – for each plate 10ml is required. Buffer must be made up in units of 20ml. Dilute 5x diethanolamine buffer (stored at +4˚C) in deionised water (18.2 MΩ setting). For each unit of 20ml, add one 20mg 4-nitrophenylphosphate tablet (stored at -20˚C) to give a final concentration of 1mg/ml. Prepare the buffer in a suitable tube wrapped in foil, to prevent exposure to light. Leave to stand at RT until required, and shake to mix before use.

6.7 Development

6.7.1 Wash the plates 6x in PBS/T. Tap them dry on blue roll.

6.7.2 Make sure the computer and plate reader are available and turned on, before developing.

6.7.2 Using a multi-channel automatic pipette, add 100µl development buffer to each well of plate one. Using the timer, wait 60-90s and then add development buffer to plate 2. Continue adding development buffer to each plate in turn at the same 60-90s interval. Cover the plates in foil and leave on the bench. Make sure there are no bubbles in any of the wells, as this can aberrantly increase the absorbance readings. If bubbles are present, pop these with a clean yellow pipette tip (use a separate tip for each bubble to avoid cross-contamination of development buffer between wells).

6.8 Reading Plates and Analysis

1. During the development period, log on to ELISA station computer.
2. Load Gen5 ELISA software.
3. Create a new experiment using an existing protocol.
4. Select the MVT PfRH5 ELISA Protocol (link below if required) and click OK.
    a. X:\S Draper\Clinical Trials\ELISA Protocols\150820 MVT PfRH5 ELISA protocol
5. Click file menu and save as.
6. Save experiment files as “MVT E xx followed by initials and the date (yymmdd) in
    a. V:\1.Malaria\1.Master files\2.VAC Studies\VAC057\12.Immunology\ELISA data\Standardised ELISAs\Experiments
7. If you have more than one ELISA plate, right click on Plate 1 and select add plates. Add the number of extra plates required.
8. Click on the + icon next to plate 1 to expand the menu, and select Sample IDs.
9. Enter IDs for samples 1-22 (typically numbered 1-22) and then click OK.
10. Right click on Plate 1 and choose custom plate layout and select Yes.
11. Double click on custom layout in the menu for plate 1.
12. For Sample 1, select 3 vertical replicates and enter the dilution used. Click on Square A1.
13. Enter the dilution used for sample 2 and then click on square A2. Repeat for Sample 3 and click on A3 and so on, until all 22 samples have been accounted for. For Samples 13-22 click on squares D1-D10. Click OK at the end.

14. Repeat these steps for any extra plates in the experiment.
15. After approximately 20minutes (for RH5), read the Abs at 405nm of Plate 1 using the Bio-tek ELx800 microplate reader. Right click on Plate 1 and then “read plate 1” or click green play button in the toolbar menu, click READ and then OK.
16. The Abs 405nm of the six wells of the internal control serum should have an average OD = 1.0. The typical total development time for RH5 ~25minutes, hence initial plate reading (in step 6.8.15) begins ~5mins prior to this.
17. Repeat step 6.8.15 until the mean OD of the internal controls wells on plate 1 = ~1.0.
18. Wait for the plate to read and then select Plate 2. Click read plate after the 60-90s interval has expired (from step 6.7.2).
19. Repeat for subsequent plates, reading each in order with the appropriate 60-90s time interval between reading each plate.
20. When you have read all the plates, save the experiment again (File menu, then save).
21. Now right click on Plate 1 and select export.
22. Now right click on Plate 2 and select export. Repeat this process for all plates in turn.
23. Once finished, exit Gen5 software.
24. Save the Excel worksheet on the V:\ drive in the correct experiment folder with the same name as the Gen5 experiment.
25. Each plate is displayed on a separate worksheet. For each plate:
26. Check the R^2 value for the standard curve is >0.994.
27. Carefully review the Abs405 data for each well. Check for no aberrant readings in the triplicate values for each sample CV should be <20%. Check the Abs405 of the blank wells is <0.15.
28. The worksheet will analyse the data and provide a readout in the bottom table for the antibody units of each sample (AU).
29. Any readings that are below the Abs405 threshold of 0.3 should be regarded as negative.
30. Any readings that are above the Abs405 threshold of 2.5 should be repeated in another ELISA assay at higher dilution.
31. Fill in the volunteer number and timepoint for each sample.
32. Copy the final data set to the RH5 MVT ELISA database found at: V:\1.Malaria\1. Master Files\VAC Studies\VAC057\12. Immunology\ELISAs\ VAC057 ELISA Database.
33. ELISA plates can be discarded once read.
34. MVT ELISA record sheets should be stored in the relevant trial ring-binder folder.

7 Associated documents

ELISA Experiment Record Sheets and Spreadsheet of MVT Exx numbers. Found at:

V:\1.Malaria\1. Master Files\5. Lab general info \ blood stage mvt \ Templates & Record sheets \ MVT ELISA record sheet

MVT ELISA database stored at:

V:\1.Malaria\1. Master Files\VAC Studies\VAC057\12. Immunology\ELISA data \ VAC057 ELISA database

8 References

1. Miura, K., A. C. Orcutt, O. V. Muratova, L. H. Miller, A. Saul, and C. A. Long. 2008. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine* 26:193-200.

9 Review History

The review history allows a record to be kept of when and who reviews the document to ensure it remains fit for purpose. Significant changes should be recorded in the 'Detail' section.

Date	**Reviewed By (Print name)**	**Version**	**Detail (significant changes from previous version)**	**Previous Version**
13/Nov/15	Sarah Silk	1.0	First written (based on ML023)	N/A

ML021: Preparation of malaria-infected blood for injection

Document Category:	Standard Operating Procedure
Code:	ML021
Title:	Preparation of malaria-infected blood for injection
Version:	2.0
Authors:	Simon Draper and Katie Ewer
Authorised By:	Adrian Hill
Section:	Jenner Institute Malaria Trials Laboratory
Number Of Copies:	1
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Table of Contents

1 PURPOSE

This SOP describes the thawing procedure for cryopreserved *Plasmodium falciparum* infected human erythrocytes for administration to clinical trial volunteers during blood-stage controlled human malaria infection (CHMI).

2 INTRODUCTION

This forms part of a set of SOPs relating to culture of blood-stage *Plasmodium falciparum,* which is classified as a Category 3 organism by the Advisory Committee on Dangerous Pathogens.

Compliance with this SOP is of importance because it allows the clear understanding and consistent reproduction of the established laboratory procedures by the person(s) performing the task, in compliance with the Jenner Containment Level 3 Code of Practice.

This procedure requires two people: an operator who performs all manipulations of the infected material and a 'scribe' who records all the relevant information, such as batch numbers of reagents and double-checks that the correct solutions are applied at each stage of the thawing process. The role of the second person is crucial as they both cross-check each stage and produce the written record of the procedure. Two further people are also needed to prepare the parasite viability assay (see SOP ML022).

3 SCOPE

Thawing of cryopreserved *P. falciparum* and preparation of the inoculum for blood-stage CHMI conducted by the Jenner Institute.

4 DEFINITIONS/ABBREVIATIONS

None

5 RESPONSIBILITIES

All staff carrying out thawing of *P. falciparum* to prepare the inoculum must follow this procedure. **Category 3 laboratory supervisor** ensures staff are competent to perform this procedure and authorises staff to work in category 3 facility.

6 PROCEDURE

6.1 Equipment

Item	Supplier
Sterile gowns (new)	
Surgical gloves	NHS
Distel	
Fast read counting slides	Immune Systems Ltd
Discard jars (sweetie jars)	Generic
Hypodermic needles – 18G	NHS
Sterile, single use syringes – 50, 20, 10, 5, 2, 1 mL	Generic
50 and 15 mL Falcon tubes	Generic
Falcon rack (new unused) and autoclaved	Generic
Plastic beakers – autoclaved	Generic
Sharps bins	Generic
Sterile field cloth	NHS
2 ice boxes and transport boxes	Generic
Aluminium foil – autoclaved	Generic
Sterile cannulae (green)	NHS
Syringe caps	NHS
Autoclaved cryovial rack	Generic
Calculator	Generic
Stopwatch	Generic
Clinic needles 10mL with Luer Lock	
Centrifuge with buckets + lids	Generic
Marker pens	Generic
Pasteur pipettes	Generic
P200 pipette and filter tips	Generic
Sterile scalpel	Generic
Spray bottles – Distel and 70% ethanol	Generic
T75 sterile flasks	Generic

6.2 Reagents

Reagent	Supplier	Catalogue Number
30% saline for injection	Churchill Pharmacy	B.P.
0.9% saline for injection	Churchill Pharmacy	B.P.
Water for injection	Churchill Pharmacy	B.P.
Blood culture set (BACTEC)	BD Diagnostics	442192 / 442193
TSA with 5% horse blood agar plates	BD Diagnostics	212099
Sabouraud's glucose agar plates	BD Diagnostics	254039
Sterile water bottles	Sigma	W3500
Ethanol	Sigma	02877-1L
70% ethanol wipes		

6.3 Procedure

6.3.1 Prepare prior to day of challenge

1. Autoclave foil, cryovial rack and falcon rack.
2. Fumigate two microbiological safety cabinets (MSC). The work undertaken in this SOP can be performed under derogated category 3. Organise with the Jenner Institute Laboratory Manager for 2 hoods to be fumigated (internally or by a contractor) – all associated records of fumigation and validation should be filed in the trial-specific folder held by the Senior Immunologist. It is imperative to ensure that the hoods are not used between fumigation and preparation of the inoculum (e.g. by locking the door of the tissue culture room). Arrange with the Lab Manager best situation depending on hoods designated for use.
3. Clean centrifuge with 1% Distel solution, then wipe over with 70% ethanol wipes. Soak buckets and lids in 1 % Distel, rinse thoroughly with water and leave to dry on blue roll.
4. Prepare balance tubes.
5. Ensure sufficient unused sterile gowns and surgical gloves are available for staff.
6. Print identification labels for syringes.

6.3.2 Prepare on day of challenge

1. ≥ 2 hours in advance: Half-fill autoclaved beaker with sterile water and warm to 37℃ in incubator covered with sterile foil.
2. Place 0.9% and 30% saline and water for injection in the fridge to cool.
3. Wipe over centrifuge buckets with 70% ethanol wipes and return to centrifuge.
4. Prepare fresh 1% Distel solution and 70% ethanol in wash bottles.
5. Half-fill ice box(es) for transport of parasites to the clinic (CCVTM), cover with sterile foil just before starting the thawing procedure. The number of ice boxes required will depend on the number of syringes being prepared for the trial. Place ice box(es) inside plastic transport box in the derogated TC room.
6. Fill a large beaker with ice for use inside the TC room (just before starting the thawing procedure).
7. Pre-cool centrifuge to 4℃.
8. Before starting work in the hood, put on sterile gown and gloves.
9. Start up both MSCs and clean with sterile 70% ethanol wipes. All equipment should be sprayed in and out of the cabinet with 70% ethanol and allowed to dry. One of the hoods will be used to prepare the inoculum and the other hood to prepare the syringes.
10. In the first hood (for the inoculum), place the falcon tube rack, cryovial rack, sharps' bin and the beaker of ice. Also put a sweetie jar lined with an autoclave bag (for solid waste) and a sweetie jar containing Distel (for liquid waste) inside the cabinet.

11. Place another sharps' bin in the second hood (used for syringe preparation).
12. Place a set of open agar plates at the back of each cabinet (one blood agar and one Sabouraud's) – labelled either 'inoculum' or 'syringe' (depending on the hood) plus the trial number, name of operator, & date.
13. Observe good sterile technique throughout the following process – open syringes and needles with minimal handling.
14. For this step use a 50 mL syringe fitted with an 18G needle. Fill 4 x 50 mL Falcon tubes with 0.9% (normal) saline for injection, label as such and place in the hood on ice. In the same manner place 40 mL of water for injection in a 50 mL Falcon tube, label as such and place on ice. *Do not touch the inside of the Falcon tube with the syringe itself. Spray hands and vials with 70% ethanol before handling but allow to dry in the hood before use.
15. For this step use a 50 mL syringe fitted with an 18G needle. Prepare 2 x 9ml of 0.9% (normal) saline for injection in 15 mL Falcon tubes and label one '1:10 dilution' and other '1:100 dilution'. Place on ice.
16. Prepare 12% saline using 30% saline and water for injection (on ice from step above) in a labelled 15 mL Falcon tube as follows: 5 mL of 30% saline + 7.5 mL water = 12.5 mL of 12% saline (extract 30% saline using a Pasteur pipette into a labelled 15mL Falcon tube, then transfer the 30% saline and water to the final 15 mL Falcon tube using a 10 mL syringe and 18G needle). Place on ice.
17. Prepare 1.6% saline using the 12% saline and water for injection (on ice from steps above) in a labelled 50 mL Falcon as follows: 4 mL of 12% saline + 26 mL water = 30 mL of 1.6% saline.
18. Discard Falcon tubes containing water for injection and 30% saline.
19. Place all solutions on ice and ensure sufficient to time to cool to 4℃. If the ice will melt during a waiting period, then securely close all tubes and these can be stored in the fridge before returning to the hood when needed on ice.
20. In the second hood, prepare the number of 10 mL Luer-Lock syringes required with a cannula attached for final inoculum. De-cap cannula, remove and discard needle in the sharps' bin and then attach to syringe. Label syringes and place on a sterile cloth in the hood.

6.3.3 Thawing procedure

1. Place the beaker of pre-warmed water in the inoculum cabinet.
2. Remove a vial of a frozen blood and record the following information on the datasheet: date of the freeze, name of the operator, the thaw date and time, and all other information on the cryovial.
3. Sterilise the outside surface of the cryoflex by wiping it with 70% ethanol.
4. Using a sterile scalpel open the cryoflex, remove the vial and rapidly thaw the vial in the beaker of warmed water.
5. Start timing.

6.3.4 Deglycerolisation

1. Transfer the contents of the thawed vial to a 50 mL sterile Falcon tube using an 18G needle and 2 mL syringe. Estimate and record the volume of the vial (e.g. 1.0 mL). It is anticipated volume will be ~1.5mL.

Vol. cryopreserved blood (mL)	12% saline (mL)	1.6% saline (mL)
1.0	0.20	12.0
1.1	0.22	13.2
1.2	0.24	14.4
1.3	0.26	15.6
1.4	0.28	16.8
1.5	0.30	18.0
1.6	0.32	19.2
1.7	0.34	20.4
1.8	0.36	21.6
1.9	0.38	22.8
2.0	0.40	24.0

2. Using a 1 mL syringe and 23G needle add drop-wise, very slowly, while mixing, 0.2 volume (e.g. 0.2 mL) of sterile 12% saline. Let stand for 5 minutes at room temperature.
3. Using a 20 mL syringe and 18G needle, add drop-wise, while mixing, 10 volumes of sterile 1.6% saline. It may be necessary to reload the syringe depending on the volume required.
4. Prepare a balance tube and centrifuge for 4 minutes at 830 *xg* in a bucket with the safety lid fitted.
5. Return to the MSC and remove supernatant using a 20 mL syringe(s) with 18G needle. Resuspend pellet by gentle tapping.

6. **Wash 1.** Add 10 mL of 0.9% (normal) saline relatively quickly drop-wise from a 10 mL syringe with an 18G needle attached while mixing gently. Use 0.9% (normal) saline from one of the 50 mL Falcon tubes on ice.
7. Using the 10 mL balance, centrifuge for 4 minutes at 830 *xg* in a bucket with the safety lid fitted.
8. Return to the MSC and remove supernatant using a 20 mL syringe with 18G needle. Resuspend pellet by gentle tapping.
9. **Wash 2.** Add 10 mL of 0.9% (normal) saline from a 10 mL syringe with an 18G needle attached while mixing gently (not drop-wise anymore).
10. Using the 10 mL balance, centrifuge for 4 minutes at 830 *xg* in a bucket with the safety lid fitted.
11. Return to the MSC and remove supernatant using a 20 mL syringe with 18G needle. Fully resuspend pellet by gentle tapping.
12. Add 10 mL of 0.9% (normal) saline from a 10 mL syringe with an 18G needle attached while mixing gently (not drop-wise anymore). Place tube on ice. [If pellet doesn't easily resuspend, have a sterile Pasteur pipette to hand].

6.3.5 Calculating volumes and numbers of parasites.

1. Remove 1mL using a 5 mL syringe with an 18G needle and mix with the 9 mL 0.9% (normal) saline in prepared tube (1:10). Mix.
2. With a new 5 mL syringe with an 18G needle, remove 1 mL of the 1:10 dilution and mix with the 9 mL 0.9% (normal) saline in prepared tube (1:100). Mix.
3. Give the 1:10 dilution to the operator setting up the parasite viability assay.
4. Using a pipette, add 20 µL of the 1:100 dilution to a cell of a plastic Fast Read Slide and count 1 large square (16 small squares). Repeat 4 times in total. Calculate the average count (***a*** = average no. RBC x 100 x 10^4 / mL). [Note this is calculated as x 100 for dilution factor and x 10^4 to get no. RBC in 1 mL).
5. The scribe should now provide the viability assay team with the value (***a***). They need just the average number of parasites counted in 1 large square (without multiplication factors etc.). Most likely need to call the category 3 malaria room on 01865 617618.
6. From the frozen 3D7 parasite inoculum used for CHMI, it is assumed that there are 211 parasites / 5.37 x 10^6 RBC. Therefore 1000 parasites (target challenge dose) = 2.55 x 10^7 RBC.
7. Therefore [2.55 x 10^7 RBC / ***a***] = ***b*** = no. of mL of stock solution required per volunteer to give 1000 parasites.
8. Calculate the total volume of parasite stock solution required = ***b*** x [no. volunteers + 4 spare] = ***c*** mL.
9. Calculate the total volume of inoculum required = [no. volunteers + 4 spare] x 5 mL per volunteer = ***d*** mL.
10. Calculate the volume of 0.9% (normal) saline required = ***d*** – ***c*** mL = ***e*** mL.

6.3.6 Separation of syringes.

1. Using a 10 mL syringe with 18G needle, remove the required volume of parasite stock solution to produce the inoculum (***b*** mL) and place in a labelled sterile T75 flask [or 50mL Falcon tube depending on volume required for the study]. Using a 50 mL and 10 mL syringe (as required) with 18G needle, add the required volume of 0.9% (normal) saline (on ice from before) to produce the inoculum (***e*** mL). Mix gently and well.
2. Close the lid of the flask/tube and move to the second hood.
3. Draw 5 mL of diluted inoculum into each prepared syringe through the cannula. Remove cannula from syringe after filling and close with a syringe cap. Place the syringes on ice covered with sterile foil in prepared ice box(es). Place ice box inside plastic crate.
4. Transport to CCVTM for administration to volunteers and hand over to clinician along with form for clinic (appended below).
5. Once the syringes have been sent to the clinic, close the agar plates in each hood, and place in a 37℃ incubator. Record results after 24 hours and 4 8 hours and report to the Senior Immunologist and Chief Investigator.

6.3.7 Other parasitological requirements.

1. If there is enough inoculum left, add 0.5mL of the inoculum into a BACTEC tube set. Arrange with the clinician for these to be cultured by the NHS microbiological laboratories. The results should be reported to the Chief Investigator and filed by the Senior Immunologist.
2. If required, add 1mL of the inoculum (or volume available) to a culture and move to Category 3. These parasites may be frozen down for relevant or trial-specific parasitological studies.
3. Provide other aliquots of the inoculum remaining for other trial-specific studies as discussed with the Senior Immunologist.

7 ASSOCIATED DOCUMENTS

Jenner Institute Containment Level 3 Code of Practice

Inoculum preparation check sheet.

Inoculum paperwork for the clinic.

8 REFERENCES

None.

9 REVIEW HISTORY

The review history allows a record to be kept of when and who reviews the document to ensure it remains fit for purpose. Significant changes should be recorded in the 'Detail' section.

Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
30-06-14	Simon Draper & Rebecca Brown	v2.0	SOP re-written and updated to reflect current practice. Appendix added.	v1.0

ML022: Blood Stage Challenge Viability Assay

Document Category:	Standard Operating Procedure
Code:	ML022
Title:	Blood Stage Challenge Viability Assay
Version:	1.0
Authors:	Simon Draper
Authorised By:	Adrian Hill
Section:	Jenner Institute Malaria Trials Laboratory
Number Of Copies:	1
Location Of Copy:	Malaria SOPs ORCRB

Contents

1 PURPOSE

To permit accurate quantification of the number of viable parasites in any sample.

In particular, for use with blood-stage controlled human malaria infection (CHMI) clinical inocula, in conjunction with the SOP for thawing of cryopreserved clinical CHMI inocula (ML021) and the current SOP for parasite quantitative PCR (ML008).

2 INTRODUCTION

A number of clinical studies have been conducted with frozen blood-stage parasite inocula originally prepared by Cheng et al in 1994 (1-4). Parasite viability has been found to be somewhat variable: full viability in Cheng et al; 31-77% in Lawrence et al; ~10% in Pombo et al; and 62% in Sanderson et al.

The dose of viable parasites given is an important input to the modelling of parasite multiplication rates (PMR) which is a major trial outcome efficacy measure.

As explained below, the quantification of the number of parasites present in the inoculum prior to freezing was subject to substantial uncertainty.

Inoculum information
The clinical trial vials each contain 1.5ml of stabilate ≈ 250ul packed RBCs.

The original parasite count for volunteer 2 = AS was 212 **per microliter of whole blood** (Lawrence Vaccine 2000) (alternative sources suggest 290 per microliter: Cheng AJTMH 1997, Allan Saul personal communication; in any case this is an approximation).

Assuming normal male haematocrit of 0.44, this implies parasite count of 212/0.44 = 482 per microliter of packed red cells.

With 482 parasites/µL of packed RBCs ≈ 120,500 parasites/vial.
This will be thawed into 10 mL of saline, i.e. 12,000 parasites/mL minus losses, e.g. to haemolysis.

This will then be diluted to 1:10 for viability assay, i.e. ~1200 parasites/mL.

Assuming that only one vial is thawed, that only the 1:10 dilution is available for the viability assay and that two 250 µL aliquots are added to T25 cultures, 8.5 mL will be available for the viability assay, i.e. ~10,000 parasites.

3 SCOPE

Thawing of cryopreserved *P. falciparum* and preparation of inoculums for blood-stage malaria CHMI for studies conducted by the Jenner Institute.

4 DEFINITIONS/ABBREVIATIONS

None

5 RESPONSIBILITIES

All staff This procedure should be performed by staff experienced in blood-stage malaria culture. It is suggested that at least 2 practice runs with non-clinical parasites should be conducted to familiarise new operators with the procedure prior to a clinical trial. Competency records from the practice runs should be included in individuals' training records.

Category 3 laboratory supervisor ensures staff are competent to perform this procedure and authorises staff to work in category 3 facility.

6 PROCEDURE

6.1 Equipment + materials

See SOP ML016 section.

6.2 Procedure

Two weeks before challenge

1. Prepare 500 mL of incomplete medium as per J132.
2. Identify a source of O+ RBCs. If NBS blood required, order O+ research RBCs from NBS, using 'Non-clinical Component Request form', for delivery three days before challenge. (If ordered for earlier delivery, would be out of date before the end of the post-challenge culture period).

Day before challenge

Prepare ***fresh*** washed O+ RBCs as per J133 (no need for any more than 5 mL).

Make 50 mL complete culture medium:
-45 mL incomplete medium
-5 mL pooled heat-inactivated filter-sterilised human serum
-50 µL of 10 mg/mL gentamicin.

Set up a stock culture as per SOP ML016 (culture maintenance). If thawing of a parasite vial is needed, use ML020. This stock culture will have to be maintained until the day of challenge for positive control wells.

Before thawing of inoculum is started

1. Make a thin smear of the stock culture to determine its parasitaemia.
2. 100 mL complete Pf culture media (as per J132) should be prepared + warmed to 37℃.
3. 50% Hct washed O+ RBCs should be available as per J133.
4. Prepare 100 mL of warm 1% Hct medium.
5. Prepare 2x flat-bottom 96 well plate labelled '1' and '2' (full-area 96wp, not the half-area GIA plates) as follows and leave in incubator until needed:

   100 µL of complete 1% Hct medium/ well in rows B, C, D, E, F.

   100 µL sterile water in rows G,H.

   100 µL sterile water in the spaces between the wells.
6. Prepare 1x flat bottom 96 well plate (labelled '3') with 90 µL of complete 1% medium in all wells.
7. Prepare 3 x T25s with 5 mL uninfected 1% Hct complete medium. Label as "Positive control", "1" and "2".
8. Add a suitable amount of the stock culture to the "Positive control" T25 to give a parasitaemia of ~0.01%.

   This will contain roughly 12.5 parasites / µL (i.e. more than the target dilution of the trial inoculum).
   Given $5.4x10^6$RBCs/µL **whole blood**
   → $(5.4x10^6/0.44) * 0.01 * 0.01 = 1230$ parasites/µL of **packed RBCs** at 0.01% parasitaemia
   → 1230 * 0.01 = 12.5 parasites/µL of **culture** at 1% haematocrit.

9. Label 2x Eppendorfs "T25-1 D0" and "T25-2 D0" and a 96 well plate labelled "Plate 1 samples frozen day 0"
10. Prepare culture chamber with wet paper towel.
11. Prepare an ice bucket.

During preparation of the inoculum

1. The 1:10 dilution of the thawed inoculum (used to prepare the 1:100 dilution for counting) should be kept on ice – see SOP ML021.

2. As soon as it arrives, a 250 µL of the 1:10 dilution should be added to T25 #1. A 100 µL sample of the resulting mixture should be put in Eppendorf "T25-1 D0". Gas flask + put in incubator.

3. The RBC count in the **1:100** dilution (X **as the average count in one large square**) from SOP ML021 should be communicated to the person conducting the viability assay ASAP to permit calculations.

   a. WRITE X HERE =

4. Calculation of volume (V mL) in which to resuspend pellet (for step 2 below):
   a. With clinical BS challenge sample:
      - Known that pre-freezing there were 212 parasites/5.37x10^6 RBC.
      - 25,450 RBC per parasite.
      - For 800 parasites/mL, want 20,360,000 RBC/mL
      - 8.5ml of the **1:10** dilution should remain i.e. total number of RBCs = 85*X*10^4
      - V = [(85*X*10^4)/20,360,000] = **X/23.95**

        WRITE 'V' HERE:

After preparation of the inocula
PLATE SET-UP

A phone call from CCVTM to ORCRB should be made at the time that the final inoculum is injected into the last volunteer. Contact numbers and back-up numbers should be provided by the trial Senior Immunologist to the clinical team. The operators for the viability assay should be contacted directly if possible, most likely on the number in the Category 3 malaria room (01865 617618) unless otherwise advised.

1. 250 µL of the 1:10 dilution should be added to T25 #2 to quantify (by qPCR comparison of growth in the two T25s) loss of parasite viability in the time between dilution preparation and injection to volunteers.
   a. Take a 100 µL sample of the resulting mixture, add to Eppendorf "T25-2 D0" and freeze.
   b. Gas the flask and put it in the incubator.

2. Resuspend the 1:10 dilution by gentle inversion to avoid it sticking to sides of Falcon, then spin down (830 *xg*, 4 min) and resuspend in the volume (**V**) of warm complete 1% Hct culture medium predicted to give 800 parasites / mL.

3. This suspension should be poured into a sterile multichannel basin.

4. Add 150 µL/well of the 1:10 dilution to wells A1-A10 of plate 2 and as many wells as possible of plate 1 row A

5. Add 150 µL/well of the 0.01% parasitaemia stock culture to wells A11 + A12 on both plates.

6. Transfer 50 µL serially from row to row down plates 1 & 2 as far as row F, discarding 50 µL from row F (i.e. all wells should end with 100 µL). Row E on these plates should, in effect, receive 100/81 µL of the parasite suspension = a predicted 0.99 parasites.

7. If there is remaining parasite suspension, add 11.1 µL to each of the wells in row A of plate 3. Mix well. Transfer 11.1 µL from these wells to each of the wells in rows B-H (**not by serial dilution – just direct transfer A → B, A → C etc**). Like row E on the other plates, rows B-H should thus receive 11.1/9 = 100/81 µL of parasite suspension, providing numerous extra replicates at ~1 parasite/well.

8. Transfer all the wells in columns 1-4 and 11-12 of plate 1 into the corresponding wells of an empty 96 well plate, which can then be frozen. Four frozen replicates at each dilution is adequate for determining day 0 background level of genome copies. Refill the empty wells with 100 µL sterile H_20.

9. Put plates in culture chamber with wet blue roll for humidity. Gas with 5% O_2, 5% CO_2, 90% N_2. Put in 37℃ incubator.

Culture

1. Culture plate(s) for 10 days, with media changes after 2, 4, 6, and 8 days (parasitaemia will be too low to necessitate 24 hourly media changes). On day 6, increase the haematocrit & add fresh RBC by using complete medium with 1% Hct uninfected RBC instead of medium.

2. Media changes – **be very careful not to remove RBC during this process**:
   a. remove plate from incubator with minimal disturbance;
   b. remove 80 µL of media from wells without disturbing the settled RBC;
   c. replace with 80 µL fresh 37℃ complete culture medium;
   d. return plate to culture chamber;
   e. gas plate with 5% O_2, 5% CO_2, 90% N_2;
   f. return to incubator.

3. Ongoing culture of the T25s should be carried out as per ML016.
   a. Change media at days 2, 4, 6, 8.

4. On day 10, freeze the culture plates, along with 250 µL aliquots from each of the T25s.

PCR

1. After thawing, samples can be removed from CL3 to CL2 for qPCR using current Jenner standard quantitative PCR assay (ML008) to obtain genome copies/ well of each plate.

- Leucocyte depletion (filtration) is not required as neither the stock culture nor the trial inocula contain leucocytes.
- Qiagen DNA extraction is needed to separate the DNA from haemoglobin etc., but given the smaller-than-usual sample volume and RBC content, reagent volumes can be reduced as follows:
  -protease – use 10 µL instead of 40 µL
  -lysis buffer AL – use 100 µL instead of 400 µL
  -ethanol - use 100 µL instead of 400 µL.
  -use the full volumes for the washes (AW1 and AW2), and elute as normal in 50 µL.
- The PCR reaction set-up should be as usual, i.e. 5 µL of the DNA eluate and the standard reaction mix, and the usual standard curve.
- The PCR plate layout requires two rows of the 96wp for standards, leaving 72 wells, i.e. enough for 24 samples in triplicate.

VIABILITY CALCULATION

1. Score post-culture wells as positive (i.e. contained ≥1 viable parasite at start of culture period) if post-culture genome copies > 100x mean of pre-culture plate results at the same dilution.

2. Choose the most informative dilution – one at which ~50% of wells are negative. Note the following values:
   N = total number of wells at that dilution
   Y = number of positive wells at that dilution

3. X, the number of parasites per well, is distributed according to a Poisson distribution:

   X~Poisson(A), P(X=0) = (N-Y)/N, want to find A, the mean number of parasites/well.

   Because of the distribution of the Poisson, $(N-Y)/N = e^{-A}$
   → $A = -\ln[(N-Y)/N]$

   Viability as % of that predicted based upon pre-freeze parasitaemia of 212 / µL = 99*A

   Actual viable parasite dose given = intended dose based upon pre-freeze parasitaemia * 0.99A

   If viability is low, similar calculations could be done with wells from rows B,C,D of plates 1+ 2.

7 ASSOCIATED DOCUMENTS

ML008 - Quantitative RT-PCR of malaria challenge blood
ML016 - Maintenance of P falciparum cultures
ML021 - Preparation of malaria infected blood for injection
J132- *P. falciparum* culture medium preparation
J133- RBC preparation for *P. falciparum* cultures

8 REFERENCES

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9 REVIEW HISTORY

The review history allows a record to be kept of when and who reviews the document to ensure it remains fit for purpose. Significant changes should be recorded in the 'Detail' section.

Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
30/06/14	Sandy Douglas and Simon Draper	2.0	Revised and updated according to current practice	1.0

Plate 1		*Serial dilutions, frozen immediately*				Cultured						Frozen immediately		
		1	2	3	4	5	6	7	8	9	10	11	12	
150ul of ~800p/ml inoculum/ 1% Hct suspension	A	120	120	120	120	120	120	120	120	120	120	3750	3750	150ul of pos control culture
3-fold serial dilutions	B	40	40	40	40	40	40	40	40	40	40	1250	1250	Serial dilutions
	C	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	416.666667	416.666667	
	D	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	138.888889	138.888889	
	E	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	46.2962963	46.2962963	
	F	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	15.4320988	15.4320988	
	G	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	5.14403292	5.14403292	
	H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.71467764	1.71467764	

Plate 2		*Serial dilutions, cultured*												
		1	2	3	4	5	6	7	8	9	10	11	12	
150ul of ~800p/ml inoculum/ 1% Hct suspension	A	120	120	120	120	120	120	120	120	120	120	3750	3750	150ul of pos control culture
3-fold serial dilutions	B	40	40	40	40	40	40	40	40	40	40	1250	1250	Serial dilutions
	C	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	416.666667	416.666667	
	D	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	138.888889	138.888889	
	E	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	46.2962963	46.2962963	
	F	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	15.4320988	15.4320988	
	G	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	5.14403292	5.14403292	
	H	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.71467764	1.71467764	

Plate 3		*Multiple replicates at c. 1p/ml*											
		1	2	3	4	5	6	7	8	9	10	11	12
	A	All receive 11ul of ~800p/ml inoculum suspension, into 90ul of medium											
	B	All receive 11ul from row A, into 90 ul of medium											
	C	All receive 11ul from row A, into 90 ul of medium											
	D	All receive 11ul from row A, into 90 ul of medium											
	E	All receive 11ul from row A, into 90 ul of medium											
	F	All receive 11ul from row A, into 90 ul of medium											
	G	All receive 11ul from row A, into 90 ul of medium											
	H	All receive 11ul from row A, into 90 ul of medium											

ML009: Collection, preparation and slide reading: Malaria Challenge Studies

Document Category:	Standard Operating Procedure
Code:	ML009
Title:	Collection, Prep & Slide Reading during Malaria Challenge Studies
Version:	4.0
Authors:	Ian Poulton
Authorised By:	Adrian Hill
Section:	Jenner Institute Malaria Trials Laboratory
Number Of Copies:	1
Location Of Copy:	Malaria SOPs file CCVTM

Collection, Prep & Slide Reading during Malaria Challenge Studies - Version: 4.0. Index: ML009. Printed: 26-Apr-2013 12:27
Authorised on: 26-Apr-2013. Authorised by: Adrian Hill. SOP Unique Reference: 626-25781654. Due for review on: 26-Apr-2015
Author(s): Ian Poulton

Table of Contents

1 Purpose

To prepare, stain and examine a thick blood film suitable for detection of malaria parasites in blood samples from trial volunteers who have been challenged with a known strain of P. falciparum as part of the malaria vaccine trial programme.

2 Introduction

This is a useful method for rapid presumptive identification of malaria parasites within the challenge model as it shows adequate staining of all stages.

3 Scope

This SOP applies to any malaria vaccine trial challenge study being conducted in Oxford

4 Definitions/abbreviations

Malaria CRF Malaria Clinical Research Fellow.

5 Responsibilities

Slide readers: Thick film preparation, reading, documentation and informing the on-call Malaria CRF of results, care of equipment, ensuring any shortages are reported promptly.

Malaria CRF: verification of positive film slides

6 Procedure

6.1 Safety

Universal precautions should be used throughout this procedure. Thick film stains are not fixed and staining them will not kill the parasites, viruses or other pathogens.

6.2 Equipment

Gloves**
Venepuncture equipment*
Protective eye wear***
Microscope slides**
Field Stain A***
Field Stain B***
Immersion oil
Microscope***
Coplin Jar***
Staining Rack***
Disposable pipettes***
Report Card***
Microscope storage Boxes***

Clinical area only, ** Clinical and Laboratory, *** Laboratory only

6.3 Method

1. Samples will be collected by the clinical staff via venepuncture using 1.1ml (vacuum) EDTA tubes in accordance with SOP VC004,

2. 35mm slide labels with the volunteers full study number printed on them and annotated with the date, time and time-point will be used to identify the specimens.

3. Each specimen will be accompanied with a standard 76x25mm glass microscope slides with a copy of the annotated label attached to one end of the slide. (see fig1) **NB**: Use only clean, grease-free slides that have been stored in a dust-proof container. The slides should be handled only on the edges to avoid fingerprints on the surface.

Fig 1 (example of labelled slide)

4. The specimen and slide will be transported to the lab in a rigid air-tight container.

5. Identify the specimen and associated slide, record details of time-point, date and time of specimen collection on the microscopy record form

6. Pipette two small drop of blood, equal distance apart on to the slide, using the corner of a second clean slide spread each drop in a circular motion 3-6 times. The resulting blood film should be thin enough to read normal print through. Once completed the slide should be laid on a clean flat surface and allowed to dry completely.

7. Once dry the slide should be dipped in the Coplin jar containing Field's stain A for 3 second

8. Remove the slide and rinse by dipping in clean water and agitating gently for 3 seconds, drain off any excess water

9. Dip the slide into the Coplin jar containing Field's stain B for 3 seconds, remove and drain off any excess stain.

10. Rinse in clean water

11. Wipe the back of the slide clean and place in an upright position within the staining rack and allow to dry.

    NOTE: If after staining, the whole film appears yellow-brown, too blue or too pink, do not attempt to examine it. Re-stain it by repeating steps 7 to 10 but only for one second at each staining step.

12. Once the film is completely dry, examine under oil immersion, The standard protocol requirement is for 200 fields at high power (1000x) to be read.

13. The microscopist will record the time and result of reading the slide on the microscopy record sheet. If any parasites are seen, the slide will be left under the microscope with the parasite centred in the field of view and the on-call malaria CRF should be contacted to verify the result as a morphologically normal parasite, the slide should not be removed until this is done. The Malaria CRF will then sign the record sheet to indicate they have seen the parasite.

14. A digital image of the parasite will be taken using the microscope camera and saved to the V drive. This will allow subsequent review to be undertaken as required.

15. Repeat samples or testing may be requested; in this case the same procedure will be followed but the results written in relevant section of the record sheet.

16. Following diagnosis, all procedures will be followed as above and again any positive slides will be reported to the Malaria CRF for visual confirmation.

7 Associated documents

Universal Microscopy Record

8 References

Health Protection Agency; Staining Procedures, BSOP TP 39,
http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1309970661136

9 Review History

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Date	Reviewed By (Print name)	Version	Detail (significant changes from previous version)	Previous Version
03/Jun/09	S. Kerridge	2.0	Re-formatted. Addition of introduction and amendment of responsibilities.	1.1
24/Sep/10	I. Poulton	3.0	Title, Insertion of paragraphs 6.2-Equipment & 6.1-Safety, Reformatting, Extension of detail for procedure. Associated documents and reference. Deletion of reference thin film preparation	2.0
25/Apr/13	N. Anagnostou	4.0	Minor clarification to text in section 6.12 and 6.13. No modification to procedure. Reference updated. Addition of b instruction to take digital image (section 6.14).	3.0

ML008: Malaria qPCR

Document Category:	Standard Operating Procedure
Code:	ML008
Title:	Malaria QPCR
Version:	7.0
Authors:	Nick Edwards
Authorised By:	Sarah Gilbert
Section:	Jenner Institute Malaria Trials Laboratory
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Author(s): Nick Edwards

Table of Contents

1 Purpose

To monitor Malaria parasitaemia levels in volunteer blood samples by means of specific, TaqMan probe based Quantitative Real Time PCR

2 Introduction

During malaria challenge clinical trials, volunteer blood samples are taken for the diagnosis of blood stage malaria parasites by thick blood film slide and quantitative assessment by qPCR. This SOP includes procedures for automated DNA extraction using the QIAsymphony SP and qPCR amplification of parasite DNA for quantitative analysis. This is to be carried out in real time during clinical trials to estimate parasitaemia levels. The data produced may be used to confirm blood film microscopy diagnosis of malaria or, in exceptional circumstances, to advise on treatment of volunteers displaying malaria-like symptoms when no parasite is observed by microscopy. (e.g. 3 positive PCR results when volunteer is still slide negative. Treatment would always remain a purely clinical decision with final decision in ambiguous cases resting with the PI.)

Volunteers give single blood samples at Day -1 or Day 0. Then twice daily from Day 6.5 to 14.5 and then single daily samples from Day 15 to 21 for most liver stage vaccine challenges (or as detailed in the specific Clinical Trial Protocol). Blood stage vaccine challenges are monitored with a single bleed at Day 1 and then twice daily Day 2 to Day 13, followed by once daily to Day 21 (or as detailed in the specific Clinical Trial Protocol). Whole blood samples have automated DNA extractions performed on them to recover all genomic DNA, including that of malarial parasites from the red cells. This is then quantified by qPCR for the 18s Ribosomal gene of *P.falciparum*.

3 Scope

All clinical trials involving experimental malaria challenge (CHMI) within the Jenner Institute at the University of Oxford.

4 Definitions/abbreviations

qPCR	quantitative polymerase chain reaction
DNA	Deoxyribonucleic Acid
NFQ-MGB	Non-Fluorescent Quencher with Minor Groove Binder moiety
PI	Principal Investigator
CHMI	Controlled Human Malaria Infection

5 Responsibilities

All staff who are competent and trained in this SOP. This includes appropriate GCP and CL3 training plus Occupational Health monitoring as required.

6 Procedure

6.1 Safety

Plasmodium falciparum is defined as a biological hazard group 3 organism by the HSE. Derogated work on *P.falciparum* is permitted in CL2 laboratories as described in the current Jenner Code of Practice Derogated CL3 document here S:\Safety\CL3 lab documents

Staff must have read this have the fact recorded by the CL3 Laboratory Supervisor. Similarly for COSHH documents C041 and C043. Staff should record this in their training records and also familiarise themselves with the associated HSE documents listed in section 8.

All work with infected blood up to the point of lysis, or subsequent recovery of un-processed whole blood must be carried out at derogated CL3 in accordance with the Code of Practice. All staff must be fully trained in this and the SOP before any work on infected blood can proceed and appropriate personal safety equipment must be used at all times.

6.2 Consumables and Equipment

Sufficient in-date QIAsymphony consumables should be purchased to cover 120% of maximum possible sample numbers. Equipment must be within service and calibration intervals as recommended by manufacturer. 2ml Sarstedt Tubes and storage boxes may be pre-labelled to facilitate speed of processing. [Note: order sufficient freezer safe printable labels to cover the expected number of sample tubes that will be stored. Also order sufficient qPCR consumables, extra tubes, pipette tips etc. (10µl filter tips in particular) and ensure that 1% Distel, 70% EtOH, lab tissue and general lab consumables over and above normal use levels are available.]

Equipment

Product	Company	Cat. No.
Bench Spill Trays for all derogated CL3 work	Fisher	PBH-891-030A
Racks to hold 2ml vacutainer tubes	VWR/Fisher	STK-660-050D
Racks to hold 1.7ml eppendorf tubes/2ml Sarstedt tubes	VWR/Fisher	As available
QIAsymphony SP System	Qiagen	Bay 2
Tube Rack carriers with 1A inserts (3B for 2ml Sarstedt tubes)	Qiagen	Bay 2
Cool adapter for Qiagen EMTR 96 well elution plates	Qiagen	Bay 2
Step One Plus QPCR machine system (2 machines)	Applied Biosystems	4376598
Benchtop centrifuge for 1.7ml eppendorf tubes	Any designated	As available
Vortex	Any designated	As available
10, 20, 200, 1000 ul pipettes	Anachem/Rainin	Trial designated
5-100 ul Biohit Electronic pipette	BioHit	Trial designated
PCR plate holder to support PCR plate during loading	Applied Biosystems	As available
Pipette filler ('Pipette Boy Acu, cable free')	Any designated	As available
Timer	Any designated	As available
Freezer Space -20ºC and -80ºC	Any designated	Clinical Freezers
Designated Schott Bottles for CL3 liquid disposal (yellow lid)	Any designated	As available
Designated sealed tins for transfer of derogated CL3 waste	Any designated	As available

Consumables

Product	Company	Cat. No.
Blood taking equipment, 2ml EDTA vacutainer tubes	*clinic will order and supply*	
QIAsymphony DSP DNA Midi Kit (144 samples at 400µl)	Qiagen	937255
Filter-Tips, 200 µl (1024) (QIAsymphony)	Qiagen	990332
Filter-Tips, 1500 µl (1024) (QIAsymphony)	Qiagen	997024
Sample Prep Cartridges, 8-well (336)	Qiagen	997002
8-Rod Covers (144)	Qiagen	997004
High Temp Autoclave bags suitable for CL3 waste	Fisher	As available
Small autoclave bags for QIAsymphony tip waste	Fisher	As available
3ml disposable Pasteur pipettes	Fisher	11537732
10ml disposable stripettes	Fisher/Corning	As available
Mol Biol grade water (10 x 50ml)	Qiagen	129114
Distel 1% solution (formerly named Trigene)	SLS	TRI1310
10" rolls Kimwipe	Lab stock	As available
Dispo Jars for pipette tips/BioBoxes as appropriate	Lab stock	As available
Treff tubes 1.5ml clear (must be certified nuclease free)	Fisher	As available
Microtube tough spot stickers (3800PK)	Web Scientific	SPOT-1000
laser print labels LCRY-1700	SLS	CRY8510
Cryobox System 100 for storage of vials in 10 x 10 array (10)	Fisher	10069130
Cryobox for storage of vials for 1.2/2.0mL in 9 x 9 array (4)	Fisher	10324741
MicroAmp Optical Adhesive Film	Applied Biosystems	4311971
MicroAmp Fast Optical 96 well PCR plate 0.1ml	Applied Biosystems	4346907
Forward and Reverse primers 80 µMol scale (Custom)	Applied Biosystems	4304971
TaqMan FAM- NFQ-MGB Probe 20 µMol scale (Custom)	Applied Biosystems	4316033
TaqMan Universal PCR master Mix (with Amperase UNG)	Applied Biosystems	4304437
Counted Parasite or Plasmid DNA standards for PCR	In house stock	x
2ml tubes -screw top (1000)	SARSTEDT	72.694.006
Book A4 Black 'n' Red 96LF Feint	Isis	D66174
Nitrile Gloves Small, Medium, Large	Fisher	As available
0.1-10ul Filter Tip, Racked (need 1 box per full PCR plate)	Anachem/Rainin/StarLab	As available
1-20ul Filter Tip, Racked	Anachem/Rainin/StarLab	As available
1-200ul Filter Tip, Racked	Anachem/Rainin/StarLab	As available
100-1000ul Filter Tip, Racked	Anachem/Rainin/StarLab	As available

6.3 Procedure Checklist before starting <u>each</u> session

- Turn on QIAsymphony SP system. When start up routine has finished (approx. 10 minutes), login (password = jenner2 unless otherwise informed) NOTE: the QIAsymphony uses a touchscreen with an intuitive interface and intelligent software and will **always** check what you tell it or if you change anything.
- Make sure all relevant CL3 and CL2 waste disposal containers and disinfectant bottles are ready for use and that the work area is free of clutter.
- Make sure all tubes for sample storage and processing are correctly labelled and ready for use. Have storage boxes ready to receive stored samples.
- Have the Trial lab book ready to record volunteer's number, time (AM or PM), date, any comments on blood volume or appearance for each run. *[During a*

challenge, this book should be kept to hand near the bench space used for processing samples and updated day by day as the challenge progresses. NO qPCR data to be recorded here during the trial period for confidentiality reasons.]

- Ensure that there are sufficient reagents and consumables available and to hand for each sample processing session. (NOTE: DSP kit reagent cartridges used in each session must be of the same lot number as the QIAsymphony will not switch lots mid-run)

6.4 Reception of blood samples

NOTE: Reception of blood tubes, any handling of infected blood must all be carried out on the spill trays provided for derogated CL3 work in Bay 2 of the Immunology lab. No paperwork or notes must contact these trays (accidental contact will require autoclaving of any potentially contaminated material) **Any** materials/consumable in contact with non-heat treated or frozen blood **must** be disinfected and autoclaved as set out in the Jenner Code of Practice Derogated CL3 document, before disposal.

1. Record the volunteer number and time of sample in the trial Lab Book. Also record time samples arrived in the lab and ideally, when the qPCR run starts. (Note any errors or comments on the processing here.)
2. Number lids and tubes clearly in a way that can be tracked back to the volunteer number recorded in the lab book (e.g. number 1-24 in lab book in the order samples are received. *[Note: if blood volume permits, small aliquots may be taken prior to processing as required for further assay development samples.]* Use a suitable holding rack for the tubes prior to loading. Do **NOT** use the QIAsymphony sample racks yet, as they can be easily knocked over.

6.5 DNA Extraction with QIAsymphony

- From the Sample Prep screen, select Wizard
- Select protocol DNA Blood 400 DSP v6 from the protocol selection list on the left (bottom of the DNA sub menu protocols list). Click the arrow button to transfer the protocol selection to the sample number window.
- Enter the number of samples to be processed on this run using the + or – keys or select the sample number to open the keyboard for number entry (you will be able to repeat this process during a run when you want to queue up the next lot of samples if not performing extractions all at the same time) Click Next.

The QIAsymphony has an optimised workflow for loading reagents and consumables once it knows how many samples it will be processing. If it ever runs short during a run, it will tell you and not proceed until you have added what it requests. In normal practice during a clinical trial, keeping the machine at maximum capacity for plastics and waste disposal is advisable.

In the Wizard sample prep mode, it always follows the W.E.R.S order corresponding to the 4 drawers: Waste, Elution, Reagents, Samples. (Individual drawers may be refilled and inventory initiated manually prior to sample setup if preferred for experienced users) In Wizard mode, it will always check exactly what is available in each drawer at each step of setup.

- Load the **Waste** drawer. The QIAsymphony will tell you how many unit boxes it needs (the plastic containers that sample prep cartridges and rod covers come in. Empty ones should be retained for waste) **IMPORTANT**: always ensure that an empty unit box is in position 4 (closest to the front of the drawer. This is the default position for dumping plastics should an error occur)
- Ensure that the 1) black tip chute is clean and in position, 2) that the liquid waste container is present and empty and 3) that an autoclave bag for the waste tips is in position at the front of the drawer. Load as many unit boxes as requested (the machine will efficiently fill part used waste boxes to maximum before they need to be discarded in autoclave waste.) Click Next.
- While instrument is scanning Waste, open and load the **Eluate** drawer with the cooling adapter in position 1 (at the back). Either open a fresh EMTR deep well plate, or use the one from the previous run if stored at 4 degrees and it has enough spaces (full lanes only)
- Carefully remove the bottom of the plate by gently waggling the sides back and forth to loosen. This frees up the bottom of the tubes and allows them to sit in the cooling adapter.
- Select the position on the screen and scan the plate barcode with the scanner. Put the plate into the cooling adapter. (It may be necessary to manually select the plate type if the code is not recognised: select Deep Well Qiagen EMTR tubes from the labware menu.)
- With a new plate, this will enter automatically, with a part used plate with samples from the previous run, the machine will recognise the number and ask if you want to continue. It should recognise the lanes used and automatically select the next available full lane. NOTE: if the plate is not recognised, a new plate name may be entered manually and the used lanes "reserved". This is crucial or the machine may not realise that some lanes have been used.
- Close the drawer and Click Next to move on the **Reagents** drawer screens. These will tell you exactly what is needed for the sample number entered.
- Load up the requested numbers of tips, rod covers and sample prep cartridges or visually check that sufficient is present (machine will check anyway.)
- **IMPORTANT**: visually check for any chips, cracks or misalignment in tip holders and unit boxes before loading (especially plastic ware alignment inside unit boxes)

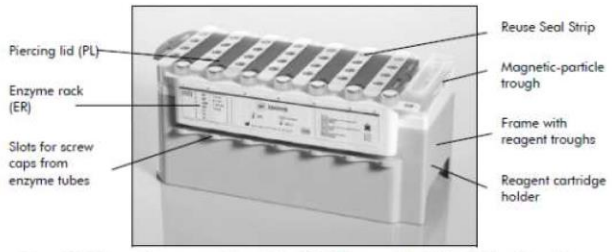

Figure 2. QIAsymphony reagent cartridge (RC). The reagent cartridge (RC) contains all reagents required for the protocol run.

- For first use of each QIAsymphony reagent cartridge (see above), the piercing lid from the kit must be fitted (only clicks on one way round.) This is VERY sharp! Also, add the enzyme rack to the cartridge assembly.

- Remove and vortex the magnetic beads compartment of the reagent cartridge for 3 minutes. NOTE: if the cartridge is already in use, ensure that the re-sealable lid is FIRMLY secure **before** and during vortexing.
- Manually remove the foil seal (carefully pierce with a 1000µl pipette tip at one end to release pressure and taking care not to spill contents, peel back the foil seal and remove any residual bits of foil) If already in use, carefully remove the re-sealable lid and replace the beads container back in the cartridge.
- Check that the cartridge has no precipitate in any of the reagent compartments and that it has not been re-used more than 4 times in total within a 2 week period from first use.(Record sample number and dates of use on the cartridge)
- If cartridge is already in use, remove numbered re-sealable strips from compartments in order and store in the white cartridge base that comes with the kit. It is important that these be re-applied to the correct reagent compartment when the cartridge is removed from the QIAsymphony.
- Place the cartridge into the grey cartridge holder and remove all the lids from the Proteinase K tubes (these are conveniently stored in a slot under the tube.)
- Place the cartridge holder(s) into the reagent drawer slot (s), do another visual check of tip holders and unit boxes and close the drawer. Click Next.

At this stage, the QIAsymphony is ready to go for the number of samples entered and these steps may be carried out up to several hours in advance of receiving samples.

- Select Tube Carrier for sample rack type and open the **Sample** rack door.
- Prior to loading into machine, **with the lid still on**, mix the blood samples by GENTLY inverting the holding rack 5-6 times – important not to cause foaming. Let the samples sit for at least 3 minutes.
- Remove the tube lids **CAREFULLY** to avoid cross contamination. Place lids open end down on a clean bit of lab tissue well away from where they could be knocked over. **VERY CAREFULLY**, remove any film/bubbles formed in the neck of the sample tube and use a fresh pipette tip to gently burst it, well away from the other samples (cross contamination avoidance). Replace the sample and discard tip as derogated Cat3 waste. This is a good opportunity for a final check of sample order before entering the details
- Load tubes into QIAsymphony sample racks with 1A inserts in order of processing (24 per rack) (NOTE: if using frozen blood samples in 2ml Sarstedt tubes, use a rack with 3B inserts. Samples must be at least 0.5ml in volume for the Blood 400 DSP protocol to succeed due to dead volume considerations, preferably >0.6ml. Samples must be thawed and room temperature equilibrated before starting run.) Do not leave loaded sample racks on the bench!
- Load the sample rack(s) by bringing the end of the rack to the line in the sample drawer (indicator light on the lane will be green). The bar code reader will slide out. Slide the rack in with one single smooth movement taking about 3 seconds, to allow the reader to scan the rack. If successful, the sample screen will show the rack as successfully loaded. Click Next.
- The screen will go to the sample ID screen.
- Click Select All and ensure that the correct Labware selection is applied. Default setting should be Sarstedt 2ml skirted tubes (for frozen samples with 3B adapters in sample rack) For fresh challenge samples in paediatric 2ml EDTA vacutainers, select the labware file **BD367836 13 x 75mm K3**. Machine will ask if you want to change labware type (note that this is a non-validated file

in a strict Forensic Casework setting that does not apply here, so we can ignore the warning message). Click Yes.

- Select individual samples, click Sample ID and enter the sample identity. Click OK and repeat for all loaded samples.
- Select All and specify the protocol to apply to all the samples. Click Next
- Highlight the Elution slot to be used and View (if Rack file exists) to check that the machine recognises any previously used lanes.
- Select Elution volume of 100µl. Click Finish (or Queue for when more than one sample rack is being setup). Machine will start final scans if not completed at this stage. NOTE: it is possible to select Batch 1 and remove so that it may be re-entered if anything incorrect is spotted at this stage (e.g wrong protocol selected, elution vol, sample ID) and to repeat the sample entry process if nothing else has changed. (Fill in an Elution rack template sheet for each plate)
- If Queueing more than one rack initially, you will then need to load the next one. Repeat the process of sample ID entry. Click Finish.
- Press RUN. Scans, reagent cartridge piercing and sample processing preparation will begin. When finished, the batch indicator will change to Completed.

NOTE: The samples may be removed for storage of any excess whole blood **after** the Lysis stage has begun during the sample run. Open Sample door and if rack indicator light is green, samples may be removed. Make sure to Queue up any further sample racks in position 2, 3 or 4 as appropriate
Using **filter tips or single use Pasteur transfer pipettes**, transfer remaining whole blood into a labelled 2ml screw cap tube. Pipette tips or pasteurs must be disinfected and both solid and liquid waste disposed of as CL3 material. 2ml tubes should be stored at -80ºC. At the end of the run:

- Retrieve the elution rack containing the purified nucleic acids from the "Eluate" drawer. You will need to Click Remove on the screen before closing it. Even if being used immediately, seal the tubes with the rubber sealing strips provided to reduce evaporation and avoid cross contamination from open lanes.
- If a reagent cartridge is only partially used, seal it with the provided Reuse Seal Strips and close tubes containing proteinase K with screw caps immediately after the end of the protocol run to avoid evaporation. (NOTE: if used again within a few hours, reagent cartridge may be left open and in position.)
- Discard used sample tubes, plates, waste unit boxes and tip waste bags (only tip waste bag need be treated as derogated CL3 solid waste. If required, download the run files from the machine with a USB stick via the Tools menu – sample names, labware details etc.
- Turn off the QIAsymphony. (NOTE: only do this at the end of the day's runs.)

Daily Clean and Maintenance Routine

The following steps should be followed at the end of each day's use of the QIAsymphony. In general, all working surfaces of the machine should only be wiped down carefully with blue roll wetted with 70% ethanol unless otherwise specified (e.g. tip waste chute and liquid waste bottle require full disinfection with Distel first)

- Open the Eluate drawer and wipe down external surfaces gently.
- Open the Reagent drawer and remove empty tip racks and unit boxes if required (re-seal with lids), wipe down external surfaces.

- Open Waste drawer. Remove liquid waste guide port (metal). Wipe with 70% ethanol and always replace with spring to the right.
- Remove waste unit boxes. Full ones should be sealed and discarded as normal CL2 autoclave waste. Partially used ones should be sealed with clearly marked WASTE lids for re-use.)
- Remove the liquid waste container and replace immediately with the clean one (remove the black rubber seal first) – this allows the drawer to be shut again. [**NEVER** circumvent the mechanical device that stops the drawer closing without a waste bottle in position!]. Add a squirt of Distel to the waste bottle to disinfect the CL2 waste, let it sit for 5 minutes and rinse down the sink with plenty of water. Further disinfect bottle with 1% Distel and 70% ethanol. Prop up on blue roll, inverted, to allow it to drain and dry.
- Remove black tip waste chute and **thoroughly** disinfect with 1% Distel and 70% ethanol. Prop up on blue roll to drain and dry. (NOTE, both waste bottle and tip chute may be cleaned with more aggressive disinfectants or allowed to soak overnight if heavily soiled, but do NOT autoclave them.)
- Wipe down external surfaces gently with 70% ethanol and blue roll and close.
- Open the cover, fold down the touch screen (be careful not to lean against the screen in the folded down position!) and wipe down all the external surfaces of the work table. Check the conveyor belt tray and magnetic rod "wings" for any drips and wipe down. Replace conveyor tray.
- Remove and wipe down tip guards with 70% ethanol if apparently clean, but use 1% Distel followed by 70% ethanol if any evaporated buffer or sample material is detected. Replace in order they were removed. Wipe down the black metal weight on the Z drive to the right of the tip guards. (NOTE, the X-Y head may be moved CAREFULLY by hand to facilitate this
- Lastly, check sample drawer for any spills, wipe down with 70% ethanol and check sample spill tray behind front cover of sample drawer.
- Fold up screen and close cover, wipe down exterior with 70% ethanol if required.

6.6 Pre-Challenge or D-1 Samples

Volunteer blood samples are normally taken on Day -1 of the challenge. These are processed and analysed in the same way as all other blood samples with the following protocol changes.

Following DNA extraction, 2 sets of qPCR are set up for each sample. One is set up as for all other samples and a second with a "spike" of known concentration positive control DNA. This is to check that no PCR inhibitors are present in the volunteer's blood. (This has been previously observed on rare occasions.)

As there should be no malaria parasite DNA in the volunteer blood prior to challenge, the first set of PCRs is essentially a negative control. The "spiked" set should contain the equivalent positive control DNA of one of the standards used to create the standard curves on each assay plate. As 15µl of sample DNA will be required for the 3 replicates of each sample, with the spiked set, introducing 1.6 µl of the 10,000 p/ml standard before mixing and dispensing spiked sample into the PCR plate, should generate a positive amplification for the spiked sample that is closely matched to the 1000 p/ml standard (effectively a 1/10 dilution in D-1 DNA). This is well within the normal standard curve range.

Any significant deviation from this expected value (30% or less than expected range) should be discussed with a senior investigator.

6.7 Q-PCR

6.7.1 Background

The Taqman probe based PCR amplifies a 133bp PCR product from the multicopy (3 per parasite) 18S (small subunit) ribosomal RNA genes of P. falciparum (Accession number:M19173). A 5' FAM labelled probe with the Applied Biosystems proprietary NFQ quencher plus MGB moiety at the 3' then binds to the PCR product and is hydrolysed on each cycle of PCR to release the flurophore that is then detected in a quantitative manner. (After Hermsen C.C, Telgt D.S.C, Linders E.H.P, van de Locht L.A.T.F, Eling, W.M.C, Mensink E.J.B.M, Sauerwein R, "Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR" Molecular & Biochemical Parasitology 118 (2001) 247-251)

The sequences of the primers and probe are:

18S Pf Forw (10µM working conc. aliquots labelled For)
5' GTAATTGGAATGATAGGAATTTACAAGGT 3'

18S Pf Rev (10µM working conc. aliquots labelled Rev)
5' TCAACTACGAACGTTTTAACTGCAAC 3'

18S Pf MGB (10µM working conc. aliquots labelled MGB)
5' FAM- AACAATTGGAGGGCAAG-NFQ-MGB 3'
(sequence from Hermsen group 08/07/08, published in C.W. Wang et al. / Parasitology International 58 (2009) 478–480*)*

(Note: Sequence primers at the 80 nmols scale and probe at 20nmols scale are ordered from Applied Biosystems.)

Each 25ul reaction contains:

Sterile Milli-Q water	*5.25µl*	
18S Pf Forw (10µM stock)	*1.0µl*	
18S Pf Rev (10µM stock)	*1.0µl*	
18S MGB (10µM stock)	*0.25µl*	
Universal Probe PCR Master Mix (2x)	*12.5µl*	
Sample/standard	*5µl*	*Total volume 25µl*

The standards for the assay contain DNA extracted from counted, synchronised Ring Stage 3D7 parasites and spiked into whole blood at 1 x 10^6 parasites per ml. This is extracted from 400µl whole blood spike (spike initially prepared in at least 5ml total volume and split into aliquots for replicate automated DNA extraction in 100µl elution). This DNA (equivalent to 10^6 p/ml start concentration) is pooled and then serially diluted in ATE buffer (DSP kit elution buffer) to generate suitable volumes of serially diluted standards as shown below (split into single use tubes with enough volume for at least 5 qPCR plates). These working standards will be checked as equivalent to identical serial dilutions of 3D7 parasites spiked into whole blood and extracted with the standard Blood 400 DSP v6 protocol as used for volunteer samples, before use in a clinical trial.

Name	par/ml equiv	Previous plasmid std equivalent
S1 (1)	100000	PT1
S2 (2)	10000	PT2
S3 (3)	1000	PT3
S4 (4)	100	PT5
S5 (5)	50	(close to PT6 40p/ml)
S6 (6)	25	(close to PT7 20p/ml)
S7 (7)	12.5	(no current plasmid equivalent)

A plasmid copy number standard curve, if used, should be from pre-prepared single use frozen aliquots and triplicate wells of each standard must be run on each assay plate along with NTC (no template control) wells. This curve should be compared with counted parasite dilutions from cultured 3D7 P.falciparum in order to establish a direct relationship with counted parasites per ml for a particular trial. (Relationship appears consistent when tested previously.) Checking quantitation of a new curve against previously used, reliable standards or a externally validated Molecular Biology QA nucleic acid control (if available) is also strongly recommended.

6.7.2 Method

[Note: Ideally, perform all qPCR setup steps away from the sample processing and DNA extraction work areas and take all practical steps to eliminate any chances of PCR contamination. Use PCR flow hood if available. In particular, ensure mastermix, primer and probe preparation is kept well away from any positive plasmid or counted parasite DNA standards. With automated extraction totally enclosing the process, PCR setup may be carried out in the same bay.]

A new working mastermix containing primers, probe, Universal Master Mix and water sufficient for the samples to be tested should be made up fresh for each day's work. Frozen components should be stored in suitable volume aliquots at working concentration in a nominated and monitored -20ºC Clinical Trials freezer and Universal Master Mix stored at 4ºC in a nominated and monitored Clinical Trial reagents fridge. Prepared Master Mix ready for use remains stable at 4ºC for at least 24 hours. (NOTE: where possible, do not store standards in the same freezer as primers and probe, certainly not in the same storage box – **extreme** care must be taken to avoid possible PCR contamination at **all** stages of this assay) Allow sufficient working master mix for each sample and standards (triplicate wells) and an excess (approx 10%) to cover minor pipetting errors. A table scaling up the reaction components for the appropriate number of rows/lanes required on the 96 well plate(s) may be drawn up to assist plate preparation.

1. Thaw enough aliquots for the amount of master mix required of 10µM primers and probe and allow **all** components to equilibrate at room temperature for at least 10 minutes. (probe should be protected from strong light as far as is practical if thawed some time before master mix setup) Also thaw out single use aliquots of the standard curve and allow them to equilibrate at room temperature for at least 10 minutes. (Briefly vortex and spin down all thawed aliquots before use. Keep standards **away** from mastermix setup area.)

2. Ensure that the Universal PCR Master Mix is gently mixed (do not vortex) when removed from 4 ºC before making up the working PCR master mix. Use

the table below to calculate the appropriate volumes to make up for each 96 well plate or part of a plate. The completed master mix can be stored at 4ºC for up to 24 hours before use. Excess primers and probe may also be stored at 4 ºC after thawing for many days, (PCR operators running the morning samples routinely make up enough complete master mix for the entire day's samples to save time for the evening operators if doing twice daily PCR.)

(all volumes in µl)		4 rows	**Half plate**	5 rows	6 rows	7 rows
	1 rxn	40 rxns	**52 rxns**	64 rxns	76 rxns	88 rxns
dH_2O (nuclease free)	5.25	210	**273**	336	399	462
18S Pf For 10 µM	1	40	**52**	64	76	88
18S Pf Rev 10 µM	1	40	**52**	64	76	88
18S Pf MGB 10 µM	0.25	10	**13**	16	19	22
Uni Master Mix 2x	12.5	500	**650**	800	950	1100
Total	20	**800**	**1040**	**1280**	**1520**	**1760**
add per well	20	20	**20**	20	20	20
DNA	**5**	**5**	**5**	**5**	**5**	**5**

[Note: for a whole plate, it is easiest to make up 2 lots of mastermix for half a plate as this allows for an excess, as well as not overfilling a single 2ml tube. Protocol assumes vertical loading of qPCR plate from a 96 well elution plate, but other layouts may be used.]

3. Each PCR plate should include 3 replicates of water as a no template control (NTC), 3 replicates of each standard and the trial samples also in triplicate. (NTC and 7 standards is 24 wells/3 lanes/2 rows – allowing up to 24 trial samples per plate) A 96 well plate support base **must** be used to stop any potential auto-fluorescence contamination from the bench surface and to assist in holding the plate stable at the dispensing and sample addition stages. (This must also be used when spinning the plate prior to running)

4. Add 20µl of PCR master mix to each of the 0.1ml wells in the 96 well plate that will be used. Use a calibrated electronic dispensing pipette to add the master mix to the bottom of each well, taking care not to contaminate the neck of the well. Cover each row of the plate when finished dispensing to prevent contamination (sterile tip box lids are suitable) The same tip may be used for dispensing with an electronic pipette as long as it is pre-wet initially by running one full take up/dispense cycle in the master mix tube before dispensing to the 96 well plate. Keep the tip within the mastermix when performing Take up, Ejecting or Auto-calibrating steps of the electronic pipette cycle. Manual pipetting is acceptable if remaining mastermix is less than Take up volume.)

5. Add the extracted DNA samples **first**, starting at the **4th lane**. Pre-wet each fresh 10µl pipette tip used by pipetting once up and down and touching the tip to the side of the tube to remove any excess. Then take up 5µl of sample and add to the centre of the master mix in wells A4 to A6 in turn, **changing tip and pre wetting for each well**, checking tip for correct uptake volume and full dispensing each time, followed by 5µl of the next sample in each of B4 to B6 and so down the plate for all samples being tested. Then move on to A7-A9 for the next lane of eluted samples. Pipette **just past** the first stop when adding samples to ensure complete sample ejection; no mixing is required in such small volumes. (A dedicated and calibrated 10µl 8 channel pipette may alternatively be used when samples are in 96 well elution plates) Cover each

completed set of 3 lanes as you go. It is **vital** not to lose track of where each sample is: positioning of sample tubes in racks and use of complete lanes of sterile filter tips in a fresh box can assist physical organisation of this stage. Stick to strict numerical order of samples if practical and note any deviation from the typical plate layout so that any adjustments needed may be made correctly on the Step One Plus machine template. (Use a PCR template sheet to manually record what was **actually** put on the plate.)

6. Change gloves (and lab coat if practical) and use a **seperate** designated 10µl pipette before handling positive DNA standards. (Change gloves frequently as a matter of course during both sample prep and PCR setup)

7. Add 5µl of the positive DNA standards in triplicate in the same manner as the samples. **Keep the sample wells covered while doing this**. Pre-wetting the tip, take up 5µl of NTC (same water used to make up mastermix) and add to the centre of the master mix in wells A1 to A3 in turn, changing tip and pre wetting for each well, followed by 5µl standard 1 in each of B1 to B3. Similarly add triplicate standard 2 to wells C1 to C3 and so on down the plate.

8. Change gloves again before adding the optical adhesive cover to the plate. Use the plastic applicator to firmly smooth the optical film onto the plate and make a good seal. Remove the side pieces of the optical film carefully.

9. Spin the plate in the appropriate benchtop centrifuge for 3 minutes at 1500 rpm to ensure the reaction mixture is evenly located in the bottom of each well. Take care not to knock the plate before it is placed in the Step One Plus. (A 96 well support base **must** be used in the centrifuge to minimise any auto-fluorescence from other material that might be transferred to the bottom of the plate. Use an identical base with the balance plate.)

10. Ensure the Step One Plus is switched on and connected to the laptop computer. (NOTE: the setup of the machine and the plate layout template should already be prepared before this stage. Templates may be stored and reused or edited as required at any time – reference to the manual PCR template sheet of what was actually loaded can be vital) Place the plate carefully in the plate drawer and ensure it is shut correctly.

The PCR programme is as follows:

AMPerase step	*50°C for 2 minutes (retain, even if non Amperase mix is used)*
Hotstart activation -	*95°C for 10 minutes*
Cycling -	*95°C for 15 seconds (denature)*
	60°C for 1 minute (annealing and extension)

Repeat 'Cycling' 45 times.

The ROX reference dye should be selected and the target should be FAM-NFQ-MGB (This is actually the default setting for the ABi Step One Plus.)

11. Select and open an appropriate previous template (.edt) file to use or to modify. (May be done in advance, e.g. while plate is spinning.)

12. Make any modifications to the plate layout required for samples being tested and check the programme parameters are correct. When ready, use "save as

template" from the save menu to save the modifications in the appropriate folder as a template file (.eds)

13. When ready to run, go to "Save as…" to save it as a run file (.eds). The green Start Run button will highlight and can be clicked when the plate is loaded.

14. Make sure that the machine begins the run correctly and that the plate drawer moves up and locks into running position. The machine display and computer should give the expected remaining duration of the run (typically 80 - 90 minutes.)

15. If another plate or plates are required to be run immediately after this, the plate(s) may be set up as above and covered with foil to be stored at 4ºC for up to 24 hours until the machine has finished the previous run.

16. [Optional: if required and the laptop is networked, enable the email notification of results to the appropriate email address for when the run is finished. This will be the address of the qPCR lead analyst who will report results direct to the PI. The machine will send the results when the run is complete and go into standby mode after a few hours. All data from all 96 wells is collected and any plate layout changes can be applied after the run if required.

17. Ensure all reagents and samples are put away appropriately (note down sample locations in freezer) and wipe down all pipettes, racks and bench space used with 1% Distel sprayed onto blue roll.

6.8 Data Analysis & Reporting

[Note: Confidentiality is very important at this stage of the trial. No volunteers or microscopists should be made aware of the PCR results. PCR Operators should not be aware of microscopy results or the vaccination status of any volunteers taking part in the trial. Data analysis should ideally be carried out by as few operators as possible (typically the lead qPCR analyst) and not viewed by other laboratory staff. The Principal Investigator should be the only person aware of all results from both laboratory and clinical sides during the trial.]

1. Once the run has finished, analysis is carried out automatically, generating the standard curve from which sample quantitation is calculated. Further manual analysis is also possible following any changes that may be required to plate setup for example. Any manual changes or analysis should be clearly documented and reported with results along with reasons for changes (e.g. adjustment of analysis guided by the QC flags generated by the software can bring a standard curve back within acceptable limits by omission of outlying replicates to allow acceptable R^2 or reaction efficiency)

2. Ensure the NTC samples do not have an amplification curve *[There may occasionally be some late cycle, amplification at very low level, typically at or below the agreed detection cut off for the assay. If more than this is detected or in more than one NTC replicate, the run should be considered potentially compromised and the assay repeated with new NTC samples after consultation with a senior investigator.]*

3. Ensure that the standard curve generated is of sufficient quality to allow reliable quantitation of samples. Reaction efficiency should normally be in the range 90-110% and R^2 value for the linear regression line fit should be greater or equal to 0.98. Deviations from this range that cannot be corrected by QC flag guided analysis adjustments should be discussed with a senior investigator before deciding to repeat the assay.

4. The concentrations given are already in parasites/ml equivalent so no further adjustment is required. The results table may be directly exported into Excel for easier organisation of data.

5. At this stage, a decision must be made on whether a sample is positive or negative (below agreed lower detection limits) for malaria parasite DNA and this information is reported directly to the Principal Investigator only (who should be the only person not "blinded" to both PCR and Thick Blood Film results)

6. The cut off sensitivity used in deciding positive or negative status for each challenge trial should be decided on and agreed with a senior investigator or the PI during the preliminary work up and checking of each trial protocol, as should status of ambiguous results (e.g. very widely different values for different replicates) prior to retest.

7. Inform the Principal Investigator of the mean PCR results as soon as possible*.

*[*By phone or email, attaching a copy of the updated Results Excel sheet where possible. Evening results during Day 6.5 to 14.5 may be analysed the following morning by agreement with the PI or arrangement can be made to have the machine email results directly if the laptop is networked.]*

6.9 Record Keeping

[Note: Confidentiality is very important at this stage. All raw data, data analysis and <u>any</u> records of qPCR results should be kept out of general view. Minimum security would be use of a password locked screensaver set to require the user's password after the minimum amount of inactivity. Operator performing data analysis and anyone with access to the data should be very aware of the potential for bias and should treat all qPCR data as confidential until after the trial is complete.]

The mean value of each triplicate sample is filled in on the electronic qPCR results spreadsheet for each run. This should be kept secure during the trial with only the lead qPCR analyst, their Senior Investigator supervisor and Principal Investigator having access. This file should be kept in a secure, backed up location and password protected during the trial. Details of time samples received, time qPCR run started and time PI informed of result should be recorded in the trial lab book. (No PCR results to be recorded here.) This is also where any anomalies should be recorded in the Comments section. Results are signed off by the operator performing the analysis and a hard copy retained in the Study Site File in a secure location at the end of the trial. (See section 7 for location of associated document templates.)

Raw data from the Step One Plus should be saved in the appropriate Experiments folder at the same location. Save copies of analysed data, until after the trial, on the Step One Plus laptop or a designated analysis station only. *[The Step One Plus laptop should remain on throughout the trial, with the primary operator logged in and with a password protected screensaver enabled to prevent casual access. Similar measures should apply to any designated computer used for analysis]*

All received volunteer blood sample information and comments should be recorded in the trial lab book. Any problems or deviations from the SOP should also be recorded. No current trial PCR results data to be entered into the lab book until the challenge is complete.

7 Associated documents

The qPCR results spreadsheet and Raw data folders are found in the appropriate folders for each trial here: V:\1.Malaria\1. Master Files\MAL Studies or VAC studies in the "Trial name"\Immunology\QPCR Results sub folder.

Jenner Code of Practice Derogated CL3 document (S:\Safety\CL3 lab documents).
COSHH C041 Work with microorganisms (S:\Safety\COSHH assessments\COSHH microbes).
COSHH C043 Work with P. Falciparum under derogated CL3 conditions (S:\Safety\COSHH assessments\Cat 3 COSHH protocols).
ML008F1 Elution Plate Template Form
ML008F2 PCR Plate Template Form.

8 References

Qiagen DSP Midi Kit Instructions
QIAsymphony SP Manual
Applied Biosystems Step One Plus user manuals
HSE ACDP Approved List (S:\Safety\CL3 lab documents)
HSE ACDP managing biological agents (S:\Safety\CL3 lab documents)

9 Review History

Date	Reviewed By	Version	Detail (significant changes from previous)	Previous
Jun 2009	N.Edwards	2.0	Updates of equipment and procedure	1.0
Dec 2009	N.Edwards	3.0	Change to probe based QPCR and new standards for quantitation	2.0
Sept 2010	N.Edwards	3.1	Update of materials and inclusion of CL3 derogated work and waste disposal	3.0
Sept 2010	S Kerridge	4.0	Standardisation of text format and issue.	3.1
Sept 2012	N.Edwards	5.0	Updated on review, with clarifications, typo corrections. Minor reformatting.	4.0
Sept 2014	N.Edwards	6.0	Updated on review, with clarifications for blood stage challenge schedule, typo corrections. Minor technical updates.	5.0
Feb 2015	N.Edwards	7.0	Major revision of SOP to cover use of QIASymphony for DNA extraction from whole blood.	6.0