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'Malaria immunology and vaccine development'

One Volume

Fiona Mary Thompson

Thesis for degree of Doctor of Medicine.

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Abstract

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

INFECTION, INFLAMMATION AND REPAIR DIVISION
SCHOOL OF MEDICINE

Doctor of Medicine

MALARIA IMMUNOLOGY AND VACCINE DEVELOPMENT

By Fiona Mary Thompson

This thesis describes work undertaken by the author at the University of Oxford. It begins by providing an introduction to malaria infection and pathophysiology, and a review of the latest attempts to produce an effective malaria vaccine. It goes on to describe the rationale behind the vaccines developed by the University of Oxford and others. A brief introduction to the process of planning and carrying out clinical trials of vaccines is then provided, and is followed by chapters describing two clinical trials, designed to test the safety, immunogenicity and then efficacy of candidate malaria vaccines. These trials were performed in Oxford, to examine two different vaccination approaches. The first intended to broaden the specificity of the vaccine induced immune response, by providing multiple antigens in one vaccine, aiming thereby to improve protection from malaria infection. The second regimen used a combination vaccine intending to induce both humoral and cellular immunity simultaneously, thereby providing enhanced efficacy against malaria infection. Neither approach was sufficient to provide protection from infection in the challenge studies described; however, some impact on the disease was detected in the second study. This is examined in detail. The laboratory work described studies background immune responses (both cellular and humoral) to vaccine antigens in a malaria exposed population, intended to support the inclusion of these antigens in the multi-antigen vaccine. The remaining chapters describe work in parasite life cycle modelling, undertaken to aid interpretation of results of these clinical trials, and finally an examination of the clinical course of malaria in the control volunteers who have taken part in the many challenge studies conducted in Oxford.

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Author’s Declaration

Author's declaration

DECLARARION OF AUTHORSHIP

I, Fiona Mary Thompson

Declare that the thesis entitled ‘Malaria immunology and vaccine development’ and the work presented in it are my own. I confirm that

This work was done wholly or mainly while in candidature for a research degree at this University.

Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.

Where I have consulted the published work of others, this is always clearly attributed. I have acknowledged all main sources of help.

Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Parts of this work have been published as:

Evidence of Blood Stage Efficacy with a Virosomal Malaria Vaccine in a Phase IIa Clinical Trial, Thompson F.M. et al. PLoS One 2008 3(1) p e1493

‘Challenging a World Killer?’ Thompson, F.M., Good Clinical Practice Journal, 2006: p. 30-33.

Signed……

Date……

Acknowledgements

Chapter 2

This was written by the author as a reference document for use in introducing new staff to the malaria vaccine clinical trials performed by the University of Oxford. The SOP on protocol development was written as part of upgrading the trial management systems in line with new clinical trials legislation.

Chapter 3

The clinical trial was carried out with other members of the Malaria Vaccine Trials team. The protocol was developed by the author, and David Porter (Clinical Research Fellow) in collaboration with Odile Leroy and Hildur Blythman at the European Malaria Vaccine Initiative, who funded the project. The study was planned, the case report forms were developed in StudyBuilder, and the clinical side of the project was carried out by the author, with David Porter and Ian Poulton (Clinical Research Nurse). This included recruitment of subjects, screening, vaccination, safety follow up post vaccination, planning the malaria challenge, and care of volunteers following malaria challenge, diagnosis and treatment of malaria infection. The samples were processed in the laboratory at the Centre for Clinical Vaccinology and Tropical Medicine(CCVTM) by Stephen Todryk, Sheila Keating, Tamara Berthoud, Susanna Dunachie, and Claire Hutchings. The malaria challenge was performed in collaboration with Professor Robert Sinden at Imperial College, London, and mosquitoes were provided by Captain Jack Williams from the Walter Reid Army Institute for Research. Blood films during the malaria challenge were prepared and read by Angela Huntcooke and Simon Correa. PCR to detect malaria parasites during the challenge was performed by Laura Andrews. The results were analysed by the author and David Porter, in addition immunogenicity results were analysed by Stephen Todryk. Estimates of parasite growth rates were performed by the author. Statistical help was provided by Nicola Alder of the Centre for Statistics in Medicine. Professor Adrian Hill supervised the study. The full list of personnel involved in this study may be found in appendix 4.

Chapter 4

This clinical trial was funded by the Medical Research Council. The author, in conjunction with David Porter and Professor Hill successfully wrote the funding application for this study. The protocol was developed by the author and David Porter, in collaboration with Rinaldo Zurbriggen (Pevion Biotec) and Gerd Pluschke (Swiss Tropical Institute). Again, the study was planned, case report forms were developed, and the clinical side of the study was performed by the author, David Porter and Ian Poulton. Samples were processed in the CCVTM laboratory by Stephen Todryk, Tamara Berthoud, Susanna Dunachie and Claire Hutchings. Some samples were shipped to Pevion for antibody analysis, this was performed by Shinji Okitsu and Nicole Westerfield. In addition Carole Long's group at National Institutes of Health, USA, performed Growth Inhibition assays. The challenge was conducted with the collaboration of Professor Robert Sinden at Imperial College, London, and mosquitoes were provided by Captain Jack Williams (WRAIR). The blood films during the challenge were prepared and read by Angela Huntcooke and Simon Correa. A number of films were re-examined after the study, along with films from a number of previous studies, by Simon Correa. PCR during the challenge was performed by Laura Andrews. Extraction and cloning of parasite DNA for sequencing was performed by the author, with help from Laura Andrews. Growth rate calculations were performed by the author. Statistical support was provided by Nicola Alder of the Centre for Statistics in Medicine. The results were collated and analysed by the author and a paper based on this work has been published [170]. A complete list of study personnel may be found in appendix 4.

Chapter 5

The lab work described in Chapter 5 was planned by the author, with assistance from Sheila Keating, Tamara Berthoud and Claire Hutchings. The work was carried out by the author, who also analysed and reported the results. This work formed the basis of an oral presentation given at the AMANET conference in Yaoundé, Cameroon by the author [171].

Chapter 6

The review of literature on parasite life cycle modelling, and selection of models was performed by the author. The models selected were programmed into Excel™ by the author, with assistance from Ben Cox, and models were run by the author. The results were analysed and reported by the author. This work is as yet unpublished.

Chapter 7

The data required for this review of the Oxford University malaria challenge safety was collected by the author with help from Ian Poulton. It was entered into an Excel spreadsheet by Ian Poulton, and the analysis and results were reported by the author.

Abbreviations

AMA-1	Apical membrane antigen 1
APC	Antigen presenting cell
COREC	Central office for research ethics committees
CEF	Chicken embryo fibroblast
CI	Confidence interval
CS	Circumsporozoite
CRF	Case record form
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
Exp 1	Exported protein 1
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot technique
EMP	Erythrocyte membrane protein
FP9	Fowl pox strain FP9
G6PD	Glucose-6-phosphate dehydrogenase deficiency
GCP	Good clinical practice
GMSC	Genetic modification safety committee
GPI	Glycosylphosphatidylinositol (a membrane anchor protein)
GTAC	Gene therapy advisory committee
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSPG	Heparan sulfate proteoglycan
IB	Investigator's brochure
IFN	Interferon
IMPD	Investigational medicinal product dossier
IRBC	Infected red blood cell
IRS	Indoor residual spraying
L3SEPTL	Vaccine insert (the polyprotein) encoding LSA3, STARP, Exp1, Pfs16, TRAP and LSA1.
LSA	Liver stage antigen

LSM	Local safety monitor
ME	Multi-epitope string
ME-TRAP	Vaccine insert encoding the multi epitope string and TRAP
MSP	Merozoite surface protein
MVA	Modified vaccinia virus Ankara
MHRA	Medicines and healthcare products regulatory agency
MHC	Major histocompatability complex
NANP	Asn-Ala-Asn-Pro repeat region of CS protein
NRES	National research ethics service (replaced COREC)
NYVAC	New York Vaccinia - Attenuated strain of vaccinia virus used as viral vector for vaccines
PCR	Polymerase chain reaction
PEV3A	Virosomal vaccine containing a mixture of PEV0301 and PEV0302
PfEMP	Plasmodium falciparum erythrocyte membrane protein
Pfs16	Plasmodium falciparum sexual stage 16
PI	Principal investigator
PP	The ‘Polyprotein’ vaccine insert (L3SEPTL)
PyCSP	Plasmodium yoelii circumsporozoite protein
RBC	Red blood cell
SIRS	Systemic inflammatory response syndrome
SIV	Simian immunodeficiency virus
SD	Standard deviation
SNP	Single nucleotide polymorphism
STARP	Sporozoite threonine and asparagine rich protein
SSP2	Sporozoite surface protein 2 (murine homologue of TRAP)
TRAP	Thrombospondin related adhesion protein
TNF	Tumour necrosis factor

Chapter 1 Introduction

1.1 Purpose

The work described in this thesis is part of the ongoing work at the Jenner Institute, in the Centre for Clinical Vaccinology and Tropical Medicine, part of the Nuffield Department of Medicine, under the supervision of Professor Adrian Hill at the University of Oxford. The work described here includes a summary of the methodology for conducting a clinical trial of a malaria vaccine, followed by two candidate malaria vaccine trials, performed in Oxford, along with some laboratory work relating to the clinical trials, and some computational modelling of malaria infection, also relevant for the clinical trials. Finally there is an analysis of safety data compiled from a large number of the studies conducted by Professor Hill's group in Oxford over the last 8 years.

1.2 Malaria Introduction

1.2.1 Burden and Importance

Malaria is a global disease, affecting approximately 40% of the world's population. It is estimated that there were 515 million clinical episodes of P. falciparum malaria in 2002 alone [1] and at least a million people die annually from the disease in Africa [2]. In most tropical and many subtropical developing countries, malaria is a major cause of morbidity and mortality, especially among infants, young children and pregnant women [2]. Malaria also represents a serious health hazard for travellers who visit malaria-endemic areas. The widespread resistance of Anopheles mosquitoes to insecticides has crippled national and regional malaria control programs based on vector control interventions. Furthermore, the increasing prevalence of chloroquine and anti-folate resistant strains of P. falciparum in Asia, Africa and South America has created a crisis in the clinical treatment of malaria in many countries. These drug resistant strains have made devising well-tolerated and effective prophylactic regimens challenging. Given these facts, there is an urgent need for new, effective interventions that can be

applied to the control of malaria. One such approach involves immunoprophylaxis by means of malaria vaccines. An effective vaccine could make an enormous impact on this problem, both for people in the developing world and for those travelling to malaria endemic countries [1, 3]. It could also contribute significantly to the longer term goal of eradication of malaria.

The scale of the problem that malaria causes across the world is gradually becoming more widely understood. Despite this, it is still shocking that a child dies every 30 seconds from this disease [2]. Most of these deaths occur in sub-Saharan Africa, where children under five are most at risk [4]. The UN acknowledged malaria in its Millennium Development Goals; goal number 6 is to 'Halt and begin to reverse the incidence of malaria and other major diseases'. At present, the main strategies employed to control malaria include indoor residual spraying (IRS), bed nets and effective treatment. IRS involves coating the walls and other surfaces of a house with a residual insecticide, which is intended to kill mosquitoes and other insects that come into contact with these surfaces for several months afterwards. This can be a highly effective measure: when used in a study in Bolivia, it dramatically reduced the annual parasite index (the number of cases of malaria per 1000 people per year) [5] and reduced malaria transmission by up to 90% [6]. The number of spray programmes declined in the 1980s, largely as a result of health and environmental concerns surrounding DDT, these are gradually being reintroduced. The use of insecticide-treated bed nets is also known to be an effective means of reducing transmission, although their efficacy depends largely on the local mosquito population, where mosquitoes bite largely at night (as the majority of African vectors do), they are more effective than where mosquitoes bite mainly in the evening (for example in South America) [7]. The prompt provision of effective antimalarial medication, particularly in areas where the parasite is resistant to conventional drugs is also important. These strategies, combined with improved diagnosis of infection, can successfully reduce the impact of disease. But, however effective these methods are, they can only hope to control malaria infection, rather than to eradicate it.

1.2.2 Life Cycle and Pathogenesis

There are over a hundred species of the malaria parasite (Plasmodium species), but only four of these species have humans as their vertebrate host [8]. These are Plasmodium falciparum, Plasmodium vivax (these first two cause most of the vast number of clinical cases of malaria), Plasmodium malariae and Plasmodium ovale. Rare zoonotic infections have been recorded from non-human primate malarias such as P. knowlesi, P. simium, and P. cynomolgi. Although each of the human malarias has distinguishing biological, morphological, and clinical characteristics, their overall biology and lifecycles are similar. Research into malaria has used a number of animal models of malaria infection, those most commonly used are the murine pathogens, P. yoelii and P. berghei, along with the non-human primate malarias mentioned above. The fight against malaria has been focussed largely on P. falciparum as this is the type associated with the highest mortality, and is the cause of the majority of malaria deaths. The Plasmodium parasite is spread to humans by the bite of an infected female Anopheles mosquito.

When a mosquito feeds on an individual, it injects saliva as it probes for blood. The saliva of an infectious mosquito contains malaria parasites – sporozoites. Originally it was assumed that these were injected directly into the blood stream, but groups have shown experimentally that removal or heat treatment of the injection site can prevent infection in animals probed upon by infected mosquitoes [9, 10] suggesting they are deposited into the dermis. The number of sporozoites injected by a single infectious mosquito was previously estimated in studies using membrane feeders at 5 – 20 [11, 12]. However, quantitative PCR analysis of the injection site after single mosquito feeds using the rodent malaria parasite Plasmodium yoelii, showed that a single infected mosquito injected between 0 and 1,297 sporozoites, with a mean of 123 and a median of 18 [13]. 20% of infected mosquitoes injected no sporozoites after 3 min of probing [13], a result also observed when salivating mosquitoes were analyzed [12]. These data demonstrate that the sporozoite inoculum itself is highly variable. Sporozoites move randomly in the skin, until they contact the vascular or lymphatic endothelium [14]. It had been assumed that sporozoites were rapidly carried away from the injection site, as some do enter the circulation within minutes of injection

[10], and the infectivity of sporozoites dissected from mosquito salivary glands and kept at 37℃ for 1 hour wanes rapidly [15]. Again, initial assumptions have proven to be incorrect, with detailed quantitative studies of the kinetics showing that 90% of sporozoites remain at the injection site 1 hour post infection [16], and they leave the site in a slow trickle between 1 and 3 hours post injection. As many as 20% of sporozoites leave the injection site via lymphatics, and some of these sporozoites begin to develop in the draining lymph node, an important site for the initiation of anti-sporozoite immune responses [14] and [16]. Once sporozoites enter the circulation, they travel in the blood to the liver. Their circulation is arrested in the liver by specific interactions between the circumsporozoite protein (CS) and the highly sulphated HSPGs of hepatocytes [17, 18].

Sporozoites will typically migrate through several hepatocytes before settling in one [19, 20], and initiating a process of asexual replication to give rise to an exoerythrocytic schizont. Up to this point, infection is clinically silent, and non-pathogenic. After about seven days the liver shizonts rupture to release many thousands (estimated at 20,000 – 30,000) of merozoites into the bloodstream. Here they attach to and invade circulating erythrocytes, where they develop through a series of morphological changes from ‘ring’ forms to trophozoites and finally dividing mitotically to produce schizonts containing up to 20 daughter erythrocytic merozoites. These are liberated by red-cell lysis and can go on to invade fresh uninfected erythrocytes, giving rise to a cyclical blood-stage infection with a periodicity of 48–72 hours, depending on the Plasmodium species. The intra-erythrocytic cycle of infection is usually fairly synchronous (particularly in P. vivax and P. ovale infections, although less so in P. falciparum) so red-cell lysis and merozoite release occur at regular intervals in infected individuals. ‘Malarial pyrogens’ released at the time of RBC lysis induce cytokine production (for example, tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1)) giving rise to the periodic ‘agues’ or paroxysms of fever that have long been a diagnostic feature of malaria infection. The asexual blood forms are the only forms of the parasite that give rise to clinical symptoms. As-yet-unknown factors trigger a subset of developing merozoites to differentiate into male and female gametocytes. When another mosquito feeds on the same individual, these sexual stage parasites are taken up by the feeding mosquito, and give rise to extracellular gametes. In the

mosquito mid-gut, the gametes fuse to form a motile zygote (ookinete), which penetrates the mid-gut wall and forms an oocyst, within which meiosis takes place and haploid sporozoites develop. Sporozoites then migrate across the gut wall and into the salivary glands of the mosquito to complete the cycle. This life cycle is illustrated in Figure 1.1.

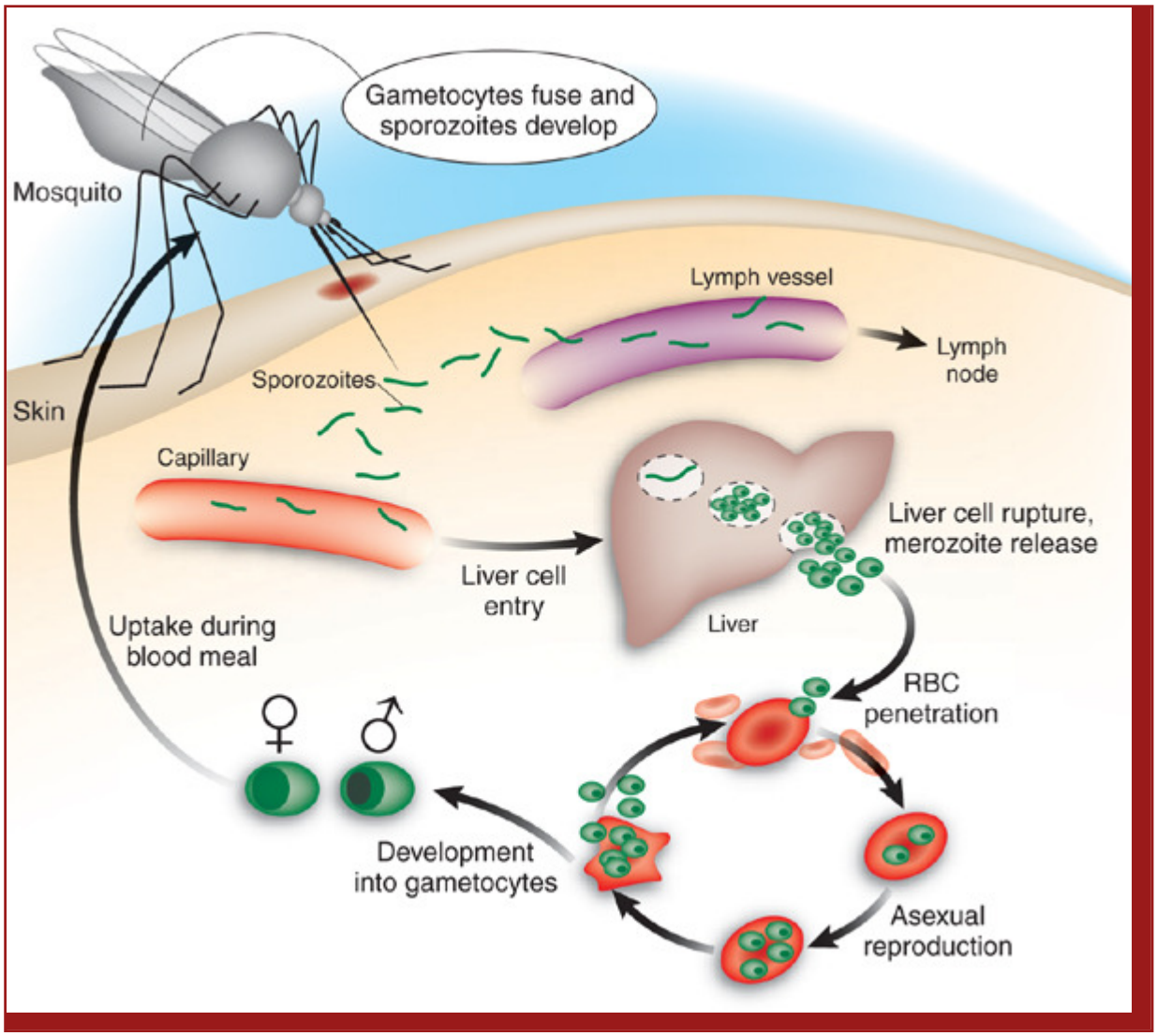

Figure 1.1: Life cycle of malaria in humans
Sporozoites are injected into human dermis through the bite of infected Anopheles mosquito. After inoculation, sporozoites migrate to liver cells to establish the first intracellular replicative stage. Merozoites generated from this exoerythrocytic phase then invade erythrocytes (RBCs), and it is during this erythrocytic stage that severe conditions of malaria occur. The life cycle is completed when sexual stages (gametocytes) are ingested by a mosquito. From [21] © Nature Medicine 2006.

1.2.3 Molecular Biology

The genome of P. falciparum was sequenced in 2002 [22], and was found to have an extraordinarily high adenine and thymine (A + T) content of around 82%. The 23-megabase nuclear genome consists of 14 chromosomes and encodes about 5300 genes.

One result of the availability of genome sequence data is the possibility for detection of genome wide single-nucleotide polymorphisms (SNPs). This process may assist in pinpointing the origin and mapping the spread of infectious diseases, to identify and track new mutations that confer resistance to drugs or vaccine-induced immunity, and potentially to identify candidate genes for novel therapeutic or immunological intervention. One such approach identified a disproportionate number of polymorphisms in genes encoding surface proteins or those associated with the cell membrane. These genes are targets for only 22% of the oligonucleotide probes used in this analysis, but account for 69% of the polymorphisms identified [23].

The major antigen on the sporozoite surface is the circumsporozoite protein (CS) which facilitates sporozoite migration to the liver and binds specifically to regions of the plasma membrane of hepatocytes exposed to circulating blood in the space of Disse [17]. An associated protein, the thrombospondin related adhesion protein (TRAP) also plays a key role in sporozoite invasion of hepatocytes [24, 25]. The surface of the merozoite contains the merozoite surface proteins (MSPs) of which a number have been identified, but for many, their role has yet to be defined. Merozoite surface proteins (MSP-1, 2, 4, 5, 8 and 10) link to the membrane via a glycosylphosphatidylinositol (GPI) membrane anchor, whilst others, such as MSP-3, MSP-6, MSP-7 and MSP-9 are soluble and are, in part, associated with the merozoite surface [26]. MSP-1 was the first Plasmodium MSP to be discovered and is the most well characterised member of the MSP family. It appears that MSP-1 is essential for red blood cell invasion and survival of the malarial parasite as strains of knock-out parasite with this gene removed are not viable [26].

Many other parasite-derived proteins are produced in the course of the life cycle, not all of which are exported to the membrane of the infected cell. Some others are secreted into the cells, and some interact specifically with the red-cell cytoskeleton modifying the host-cell environment in favour of the parasite [8].

1.2.4 Molecular Pathology

The cause of malaria was unknown for centuries, but it was noted to be characterized by periodic fevers. Ronald Ross first confirmed the presence of oocysts within the mosquito gut, and recorded that the parasite was transmitted through the mosquito bite [27]; work which won him the Nobel Prize for Medicine in 1902. It was subsequently realized that the bouts of fever generally coincided with the release of new merozoites into the blood, as each cycle of replication was completed (observation published by Golgi in 1889 [28]). Initially, it was assumed that the release of infected cell contents that occurs at this time was responsible for fever induction, it was not until more recently that it was proven that components of the infected cell such as the lipid, glycosylphosphatidylinositol (GPI) anchor of a parasite membrane protein could directly induce the release of cytokines such as TNF-α and IL-1 from macrophages [29]. GPI has subsequently emerged as a central toxin that induces the expression of many host genes implicated in malaria pathogenesis [30]. GPIs anchor proteins in the membrane of virtually all eukaryotic cells [31]. A considerable fraction of GPI in parasites appears at the plasma membrane without protein attachment as free GPI [32-34]. Although evolutionarily conserved, species-dependent branching of the core molecular structure results in unique GPI molecules comprising epitopes that can be recognized by the mammalian immune system. Serological studies using GPIs purified from P. falciparum have indicated that sera from malaria-endemic regions contain naturally occurring anti-GPI antibodies [35-37]. Purified GPI induces the expression of many genes that are implicated in malaria pathogenesis: for example, genes that encode pro-inflammatory cytokines – such as tumour-necrosis factor (TNF), interleukin-1 (IL-1) and IL-12 [37, 38] – inducible nitric-oxide synthase [39], and various adhesion molecules that are expressed at the surface of the vascular endothelium and are recognized by P. falciparum EMP1 [40], which increases endothelial-cell binding by parasitized RBCs [40]. In a sepsis-shock

model, GPI alone is sufficient to cause symptoms that are similar to those of acute malaria, such as transient pyrexia, hypoglycaemia and death of recipients as a consequence of TNF-mediated coagulopathy, as seen in the malarial shock-like syndrome [38]. Parasite GPI-glycan appears to play a similar role in other parasitic diseases, such as trypanosomiasis and toxoplasmosis [41, 42], in which they have also been shown to induce TNF-α. Measurements of TNF-α in children suffering from malaria demonstrated that very high levels of this cytokine were associated with severe disease [43] and cerebral malaria with a poor outcome [44], although the correlation was not sufficient to be a useful prognostic indicator.

It has been demonstrated that more mature parasites within erythrocytes are differentially sensitive to physiological increases in temperature [45], so that the effect of fever is both to limit parasite multiplication and to maintain synchronous development. More recently, Oakley et al. have confirmed that cultivation of P. falciparum parasites at 41℃ leads to parasite death in a time-dependent manner [46], and suggested that an apoptosis-like cell death mechanism might be induced in response to febrile temperatures. However, this hypothesis was not borne out by analysis of differential gene expression in these parasites.

The pathology associated with malaria infection is largely related to asexual parasite multiplication in the bloodstream and in the case of the most virulent human malaria, P. falciparum, this has been linked to the effects infection has on parasitised erythrocytes [47]. Although sporozoites can and do induce some anti parasite activity, the relatively small numbers of parasites involved in the liver stages do not appear to be related to any significant symptoms. Circulating gametocytes also do not appear to be related to any adverse effects. Infection with P. falciparum can have a wide range of consequences, from the relatively benign fever through to the potentially fatal severe anaemia and cerebral malaria. Metabolic acidosis, often manifesting as respiratory distress, is an important component of the severe malaria syndrome [48, 49]. Metabolic acidosis has been demonstrated to be the best independent predictor of a fatal outcome [48-51].

The effect that seems to underlie the pathology of most manifestations of severe falciparum malaria is the result of the appearance of parasite encoded proteins on

the red cell surface. These proteins mediate adherence to a variety of host tissues, including the vascular endothelium, resulting in sequestration of infected cells. Only red cells that have been infected for longer than 16 hours exhibit these receptors, resulting in high levels of circulating immature parasites, and sequestration of more mature forms [47]. This process occurs in all infections, and is thought to be an important factor in the pathogenesis of severe disease where concentration of organisms in particular organs such as the brain occurs. Binding of sequestered parasitized erythrocytes to uninfected red cells to form erythrocyte rosettes is linked to disease severity [52]. The distribution of infected RBCs found in tissue sections suggests that the chief sites of infected cell sequestration correlate with specific organ dysfunction, for example in the cerebral circulation leading to cerebral malaria [53].

It is assumed, but not formally proven, that the microvascular obstruction secondary to cytoadhesion of parasitised RBCs [54], rosetting [55] and reduced RBC deformability [56] leads to a reduction in local blood flow, or obstruction of small vessels and this results in reduced perfusion and tissue damage. It has also been suggested that the sequestered cells exert a direct toxic effect, triggering the activation of host immune factors that include cytokines, such as TNF and pro-inflammatory interleukins, oxygen free radicals and nitric oxide, which result in damage to host endothelium and tissues [57]. This results in a host response syndrome similar to the systemic inflammatory response syndrome (SIRS) or sepsis syndrome [58].

The detailed mechanisms by which sequestered cells result in the specific symptoms seen in cases of severe falciparum malaria are still being elucidated, however, the molecular mechanisms leading to sequestration are becoming clearer. Specific receptors and parasite molecules involved in adhesion have been identified. Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) plays a central role in the pathogenesis of malaria; mediating infected cell cytoadherence to a range of ligands on endothelial cells, placental cells, and other red cells leading to rosetting, and on infected red cells and platelets leading to auto-agglutination, which, as described above, are related to disease severity.

Despite the life-threatening nature of severe falciparum malaria, and the enormous number of childhood deaths that it causes in sub-Saharan Africa, the mortality rate of all malaria infections is relatively low. In holoendemic areas, infections in children are universal and constant, yet only a small proportion of those infected show clinical symptoms at any one time and only a fraction of these go on to develop severe illness. This is probably only partially explained by the known innate resistance factors and acquired immunity, and so it is likely that unidentified factors are also important in determining how far individual infections progress from parasitaemia to clinical illness and finally to severe disease.

1.3 Immunology of Infection

Individuals living in areas where malaria is endemic do acquire some level of immunity to infection. In other infections, individuals rapidly become resistant to subsequent infections with the same organism. In contrast, immunity to malaria infection is acquired over several years and repeated episodes of clinical malaria. This is evident from the fact that the burden of disease falls on young children. Older children and adults are resistant to severe morbidity and death [59], though remaining susceptible to infection. These individuals are often parasitaemic, but are protected from clinical symptoms of disease. It is therefore important to define precisely what we mean by susceptibility to malaria. As outlined by Marsh et al. [60], parasite prevalence, prevalence of clinical malaria and prevalence of severe life threatening malaria must all result from exposure to infection and be a measure of susceptibility; all show evidence of acquisition of resistance with increasing age, but each indicator has a quite different relationship with age – see Figure 1.2.

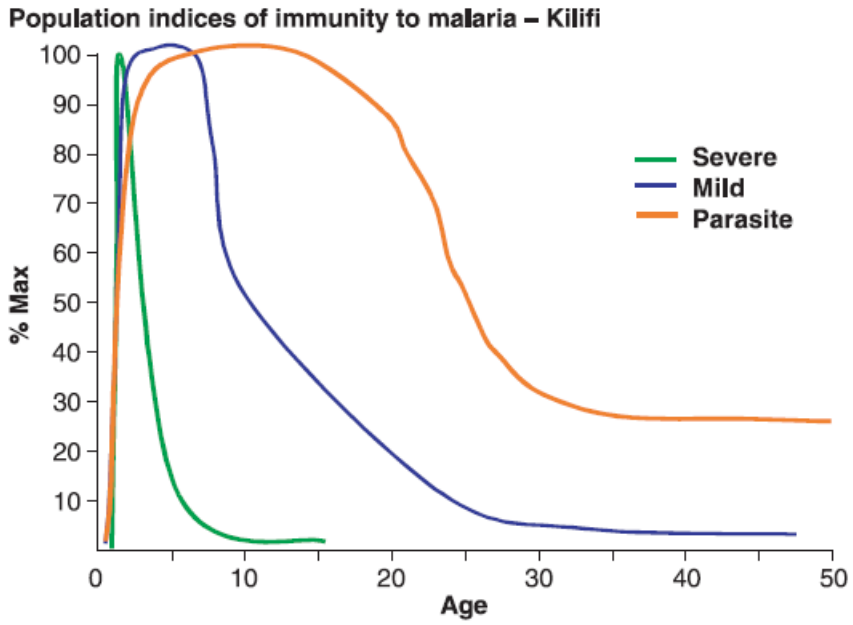

Figure 1.2: Population indices of immunity to malaria – Kilifi.
The figure shows representative data from a number of studies in Kilifi District on the coast of Kenya. The age pattern of asymptomatic parasite prevalence and the period prevalence of both mild and severe clinical malaria are shown in relation to maximum prevalences recorded. (Taken from Marsh et al. [60]).

The mechanisms responsible for protection against severe malaria are not necessarily the same as those that protect against infection per se, or against mild episodes of disease. Field studies of immune responses and mechanisms are almost entirely restricted to examining these outcomes for logistic reasons, and this remains a major problem with research in this area [60].

1.3.1 Pre-erythrocytic Immunity

Immunity to the pre-erythrocytic stages of malaria infection may act at any point from the injection of a sporozoite into the blood stream of an individual, to its invasion of a hepatocyte, and the development of an intrahepatic schizont. In individuals living in endemic areas, responses to the pre-erythrocytic stages develop relatively late on in life, compared with responses to the blood stages [60]. The importance of immune responses to this stage of the life cycle is demonstrated by the observation that immunization with radiation-attenuated

sporozoites induces sterile protective immunity against parasite challenge [61, 62]. This immunity is targeted primarily against the intrahepatic parasite and appears to be sustained long term even in the absence of sporozoite exposure [62, 63]. The mechanisms are multi-factorial, encompassing both cell mediated and antibody mediated immunity [63, 64].

Extensive polymorphisms in the circumsporozoite protein exist in wild parasites and this is thought to indicate that it may be under direct immune selection pressure. However, studies have suggested that these polymorphisms are not necessarily a result of immune selection of parasites [65]. Possible alternative mechanisms for their development include adaptations to structural differences in salivary gland receptors of different mosquito vector species [66] or to hepatocyte receptors in different human populations [67]. Individuals in endemic areas are undoubtedly exposed to sporozoite proteins on a more or less regular basis, but the short time to re-infection in adults in all studies indicates that any naturally induced immunity to these antigens is not particularly effective [60]. Possible reasons for the low levels of naturally induced immunity compared to those induced experimentally to these stages include the difference in numbers (natural infections deliver 1-100 sporozoites per bite [68], whilst each individual was vaccinated with hundreds of thousands of irradiated sporozoites in order to achieve protection [62]), and the possible persistence of irradiated sporozoites in the liver (in experimental models attenuated sporozoites were present up to 6 months after vaccination) [69]. Irradiated sporozoites have also been shown to induce hepatocyte apoptosis shortly after infection [70], dendritic cells may then phagocytose apoptotic infected hepatocytes containing parasite antigens, providing a pathway for presentation of sporozoite antigens to the immune system. In animal models of the irradiated sporozoite system, the CS protein has been shown to be immunodominant – responses against it are estimated to be responsible for 90% of the induced protection, although in this system, an immune response against PyCSP is not required for protective immunity [71].

In theory, antibodies against the circumsporozoite protein could prevent the initial event in the infection cycle and protect against disease. High titres of antibodies to CS are induced by irradiated sporozoite vaccination, and are largely directed

against the immunodominant repeat $(NANP)_n$ region of CS, with small numbers of low level responses to other regions. In contrast, in individuals from an endemic region (in West Africa) high level antibody responses to several non repeat regions of CS were seen. Responses to the repeat region were also seen, but these were lower level responses, and were not present in children [72]. It therefore seems unlikely that this mechanism is important in protection from natural infections. Antibodies to other sporozoite antigens such as TRAP [73], liver stage antigen 1 (LSA-1) [74] and STARP [75] are detectable following natural exposure to malaria infection, and these may have a role in blocking sporozoite invasion. However there is no clear evidence from field studies that the presence, or levels, of antibodies recognizing the sporozoite are related to protection against infection or disease [60].

Cell mediated immunity also has a significant role in the protective response. Immunisation of mice with irradiated sporozoites induces protection against malaria challenge [76]. Further studies showed that protection induced in this way was abrogated in T cell deficient mice, whilst B cell deficient mice were still protected [77]. It was subsequently shown that CD8+ T cells specific to an epitope within the CS protein were capable of conferring protection on mice [78]. This protective immunity to Plasmodium berghei sporozoite challenge could be transferred to other mice by transferring the T lymphocyte clones that were induced by irradiated sporozoites. T lymphocyte recognition of circumsporozoite protein-derived peptide on infected hepatocytes led to lysis of the infected cell and parasite death [79]. More recently, Doolan et al. [80] examined the responses that led to protection in inbred strains of mice, and established a marked diversity of T cell-dependent immune responses. They demonstrated a total of five distinct mechanisms of protective immunity, all of which were absolutely dependent on CD8+ T cells [80]. They have gone on to confirm IFN-γ as a critical effector molecule and to show that IL-4 secreting CD4+ T cells are required for induction of the CD8+ T cell responses, and Th1 CD4+ T cells provide help for optimal CD8+ T cell effector activity [63]. In natural infection, the draining lymph node may be the site at which the immune response to the sporozoite stage is initiated and it has been recently demonstrated that a sporozoite-specific cytotoxic T-cell (CTL) response is evident in the draining lymph node 2 days after intradermal

immunization with irradiated sporozoites [81]. When the draining lymph node was surgically ablated to prevent early local priming, the number of sporozoite-specific CTLs in the liver was significantly reduced, highlighting the importance of skin-draining lymph nodes in the initiation of the immune response to sporozoites during natural infection [81]. In humans there are several pieces of indirect evidence for a T lymphocyte role in protection. Firstly, human leukocyte antigen (HLA) class-I restricted T lymphocyte responses have been demonstrated in both residents of endemic areas repeatedly exposed to P. falciparum, and in malaria-naïve volunteers protected from sporozoite challenge by irradiated sporozoite immunization [82-84]. T cells specific to P. falciparum epitopes were shown to be present in low levels in adult Gambians [85, 86]. Secondly, HLA-B*53, which restricts the immune response of CD8+ T lymphocytes, and HLA-DRB1*1302 which restricts the immune response of CD4+ T lymphocytes, were associated with resistance to severe malaria in a large case-control study of Gambian children [87]. Cytotoxic T lymphocytes to a conserved P. falciparum epitope restricted by HLA-B*53 have been identified and these derive from the Liver Stage Antigens (LSA) LSA1 and LSA3 [83, 88]. Thirdly, some P. falciparum T lymphocyte epitopes are in highly polymorphic regions in which all nucleotide substitutions encode amino acid changes. The observation that no synonymous changes occur argues strongly that these regions are subject to selection pressure from T lymphocytes. This is supported by the identification of altered peptide ligand antagonism of T lymphocytes as an immune escape mechanism [89].

Components of the innate immune system, including gamma-delta T cells, natural killer cells and natural killer T cells, also play a role in protection [63]. The precise nature of pre-erythrocytic stage immunity in humans, including the contribution of these immune responses to the age-dependent immunity naturally acquired by residents of malaria endemic areas, is still poorly defined. A CD4+ T cell response to an epitope from the CS protein has been strongly associated with protection both from infection and disease [90] in adults in The Gambia. However, it is likely that in the normal human population a variety of different mechanisms co-exist, with different individuals being protected by different mechanisms.

1.3.2 Erythrocytic Immunity

The targets for responses against the blood stages of malaria infection are the emerging merozoites, and infected red blood cells. The process of invasion of red blood cells involves many parasite proteins, including the merozoites surface proteins MSP1 to 9, AMA-1[91-93] and some secreted proteins [94]. Antibodies to these proteins may prevent invasion of merozoites, although relatively few studies have confirmed this [95]. Some studies have concluded that antibodies against AMA-1 [96] and MSP-1 [97, 98] are associated with protection from clinical malaria, although this is not supported by others [99, 100]. These proteins are found to be highly polymorphic, which facilitates immune evasion by the parasite, and antibodies with slightly different specificities may be expected to vary in their ability to prevent invasion and protect against infection. It is possible that measuring absolute levels of antibodies is not helpful, and qualitative assessment of function may be a better measure of protection by these antibodies. Functional assays are not without their complications, as demonstrated by the studies that showed that purified antibodies reported to inhibit parasite growth in vitro are not associated with immunity from clinical malaria [101, 102].

The infected red cell displays a number of parasite proteins on its surface, again providing putative antigens for a host immune response. Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is a potentially important family of target antigens, because these proteins are inserted into the red cell surface and are prominently exposed. They are also highly polymorphic and undergo clonal antigenic variation [54], a mechanism of immune evasion maintained by a large family of var genes. Other highly polymorphic proteins may also be expressed on the cell surface, products of other multigene families such as rifs or stevor [103]. Antibody responses to some of these variants, but not others are associated with protection from malaria [104]. The association of responses to particular antigens and protection seems to vary with time and geographical setting. Responses to a restricted subset of these variants are associated with protection from placental malaria [105, 106]. The response to these gene families appears to be closely linked to the pathogenesis and severity of malaria infection [107].

Whilst antibody responses to the erythrocytic stages are clearly important, they are not the whole story. T cell immunity against blood-stage antigens can induce protection against P. yoelii murine malaria [108]. In humans, non-immune volunteers repeatedly challenged with low doses of blood stage parasites and then treated were immune to subsequent challenge [109]. These individuals did not have any detectable antibody response to schizont-stage (3D7 strain) soluble antigen or to recombinant MSP1, MSP2 or AMA1. However, cellular responses, including proliferative responses, interferon gamma production and raised concentrations of nitric oxide synthase, characteristic of a Th 1 type immune response were detected [109]. It is not clear what role responses like these may play in endemic areas.

1.4 Vaccines for Malaria

1.4.1 Background

Vaccines have classically comprised either attenuated or inactivated pathogenic organisms, or antigens derived from them, delivered in such a way as to provide a 'depot' of antigen together with an immunological adjuvant that will initiate an immune response via innate pathways. In applying this approach to malaria vaccine development, mature sporozoites were inactivated within mosquito salivary glands by irradiating the mosquitoes one hour before immunization, by exposing them to 15,000 rad of gamma radiation from a ^{60}Co or ^{137}Cs source. Humans 'immunised' by the bites of these mosquitoes were protected from subsequent malaria infection [61, 62]. These studies demonstrated that it was possible to achieve protection from malaria by vaccination. However, until recently it has been considered clinically and logistically impractical to immunise large numbers of people with irradiated sporozoites, primarily because individuals needed to receive more than 1,000 immunising bites before they were protected [62]. However, this approach is being investigated further by Hoffman et al., who have established a biotechnology company (Sanaria, [110]) to develop irradiated sporozoite vaccines, aiming to convert the current protocol for whole-parasite vaccination into a more practical parenteral immunization regime with cryopreserved parasites [111].

The first clinical trials using a purified protein malaria vaccine began 20 years ago [112], but so far only volunteers immunized with vaccines based on the circumsporozoite protein have been reproducibly protected against P. falciparum sporozoite challenges. However, the protective immunity induced by the best circumsporozoite-protein vaccine is far lower [113, 114] than that induced by radiation-attenuated P. falciparum sporozoites [62]. The main explanation for this difference is thought to be related to the huge number of antigens presented in a whole parasite vaccine, compared to one single antigen contained in the vaccine [115]. Many approaches have therefore focussed on identifying other sporozoite antigens that may be important targets for protective immunity. Opinion is divided as to whether protective immunity relies on one, or many proteins, and whether the best vaccine will be one that combines several antigens or one that targets a single, immunodominant protein.

The malaria vaccine candidate currently at the most advanced stage of development is a sporozoite vaccine, based on the circumsporozoite protein, and is named RTS,S. It consists of a protein particle vaccine in a complex adjuvant [116] (formulated with a variety of adjuvant systems, including AS01 and AS02). It is the result of a long-term collaborative programme between the Walter Reed Army Institute of Research and GlaxoSmithKline Biologicals, Rixensart, Belgium [117], and has been shown to confer complete or partial protection against experimental infection [113, 116]. Short-term protection against infection was shown in immunised adult men in Gambia in 1998 [118]. A study in 2004 immunised children in Mozambique aged 1 to 4 years, this trial established that RTS,S reduced the risk of P. falciparum infection, uncomplicated malaria, and severe disease [114], and that this protection lasted for at least 18 months [119]. More recently, a phase I/IIb trial in infants in Mozambique has been reported [120], and although this trial was designed mainly to assess safety, it also had sufficient power to assess efficacy against new infections. Vaccine efficacy for new infections was 65% over a 3-month follow-up after completion of immunisations [120], considerably higher than previously reported efficacy, although confidence intervals overlap, so it is not a statistically significant change. Importantly, for the first time in this study, there was evidence of an association between vaccine

induced anti-circumsporozoite antibodies and a reduction in the risk of infection. These findings clearly need to be corroborated, and phase III studies across Africa are planned.

Other approaches to generating protective immunity against sporozoites have been explored, these are described in more detail in [121]. Particularly relevant to this thesis is a product developed by the Swiss biotechnology company Pevion Biotech. Ltd. This is a virosome based vaccine including two P. falciparum peptides. Virosomes (see Figure 1.3) are influenza derived virus-like particles, they incorporate the influenza virus haemagglutinin and neuraminidase proteins to facilitate antigen processing. There are currently two licensed virosomal vaccines: Inflexal V (Influenza) & Epaxal (Hepatitis A).

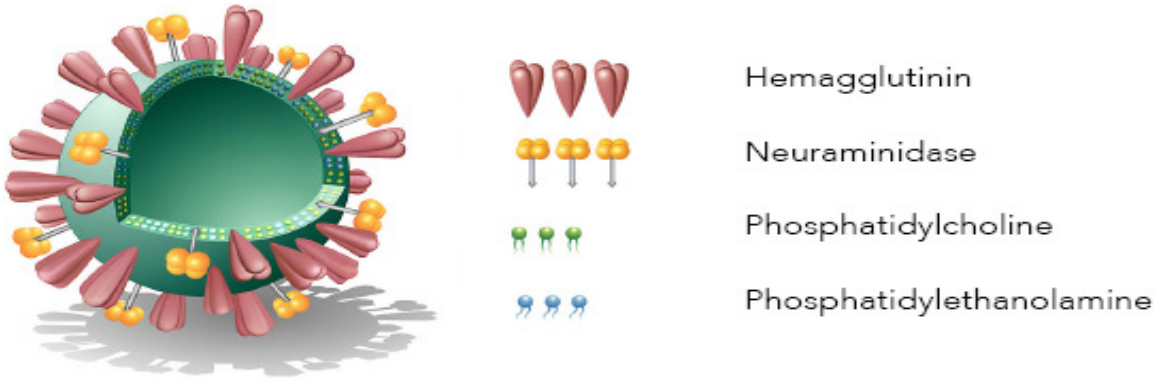

Figure 1.3: Virosome
Diagrammatic representation of a virosome, used in the production of the Pevion malaria vaccine.

Cyclized, conformationally constrained peptides with lipid tails can be incorporated into the membrane of these immunogenic liposomes [122]. A Phase I clinical trial of a malaria vaccine using virosomes incorporating the Asn-Ala-Asn-Pro (NANP) repeat from CS protein or part of domain III of the blood-stage protein AMA1 showed good safety and antibody immunogenicity [123]. One of the studies described in this thesis used a vaccine prepared from a mixture of these two virosomal formulations, and the vaccine is described in more detail in Chapter 4.

1.4.2 Development of a T Lymphocyte Inducing Vaccine

In light of the important role of T cell mediated immune responses described above, an alternative rationale for the design of pre-erythrocytic vaccines targeting T cell immunity has been adopted by other groups. Although CD8+ T cells are important in most host–parasite combinations in mice, CD4+ T cells may also be required for protection [64, 80]. Results from adoptive-transfer experiments with T cell clones specific for the CS protein or TRAP (thrombospondin-related adhesion protein, also known as SSP2) in mice [78, 124], and evidence of an HLA class I association with malaria resistance in Gambian children [83], have also encouraged pursuit of this cell mediated mechanism. A peptide-based approach using allele-specific motifs was applied to several other class I HLA molecules. In total fourteen class I epitopes were identified in six pre-erythrocytic P. falciparum antigens [82]. In a series of immunisation studies in mice, various potential vaccine types (recombinant particles, peptides, plasmid DNA, numerous adjuvants and recombinant vectors such as recombinant BCG, Salmonella, adenovirus, MVA and FP9), encoding malaria epitopes and antigens, were compared [125, 126]. Most of these approaches induced only modest levels of T cell immunogenicity that invariably failed to protect against sporozoite challenge.

However, a priming immunisation with plasmid DNA encoding an entire P. berghei pre-erythrocytic antigen followed by a booster immunisation with a MVA vector carrying the same antigen induced complete protection [126] in strains of mice highly susceptible to sporozoite challenge [127]. Protection was associated with very high frequencies of splenic peptide-specific interferon-γ (IFN- γ) secreting CD8+ T cells, and the level of these cells measured in IFN- γ enzyme-linked immunospot (ELISPOT) assays correlated well with protection. The US Navy group have reported similar results for P. yoelii malaria using NYVAC (a highly attenuated vaccinia virus, New York Vaccinia) as the boosting vector [128].

The highest T-cell immunogenicity and efficacy has been achieved with heterologous prime–boost approaches, in which a pox virus vector is used as the boosting agent [127, 129-133]. In these vaccine regimens, two different vectors are usually used, each encoding the same malaria antigen. Plasmid DNA, and

vectors derived from pox viruses such as the attenuated orthopoxvirus, modified virus Ankara (MVA) and an attenuated strain fowlpox virus (FP9), as well as adenoviruses have been used in these regimens. Part of the enhanced T cell immunogenicity of these systems can be attributed to the avoidance of the effect of anti-vector immunity that tends to impair the boosting capacity of other vaccines. Other factors are probably also important, including the ability of some of these viruses to boost pre-existing T cell responses.

Viral vectored vaccines act as an attenuated pathogen with intrinsic innate-stimulatory activity, and encode an antigen of choice against which an immune response is desired. The antigen is incorporated into the DNA sequence of the virus, and the vector then expresses the antigen as though it were a viral protein (i.e. within the infected cell cytoplasm). From here the antigen is processed via the endogenous pathway and epitopes are presented on major histocompatibility complex (MHC) class I molecules to CD8+ T cells [134]. Dendritic cells (DCs) are professional antigen presenting cells (APCs), and are therefore able to acquire the antigen by direct infection with the virus. Alternatively, a process of cross-priming can occur – infected macrophages providing a source of antigen, which is taken up from infected or dying cells and debris. Antigen taken up in this way enters the alternative exogenous processing pathway, and it is presented by MHC class II to CD4+ T cells [135]. Poxviruses, and in particular vaccinia, were the first viruses to be investigated as antigen-encoding vectors. This followed the observation that vaccinia was a highly immunogenic vector for generating cell-mediated immunity in the form of T cells [136]. Following vaccination with vaccinia, the migration of infected APCs to draining lymph nodes is a rapid process, peaking at 6 hr and declining rapidly thereafter [134]. It was concluded that early antigen expression is essential for CD8+ T cell induction, but these cells need only a brief contact with antigen to proliferate [137]. These viruses also contain pathogen associated molecular patterns, which may be recognised by various pattern recognition receptors, including members of the Toll-like receptor family. This provides a mechanism for stimulation of the innate immune response. The characterization of the innate immune responses generated by infection of cells by pox viral vectors is the subject of current investigation in the author's laboratory [135].

Studies by various groups have now found that prime-boost using poxviruses is highly immunogenic for CD4+ and CD8+ T cell induction against, tuberculosis [138], HIV [139, 140], and Ebola (DNA/adenovirus) [141] and cancer [142].

1.4.3 Malaria Vaccine Studies at the University of Oxford

A series of Phase I/IIa clinical studies have been carried out at the University of Oxford since 1999. These have assessed the prime-boost immunisation approach in healthy, malaria-naïve human volunteers. These studies, using a variety of vaccines, encoded either by DNA, or viral vectors, have demonstrated safety and immunogenicity, with significant ex vivo ELISPOT responses [133, 143, 144]. Following immunisation subjects are invited to participate in a subsequent challenge study, where they are infected with the 3D7 strain of P. falciparum under close observation to assess vaccine efficacy.

In this work, the most extensively studied T-cell antigen has been the sporozoite antigen TRAP fused to a multi-epitope string of mainly CD8+ T-cell epitopes from six pre-erythrocytic antigens [145]. Most T-cell responses induced by these vectors are specific for the TRAP component, with the smaller multi-epitope string being less immunogenic [146]. These vaccination regimens induce mainly CD4+ T cell immune responses against TRAP. Boosting plasmid DNA with an MVA vaccine vector encoding ME–TRAP amplified T cell responses and induced a significant delay in time to parasitaemia in sporozoite-challenge studies [146].

More recently, the vector FP9 was incorporated into this strategy. Using two pox viruses in this heterologous prime boost system resulted in complete protection against heterologous malaria challenge in two out of five vaccinated subjects who had received two doses of FP9 ME-TRAP followed by one dose of MVA ME-TRAP [132]. Furthermore, several immunisation regimes using the ME-TRAP insert have led to highly statistically significant delays in time to patient parasitaemia. As in all studies with CS protein and ME–TRAP vectored vaccines, antibody levels induced by this regime were low or absent and were not associated with protection. However, both ex vivo and cultured IFNγ ELISPOT assays, which measure mainly effector and central memory T cells, respectively, showed association with

protection [132, 147]. The ME–TRAP vaccine that was delivered using an FP9–MVA regime has been found to be immunogenic in Gambian [148] and Kenyan adults [149]. These findings led to a Phase IIb efficacy trial in Kenyan children to assess protection against febrile malaria, but this study did not demonstrate any efficacy [150]. A prime–boost vaccination regime, using recombinant viral vectors, may provide one avenue against infections such as the liver stage of malaria. Evidence suggests that effective regimens will include a prime with a vaccine which generates good memory responses, followed by a boost with a vaccine that generates a strong secondary effector and central memory response.

1.5 Vaccine Vectors

1.5.1 Modified Vaccinia Virus Ankara (MVA) Vector

Modified Vaccinia virus Ankara (MVA) is derived from the vaccinia virus. The successful worldwide eradication of smallpox by vaccination with live vaccinia virus highlighted it as a candidate vaccine vector. However, the small but definite risk of live vaccinia virus (both to researchers and future patients) led to the development of several strains of attenuated virus, both during the smallpox vaccination campaign, and more recently. MVA is one of these strains, and recombinant MVA is attractive as a vector for several reasons, for safety - particularly because the host-range restriction of MVA is extremely attenuated compared with other vaccinia viruses, it has demonstrated immunogenicity, and avirulence in animal models as well as an excellent safety record as a smallpox vaccine [151].

MVA was originally derived from the vaccinia strain Ankara by over 500 serial passages in primary chicken embryo fibroblasts. The passage attenuation of the virus resulted in around 31 kb of genomic deletions. These comprise six major genomic deletions compared to the parental Ankara genome and as a result, MVA is severely compromised in its ability to replicate in mammalian cells. No replication has been documented in non-transformed mammalian cells [151]. Thus, MVA has a large recombinant DNA capacity to accommodate large antigens, such as those from malaria.

MVA also showed no cytopathic effect or plaque formation in cells of human origin. In irradiated mice MVA did not elicit any morbidity or lethality even when administered at high doses intra-cerebrally, indicating its safety even in immuno-compromised organisms [152]. The viral genome has been proven to be stable through a large series of passages in chicken embryo fibroblasts. Using restriction enzyme analysis virtually no difference was observed between passage 500 – 572 [152].

Apart from studies in mice, rabbits and elephants [153] MVA has been shown to be safe in humans. From 1972 until 1980 (the end of compulsory smallpox vaccination) MVA was licensed in Germany [153] and was included in the official immunisation schedule [154]. In a large field study carried out in Germany in the late 1970s, over 120,000 previously unvaccinated individuals were vaccinated with MVA (0.2 mL) administered either intra-dermally or subcutaneously. The study population included high-risk groups (e.g. people suffering from allergies, elderly people, alcoholics). Given intra-dermally, a red nodule of up to 4 mm in diameter was observed at the injection site at day 4 or 5. Only a small proportion showed any systemic side effects, such as fever over 38.5°C [152]. MVA proved to be non-contagious and avirulent. Viral replication is blocked late during infection of cells but importantly viral and recombinant protein synthesis is unimpaired even during this abortive infection. Replication-deficient recombinant MVA has been viewed as an exceptionally safe viral vector. When tested in animal model studies recombinant MVAs have been shown to be avirulent, yet protectively immunogenic as vaccines against viral diseases and cancer [152]. Recent studies in macaques severely immuno-suppressed by SIV infection have further supported the view that MVA should be safe in immuno-compromised humans [155].

Compared with vaccinia, MVA lacks certain immune-suppressive signalling molecules and soluble receptors, making it potentially more immunogenic [151]. It does however retain the ability to induce type I IFN expression in infected cells and this may have a profound contribution to an enhanced generation of memory T cells by direct cytokine action [156]. MVA gene expression in mature and

immature dendritic cells (DCs) is restricted to viral early gene transcription. In general MVA is more efficient at infecting immature DCs as measured by transgene expression, however both serve as functional antigen presenting cells (APC) to stimulate class I T cell responses [157]. Although MVA infection of murine DCs in vitro reduces MHC class I expression and T cell stimulatory capacity, and causes cell death, MVA-infected murine DCs retained their ability to generate a CTL response in vivo, [158], perhaps by providing a source of immunogenic antigen for cross-priming. Indeed, mice administered MVA showed an overall enhanced T cell stimulatory capacity of their DCs. Furthermore, co-administration of MVA with antigen was shown to generate enhanced T-cell and antibody responses, suggesting that the virus has potent adjuvant activity [159].

All MVA constructs are made by ligating the selected sequence into the vaccinia shuttle vector pSC11, placing it under the control of the vaccinia P7.5 early/late promoter. This vector also encodes a copy of the β-galactosidase gene to allow plaque picking [160].

1.5.2 Fowlpox Strain FP9 Vector

Fowlpox virus is a member of the avian poxvirus family and is an obligate pathogen of chickens causing cutaneous or mucosal symptoms. FP9 is a highly attenuated form of an avian pox virus derived by 438 serial passages of the wild-type fowlpox virus HP-1 [161], giving rise to 25 kb of genomic deletions. It was originally developed and used as a vaccine for poultry, but its ability to infect mammalian cells and produce proteins without replicating [162] led to interest in its use as a recombinant viral vector for mammalian, including human, vaccination.

FP9 was shown to be an effective vector, delivering antigen to both human and murine dendritic cells. Murine DCs infected with fowlpox vectors were shown to be potent at stimulating transgene-specific CD8+ T cells for up to 3 days and also up-regulated MHC and co-stimulatory molecules [163]. Human DCs were able to express the transgene for up to 20 days [163]. This suggests that fowlpox may be less cytopathic than MVA and allow for longer transgene, and hence antigen,

expression. The FP9 genome has now been sequenced and found to have 118 mutations when compared to the pathogenic US fowlpox strain [164].

FP9 recombinants are constructed using an established protocol [165]. The DNA sequence of interest is ligated into the SmaI cloning site of the Fowlpox shuttle vector pEFL29 [165] placing expression of this gene under the control of the vaccinia virus P7.5 promoter. The pEFL29 plasmid also encodes a copy of the β-galactosidase gene under the control of the FP4b fowlpox late promoter, allowing identification of recombinant viruses by X-gal staining as described previously for vaccinia virus [160]. Recombinant viruses are prepared by in vitro recombination of the shuttle vector with the FP9 fowlpox strain in primary cultures of chick embryo fibroblasts (CEF). Recombinant viruses are repeatedly plaque purified in CEF monolayers until homogenous.

1.6 Contribution of this Thesis

The work in this thesis comprises a variety of laboratory based studies, computational modelling and clinical trials. A summary of each chapter follows.

1.6.1 Clinical Trials

Chapter 2 beings by outlining the basic methods required for performing small scale clinical trials for the testing of candidate malaria vaccines. Although Professor Hill's group at the University of Oxford has been conducting similar studies for some years, some changes needed to be made to comply with new legislation. This work was required because of the introduction of the Medicines for Human Use (Clinical Trials) Regulations [166] in 2004, requiring that all trials be conducted according to strict principles of Good Clinical Practice. The chapters that follow describe two such clinical trials, namely VAC027 and VAC030. These were both Phase I/IIa clinical trials, conducted in Oxford, in healthy, malaria naïve volunteers.

1.6.2 VAC027

VAC027 was a study consisting of both a vaccination and a challenge protocol. The vaccines used were novel candidate malaria vaccines, found to be immunogenic in mice [167]. MVA and FP9 were used as vectors, and an insert coding for 6 malaria antigens (the 'polyprotein') was designed. The idea of combining multiple antigens into one vaccine has been used before [168] with some success (of 35 volunteers challenged, one was protected from malaria and there was a delay in time to infection in the other vaccinated volunteers). The hypothesis behind the design of the polyprotein vaccine was that multiple antigens would broaden the target antigen range of vaccine-induced immunity and increase the number of potential epitopes available for immunogenetically diverse human populations. Also, inducing responses against multiple antigens should prevent the development of escape mutants of P. falciparum. As described earlier, the mechanism for protection in irradiated sporozoite immunisation clearly involves multiple antigens, and so a multi-antigen vaccine is a logical step. The main focus of this trial was to establish the safety and immunogenicity of the new vaccines, but two groups of immunised volunteers went on to participate in a subsequent challenge study to assess efficacy. Chapter 3 describes the conduct of this trial and the methods that were specific to this study. The outcome measures used for this study, of safety, immunogenicity and efficacy are then detailed, and the results for each outcome are reported.

1.6.3 VAC030

VAC030 was designed to test a novel approach to improving the immunogenicity of vaccines – combining existing vaccine strategies with the hypothesis that they might work synergistically. This was suggested as a strategy following some animal studies that combined partially protective antibody and T cell inducing vaccines, and found evidence of substantially increased protection [169]. The vaccines used in the human trial were FP9 and MVA ME-TRAP, a vaccine with a well characterised response, which has been shown to be partially protective [132]; and the virosomal vaccine PEV3A. PEV3A consists of a combination of two virosomal formulations, one containing a peptide derived from the (NANP) repeat

of the CS protein, and the second containing a peptide from the blood stage antigen AMA-1. The idea of combining an antibody inducing and a T cell inducing vaccine regimen also appeals as inducing responses in both humoral and cell mediated arms of the immune system would intuitively improve the efficacy of such a vaccine. This trial was designed to test the efficacy of these two vaccine regimens in combination. In Chapter 4, the planning, administration and specific methods for this study are reported, along with the results for each of the outcome measures. Additional analyses performed after the trial was completed are also reported in this chapter. A report of this study, written by the author, has been published [170] and is included as Appendix 5.

1.6.4 Lab Work

Chapter 5 contains a description of laboratory studies, undertaken to further investigate the antigens used in the polyprotein vaccine of VAC027. The aim was to investigate the presence and size of immune responses to these antigens in an African population, naturally exposed to malaria infection. Both cellular and antibody mediated immune responses were examined. Responses were correlated with time to malaria infection in an attempt to identify which antigens might be important in mediating protection. This approach, though limited [60], can provide indications of potentially important antigens, and may inform further vaccine development. The work described in this chapter was presented by the author, at the Multilateral Initiative on Malaria meeting in Yaoundé, Cameroon in November 2005 [171].

1.6.5 Parasite Life Cycle Modelling

Chapter 6 reviews the literature on parasite modelling, describing the most relevant methods and their relative advantages and disadvantages. Two of these models are then selected, as the most relevant for the studies described here, and used to perform a comparison, using data from the clinical trial VAC030. One model is selected as providing the most accurate estimation of both parasite growth rates, and the estimated numbers of infected hepatocytes. This model is then used to analyse data from VAC027, and from other clinical trials conducted

by the University of Oxford. Although multiple models of parasite growth within the human host have been published, no such comparison, using data obtained in vivo, has been performed previously.

1.6.6 Challenge Safety Data

The final chapter of this thesis takes advantage of the unique data set that performing malaria challenge studies provides. In every challenge study, a group of unvaccinated, malaria naïve, control volunteers are infected with malaria. The data collected from these volunteers provides an ideal opportunity to study early malaria infection. The recorded symptoms from control volunteers from each challenge study performed in Oxford to date are collated to provide an account of common symptoms, and the relationship of symptoms to parasitaemia is examined. A recent paper published by the US navy group [172], has also undertaken such a review, although the data set described in their paper is limited, and includes both vaccinated and unvaccinated individuals. This chapter has the advantage of describing a larger data set of only unvaccinated control individuals. A comparison of the Oxford data and the published US Navy data is reported.

Chapter 2 Vaccine Trials Background

2.1 Introduction

This chapter briefly reviews the methods used to perform clinical trials of novel malaria vaccines. The purpose is to describe the preparation, procedures, and administration required to undertake a successful small scale clinical trial of a vaccine. These procedures were used to conduct both of the trials described later in this thesis. The topic of vaccine development and study design is complex, and beyond the scope of this thesis, but an introduction is included here to put the rest of the work in context.

2.2 Vaccine Development

A Phase I clinical study tests a new vaccine or drug in humans for the first time. Phase I studies are therefore carried out in healthy volunteers, with the aim of determining the safety of the new product. If the Phase I study is successful, the vaccine or drug proceeds on to Phase II studies. In malaria vaccine development, a phase IIa study tests efficacy in healthy volunteers, whilst a Phase IIb looks at efficacy in a population where malaria is endemic, again in healthy volunteers. Phase III studies are larger, field trials, performed in the target population for the vaccine, designed to demonstrate efficacy in a variety of settings. Phase IV studies are undertaken once a drug or vaccine is licensed by a regulatory authority, these involve long term safety data collection.

The malaria vaccine studies performed in Oxford by Professor Hill's group are commonly Phase I/IIa clinical trials. These studies aim primarily to look at the safety of a vaccine in humans. As a first step it is important to evaluate a new vaccine in completely healthy individuals, before it can be given to those with other illnesses. A vaccine is administered to a small number of healthy volunteers, who

are then followed closely, and any side effects or adverse reactions recorded. A new vaccine is usually tested at a variety of different doses and safety is assessed by monitoring routine serum biochemistry and haematological parameters. Blood samples taken from volunteers in these Phase I studies also provide an opportunity to look at any immune responses that the vaccine may have produced.

Despite decades of research in this area, there is no single immunological correlate of protection from infection with malaria. In order to test new vaccines, vaccinated volunteers are therefore challenged with malaria infection. A Phase IIa malaria challenge study provides a way of obtaining biologically relevant data concerning the efficacy of the candidate vaccine. Previously, challenge studies for malaria and other infectious diseases have provided important information about innate and acquired immune responses to infectious pathogens. They are now also used to demonstrate protection against a defined strain of organism in a controlled setting. In this case, a volunteer is given the potential vaccine and then infected with malaria. Immunity to the disease is monitored and if parasites are observed the volunteer is swiftly treated with effective therapy before the disease is established. By this means, efficacy can be assessed in a small number of volunteers more rapidly than through natural exposure. Data obtained in this way can provide compelling evidence that would otherwise require much greater numbers of volunteers in Phase II-III studies. The specific issues raised by these sorts of challenge studies are discussed in section 2.3, and in more detail in [173].

Once a vaccine has gone through these stages, and there is some evidence of 'proof of concept', it can be taken on into larger Phase IIb and Phase III studies. These are studies that take place in areas where malaria is endemic, for instance in Africa and India. Vaccines are generally field-tested first in adults in endemic areas, but because most adults have substantial immunity from natural exposure and develop malarial disease less often than children, this might be an insensitive means of testing partially effective vaccines. Children and infants, particularly in Africa, represent the most important target population for new vaccines and efficacy against clinical malaria can be measured in samples of a few hundred children. Two measures of efficacy are commonly used: a reduction in disease incidence rate and a reduction in overall cases per unit time. For vaccines with a

short duration of efficacy, these numbers can differ significantly. Opinions also differ on the importance of demonstrating efficacy against severe malaria before a vaccine is licensed. This would involve studies of many thousands of children, but as, for some vaccines, efficacy might be greater against severe malaria than uncomplicated malaria [114], this approach may be necessary.

2.3 Challenge Studies – Ethical Issues

Malaria is unusual in that the efficacy of vaccines can be tested in small numbers of individuals as part of a phase IIa study, by infection with Plasmodium falciparum. The deliberate infection of healthy volunteers with a potentially fatal disease raises concern about the potential for causing harm, and the lack of any tangible benefit for the volunteer taking part. Guidelines produced by the Royal College of Physicians on this issue introduce the concept of 'minimal harm', and stipulate that - "However valuable the research, the degree of risk of harm can be no more than 'minimal'" [174]. The potential risks to volunteers must be within acceptable limits, as assessed by an Ethics Committee – the key issue to consider is whether the study puts those participating at an unacceptable level of risk. All studies must also obtain approval from the MHRA. A malaria challenge study is considered as an additional protocol within the Clinical Trial Authorization package, as it is an outcome measure to determine the efficacy of the vaccine. The risks involved in these trials may be minimized by careful consideration of protocols for vaccination and challenge, with emphasis on ensuring safety of participants.

These clinical challenge models benefit from the ability of drugs to effect sterile clearance of fully characterized, drug-sensitive P. falciparum parasites. Insectary-reared Anopheles stephensi mosquitoes are fed with blood containing gametocytes of the 3D7 strain of P. falciparum and five infectious bites (containing sporozoites) are administered to each vaccinee or non-vaccinated control. The method is discussed in section 2.4.5, and in detail in chapter 7 of this thesis. At least two endpoints are of interest: the proportion of vaccinated volunteers who are fully protected and the delay in time to infection, assessed by blood-film microscopy, in partly protected vaccinees. All non-vaccinated volunteers should

develop malaria with a mean time to patency of about 11 days. A substantial reduction in the number of sporozoites or liver-stage parasites will delay the mean time to patency, providing a measure of partial efficacy.

The selection of appropriate volunteers is particularly important for these challenge studies. The primary determinant of inclusion/exclusion criteria should be the safety of the volunteers, taking into account scientific and social issues (such as previous malaria infection). The studies are also constrained by the limitations of language, to recruit only volunteers who are fluent in English - safety is the prime concern, where signs and symptoms of early disease are likely to be subtle and may be missed if communication with study staff is suboptimal. The process of obtaining informed consent is vital, volunteers must clearly understand the potential risks involved in taking part in one of these studies. Long, detailed information sheets reflect the complexity of the trials, and the amount of important information required for a volunteer to give their fully informed consent. The study is explained with an emphasis on their safety to each prospective volunteer in great detail before they agree to participate. The consent form makes it clear that while volunteers are free to withdraw at any time, if they do so after being infected with malaria, they must take a course of anti-malarial medication.

2.4 Performing Vaccine Trials

Once a research question has been defined, and it is decided that a clinical trial is required to provide an answer, trial planning begins. The MRC and the Department of Health have jointly designed a website, the Clinical Trials Tool Kit [175] http://www.ct-toolkit.ac.uk/ and this provides an overview of the necessary steps. They have produced a flow chart to guide researchers through the process, a copy of which is included in Figure 2.1.

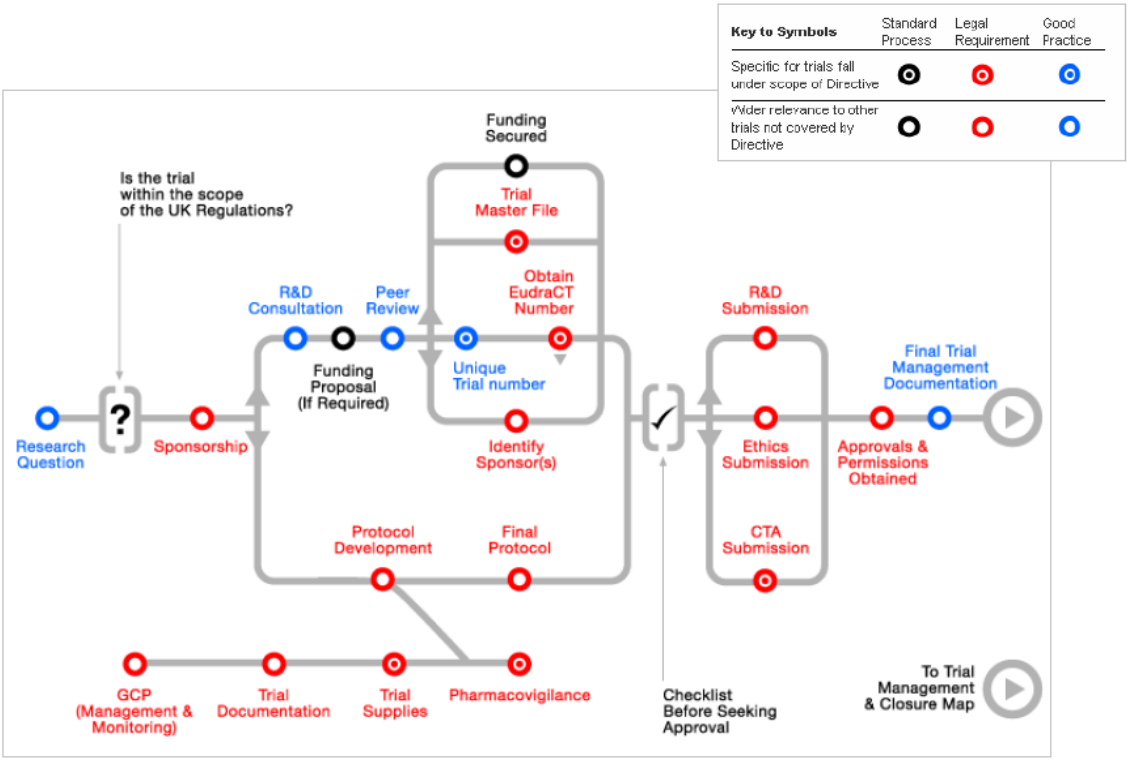

Figure 2.1: Flow chart

Flowchart for planning a new study, from Clinical Trials Tool Kit [175].

Before detailed planning can start, the roles of the key parties need to be defined. Under the 2004 Medicines for Human Use (Clinical Trials) Regulations [166], a Sponsor, and an Investigator must be appointed, each having specific responsibilities to fulfil. A source of funding also needs to be identified, and it is usually necessary to have a clear idea of the outline of the trial and main objectives in order to secure funding.

The design of a clinical trial is determined by the main objectives of the study, and the procedures used to measure these outcomes. The primary objective of each study is agreed upon by the Investigators and Sponsors of the trial, often following a process of negotiation. Once these principal objectives and outcome measures have been agreed, then a trial protocol can be written. Each clinical trial that is performed requires a clear and detailed protocol. This describes what is to be tested, and exactly how it is to be tested, what the expected outcomes might be, and how the Investigators will know if the trial has been successful. The protocol

must specify all the procedures to be carried out during the trial, and refer to standard operating procedures where applicable. A template protocol was designed by the author to assist in protocol development for malaria vaccine studies, to ensure all essential information has been included. This template is attached as appendix 1. Multiple other documents are also needed, including information sheets, consent forms, GP information, adverts, Investigators Brochure (IB) – a document containing information concerning the vaccine (or other medicinal product) and/or Investigational Medicinal Product Dossier (IMPD), which contains more detailed information concerning the design and manufacture of the vaccine, quality control procedures, conditions for storage and use. The information required for each of these documents must be collected and collated, and each document drawn up according to the relevant guidance (either European guidance, or that issued by the UK regulatory authority, or the National Research Ethics Service), in preparation for submission.

All clinical trials that are carried out in the UK must be conducted in accordance with the Declaration of Helsinki [176] and the principles of Good Clinical Practice (GCP) [177]. The World Medical Association developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. This document [176] is publicly available and on the internet at http://www.wma.net/.

The International Conference on Harmonisation (ICH) tripartite Good Clinical Practice (GCP) guideline [177] (http://www.ich.org/) was amended and finalised on 10th June 1996 and also adopted by the European Union Committee on Proprietary Medicinal Products (CPMP) in June 1996. This is an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. Compliance with GCP provides public assurance that the rights, safety and well being of the trial subjects are protected, and that the clinical trial data are credible.

2.4.1 Gaining Approval – MHRA, NRES

Under the Medicines for Human Use (Clinical Trials) Regulations [166], all clinical trials carried out in the UK, or by a UK sponsor, must be approved by a recognised ethics committee, such as one of the Oxfordshire Research Ethics Committees (Committee A, B or C), and by the Medicines and Healthcare products Regulatory Agency (MHRA). Prior to applying for approval all trials must registered on a European database of clinical trials, the EudraCT database, when they are issued with a EudraCT number. Each protocol also requires an ethics reference number, usually supplied when the protocol is registered for submission.

The National Research Ethics Service (NRES) replaced COREC on the 1st April 2007. The studies outlined in this thesis were completed before this date. Application for ethical approval requires completion of a standard on-line application form [178], and submission of this along with the trial protocol, and other supporting documentation to a recognised ethics committee. The supporting documentation required include: volunteer information sheets, consent forms, and any advertising material, along with an Investigators Brochure. As the clinical trials included in this study were both Phase I/IIa studies, involving healthy volunteer subjects, it was possible to submit directly to the Oxfordshire Research Ethics Committee A, who were specifically designated a Phase I committee. The ethics committee consider the ethical aspects of the research, from the information provided to potential participants, to the conduct of the trial, and the potential impact of the research.

Submission to the MHRA for a Clinical Trial Authorisation (CTA) can be performed in parallel with the ethics process. Again, this submission requires completion of an online application form and the provision of supporting documentation. The MHRA requires detailed information about the vaccine (or other medicinal product) to be used in the study, including information concerning its manufacture, storage, use and disposal after the study. In addition the MHRA will consider the study protocol and other documentation.

Following consideration by the relevant authorities, the study may receive a favourable ethical opinion, and a Clinical Trial Authorisation. No study related procedures can be carried out before both of these approvals have been received.

2.4.2 Gaining Approval - GMSC and GTAC

Whilst all clinical trials must be approved by the MHRA and a recognised ethics committee, there are some additional requirements for the malaria vaccine studies described in this thesis. This is because some of the vaccines used in the studies described are based on live viruses, which have been genetically modified.

Genetic modification means the altering of the genetic ('heritable') material of an organism using a method that does not occur naturally by mating and / or recombination. Under the Contained Use Regulations [179] all genetic modification work is controlled by requirements of the Health and Safety Executive. All work involving Genetically Modified Organisms (GMOs) which is carried out must be assessed and appropriate containment measures approved, by the local NHS Trust's Genetic Modification Safety Committee (GMSC). 'Work' with GMOs includes (but is not limited to) their culture, storage, transport, destruction or disposal.

A risk assessment is needed, and the results must be submitted to the GMSC for review. The committee reviews the project in two phases, looking at the containment level required to undertake the project, and the feasibility of providing that containment in the proposed premises. The approval of the local GMSC must be submitted to the MHRA before they will issue a Clinical Trial Authorisation.

In addition, the Gene Therapy Advisory Committee (GTAC) is legally required to give approval for any clinical trials involving 'gene therapy'. The definition of gene therapy in the European legislation is "… [a] gene therapy medicinal product shall mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either in vivo or ex vivo, of a prophylactic, diagnostic or therapeutic gene (i.e. a piece of nucleic acid), to human/animal cells and its subsequent expression in vivo. The gene transfer involves an expression system

contained in a delivery system known as a vector, which can be of viral, as well as non-viral origin. The vector can also be included in a human or animal cell." [180]. This has been interpreted very broadly by the Department of Health, and GTAC's definition of gene therapy is as follows:

"The deliberate introduction of genetic material into human somatic cells for therapeutic, prophylactic or diagnostic purposes."

This includes techniques for delivering synthetic or recombinant nucleic acids into humans:

genetically modified biological vectors (such as viruses or plasmids)

genetically modified stem cells

oncolytic viruses

nucleic acids associated with delivery vehicles

naked nucleic acids

antisense techniques (for example, gene silencing, gene correction or gene modification)

genetic vaccines

DNA or RNA technologies such as RNA interference

xenotransplantation of animal cells (but not solid organs).

Trials using virally vectored vaccines to induce an immune response are therefore classified as gene therapy. GTAC therefore needs to be informed of the live, virally vectored vaccines to be used. More recently, GTAC have been designated a recognised ethics committee by the NRES, and so the two requirements of ethical and GTAC approval are combined into one application.

2.5 Trial Documentation

2.5.1 Trial Master File

The process of applying for and gaining approval for a given clinical trial requires the collation of large amounts of relevant information concerning the investigational vaccine, and the methods to be used to assess its safety, and where appropriate, efficacy. The guidelines for GCP lay out the essential documents for the conduct of a clinical trial. These are not all required for submission to the regulatory authority, or to the ethics committee, but must nevertheless be produced and stored. The 'Essential Documents' are those which

“individually and collectively permit evaluation of the conduct of a trial, and the quality of the data produced” [177]. These documents serve to demonstrate the compliance of the Investigator, Sponsor and Monitor with the standards of GCP and applicable regulatory requirements. They are collected within a Trial Master File, which is held by the Investigator.

2.5.2 Case Report Forms

Before the study can begin, the Investigator must have a method in place for collecting all the data required in the protocol. Each subject in the study must have a case record form, which contains all the information relating to that subject, and has space for entering details concerning the vaccines administered, as well as any adverse reactions or other events that occur throughout the study. These can be either paper or electronic, although specific requirements are made of electronic CRFs, so that any changes made to the data can be tracked, and the individual making the change, and the date on which it was made, can be traced. For the trials described, the Investigators elected to use an electronic system, capturing data from each study visit directly onto a web based database. The software was provided by a company called StudyBuilder™, and the case report forms for each study were designed and built by the Investigators. This was a process that required substantial testing and reformulation until a satisfactory set of forms were produced. These were then used for the study, and all data was stored securely on a server owned by the University of Oxford. The advantage of this system was that all the data could be exported directly into a spreadsheet such as Microsoft Excel ™ for analysis. It was also intended to be flexible, allowing the Investigators to design different forms for each trial, and accessible over the internet, so data could be added from wherever a study visit or procedure was taking place. Unfortunately, although the system was flexible, designing the forms and questions required a considerable amount of computer coding expertise, and the interface provided was not user friendly. It provided a secure method for data capture for the two studies described here, but will not be used within the group for any future studies.

2.5.3 Trial Monitoring

Another of the stipulations of GCP is that trials are adequately monitored. This process is performed by an appropriately trained Monitor, who is appointed by the Sponsor of the study. The Monitor visits the study site before the trial begins, to ensure all the trial related documentation is in place, and the facilities are adequate, and that the vaccine or other investigational product is ready and correctly labelled and stored. The Monitor may then visit on a number of occasions during the trial, and then again, once the trial is over. The Monitor's role is clearly defined in GCP: they are required to ensure that the trial is conducted and documented properly. They need to verify independently that the trial is carried out according to the protocol, and that any deviations from the protocol are appropriately documented and reported. They ensure that all essential documents are contained within the trial master file, and identify any errors or omissions. Both trials described in this thesis were monitored by an independent research monitoring company; Appledown Ltd.

2.6 Trial Management

The clinical trials tool kit website [175] also includes a flow chart to provide an overview of the processes involved in actually running a clinical trial, once all the approvals have been obtained. This involves recruitment and screening of volunteers, safety and progress reporting. This is shown in Figure 2.2. Once the trial is over, a final report must be completed and submitted to the MHRA and the ethics committee.

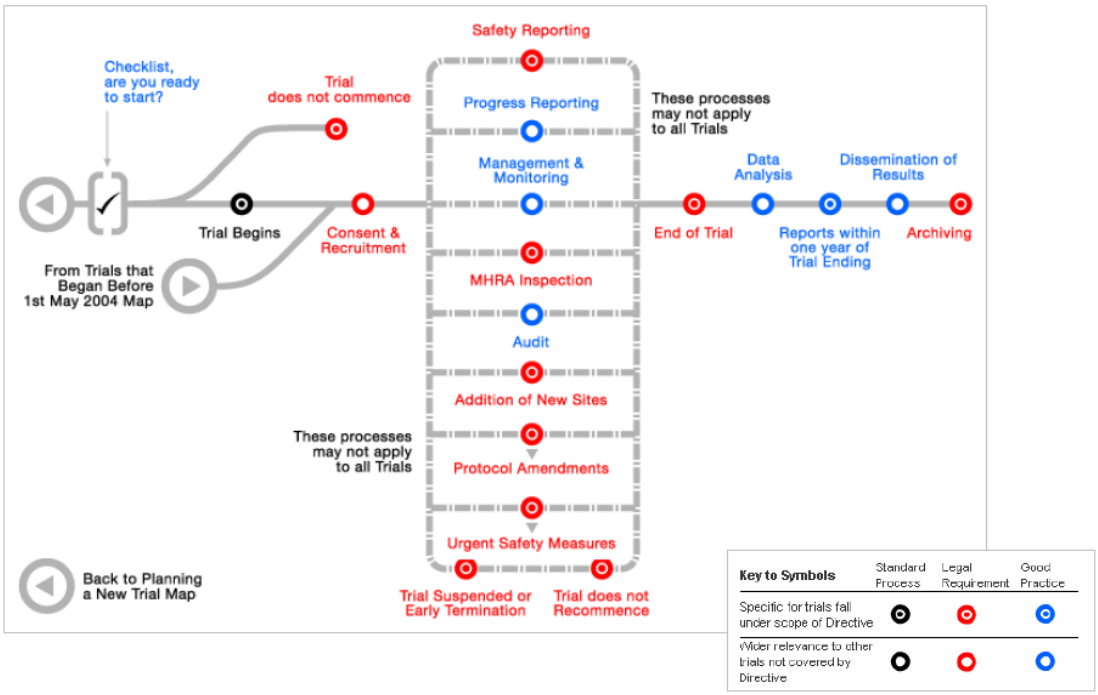

Figure 2.2: Trial management

Flow chart from Clinical Trials Tool Kit [175].

2.6.1 Recruitment and Screening

Recruitment of healthy volunteers for these studies was by widespread poster campaign in universities and colleges, libraries, health centres and places of worship. Advertisements were also taken in the local press and radio. Subjects are initially screened by telephone and then scheduled for a formal screening visit. At the screening visit, the volunteers are counselled about participation in the study, including the following general principles:

- participation in the study is entirely voluntary
- refusal to participate involves no penalty or loss of medical benefits
- the subject may withdraw from the study at any time
- the subject 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 for participating
- the volunteer's GP will be contacted to corroborate their medical history

If the volunteer agrees to undergo screening, written informed consent was obtained. Formal screening involved checking inclusion and exclusion criteria, a brief medical history, a physical examination, full blood count, kidney and liver biochemistry, urinalysis, serological studies for human immunodeficiency virus (HIV), hepatitis B and hepatitis C, human leukocyte antigen typing. Females also had a urine pregnancy (β-hCG) test.

2.6.2 Obtaining Consent

Information sheets must be provided to potential volunteers at least 24 hours prior to the screening visit, and it is necessary for them to have read the information. At the screening visit, the trial is described again in detail to the volunteer, who is then given the opportunity to ask questions about the study. They are then given some time to consider whether or not they want to take part, usually a period of 10 – 15 minutes, but longer is allowed when necessary. If they do decide to participate, they sign and date two copies of the consent form, one for them to take away and keep, and one to be kept in the study file. These forms are countersigned by the Investigator. Copies of the consent forms for the trials included in this thesis may be found in appendix 2.

2.6.3 Selection of the Trial Population

Selection of volunteers to participate in these studies is important, in order to ensure that the risks to the volunteers are minimised, and that any immunogenicity data can be interpreted correctly. Inclusion and exclusion criteria do vary from one trial to another. All volunteers selected were healthy, malaria naïve individuals, between the ages of 18 and 55 years. Detailed inclusion and exclusion criteria may be found in the relevant chapters of this thesis.

2.6.4 Evaluation Criteria

2.6.5 Primary Evaluation Criteria: Safety

As the malaria vaccine studies performed from the Centre for Clinical Vaccinology and Tropical Medicine in Oxford are largely Phase I studies (with some Phase I/IIa studies), collection of safety data relating to the vaccines used is vital.

Following the incident at Northwick Park Hospital, where several healthy volunteers were given a dose of a new product one after the other, and all of them developed serious reactions, some of them life threatening, it was decided to modify the procedures used when giving a vaccine for the first time in humans. The first dose is given to one individual only, who is then evaluated before doses of the same vaccine are given to other volunteers.

Safety is assessed by recording any immediate reactions (within 30 minutes after each injection, with an emphasis on allergic reactions) to the vaccines, and then local and systemic reactions occurring from day 0 to day 28 after each dose. All subjects are observed after each vaccination, and provided with a diary, to complete for 7 days. They are followed up after each vaccination, and at each visit they are questioned about the effects of the vaccine. The volunteers are always asked about a number of expected side effects related to vaccination – these are solicited adverse events, and may be local (related to the vaccination site) or systemic. Any other side effect or symptom is recorded as an unsolicited adverse event.

Biological safety is also assessed by measuring the following, full blood count (FBC) and routine biochemistry - urea, potassium, sodium, ALT, total bilirubin, alkaline phosphatase, albumin, creatinine - one week and four weeks after each vaccination.

The trials have an appointed Local Safety Monitor (LSM). The LSM is a local Consultant in Infectious Diseases, based on the Infectious Diseases ward adjacent to the study centre, who has a special interest in, and wide experience of malaria. He is independent from the research group. The LSM was consulted in order to

assist in decisions about the safety of vaccine doses, and also in case of any uncertainty concerning volunteer safety, and is empowered to stop the study if necessary.

2.6.5.1 Definitions

An adverse event is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An adverse event can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), for example, symptom or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product).

A serious adverse event is any untoward medical occurrence that at any dose:
results in death
is life-threatening
requires inpatient hospitalisation or prolongation of existing hospitalisation
is a congenital anomaly/birth defect
results in persistent or significant disability/incapacity

Other non-serious events may be considered serious and reported immediately, i.e., events that required intervention to prevent one of the outcomes listed in the definition above. Any Serious Adverse Event (SAE) occurring from inclusion of the subject until the end of the study was also recorded. The relationship of any adverse event to the study vaccine is recorded by the Investigator, using the following definitions: definitely related, probably related, possibly related or not related.

2.6.5.2 Parameters

The solicited clinical signs and symptoms are listed in
Table 2.1. Volunteers are asked about these at each clinic visit, and also on the diary card that they complete daily for the first 7 days after vaccination.

	Adverse events
Local (injection site)	Pain at the injection site
	Redness at the injection site
	Swelling at injection site
	Warmth at the injection site
	Itch at the injection site
	Scaling/Pustules at site
General	Documented fever (oral temperature > 37.5° C)
	Symptoms of feverishness
	Malaise
	Arthralgia
	Headache
	Myalgia
	Nausea / vomiting
	Other (specify)

Table 2.1: Adverse events
Solicited local and general adverse events documented in the CRF.

2.6.5.3 Method and Timing of Measurement

The safety criteria are assessed during 30 minutes following the vaccination, and during the 28 following days. All volunteers are assessed by an Investigator 30 minutes after the vaccination, and then again on day 2, day 7 and day 28 after vaccination. At each visit vital signs are measured together with any local reactions at the injection site. These include those signs listed in
Table 2.1. All local reactions are considered causally related to the vaccination. The largest diameter of any redness is recorded in millimetres. The largest diameter site of local swelling is also recorded in millimetres. Severity of these local findings is graded using the scale provided in the following tables. Both measurements are taken as the largest diameter through one injection site, as on some occasions volunteers receive more than one injection for each dose of vaccine.

Grade	Swelling, diameter [mm]
Grade 0	0
Grade 1	< 20
Grade 2	20 – 50
Grade 3	> 50

Grade	Redness, diameter [mm]
Grade 0	0
Grade 1	< 50
Grade 2	50 – 100
Grade 3	> 100

Table 2.2: Grading for swelling and redness
Grading scales for swelling and redness.

Study subjects are asked to indicate the maximum degree of pain they experienced at the injection site using a scale ranging from 0 to 3 as described in Table 2.3.

Grade	Description
Grade 0	No pain at all
Grade 1	Painful to touch, no restriction in movement of arms, able to work, drive, carry heavy objects as normal
Grade 2	Painful when limb is moved (i.e. restriction in range of movement in arm, difficulty in carrying objects)
Grade 3	Severe pain at rest (i.e. unable to use arm due to pain.)

Table 2.3: Grading pain
Scale for grading pain

At each visit subjects are requested to report local and general side effects they might have experienced since they last were seen. The Investigator assesses the severity of the solicited signs and symptoms using the key provided in Table 2.4.

Grade	Description
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
Grade 0	Serious, life-threatening: Extreme limitation in activity, significant assistance required; significant medical intervention/therapy required, hospitalisation or hospice care probable

Table 2.4: Grading other adverse events.
Scale for grading severity of adverse events

Adverse events likely to be related to the vaccine, whether serious or not, which persist at the end of the trial are followed up by the Investigator where possible until their complete disappearance.

The outcome of adverse event occurring within 30 days post-vaccination was assessed as:
recovered/resolved
not recovered/not resolved
recovering/resolving
recovered with sequelae/resolved with sequelae
fatal (SAEs only)

At visits 7 days and 28 days after each vaccination, volunteers provide a blood sample. This is sent for routine laboratory analysis, in order to demonstrate the biological safety of the vaccine. Any clinically significant deviations from the normal ranges are recorded as adverse events.

2.6.6 Secondary Evaluation Criteria: Immunogenicity

As well as assessing the safety of the vaccines in these studies, measuring their immunogenicity is also important. Although there is no defined immunological correlate of protection from malaria, a vaccine with only low levels of measured immunogenicity is less likely to be effective at preventing infection.

2.6.6.1 Definition

An assessment of the magnitude of the specific vaccine-induced cellular and humoral immune response was made in volunteers at baseline and following each vaccination.

2.6.6.2 Parameters

The ex-vivo interferon gamma (IFN-γ) ELISPOT assay is performed to measure cellular responses to the vaccine insert at baseline and post-vaccine administration as detailed in the study protocol. These measurements are usually performed prior to vaccination and on day 7 and day 28 after vaccination. Antibody measurements, by ELISA may also be performed to assess any vaccine induced antibody response.

2.6.6.3 Method and Timing of Measurement

The ELISPOTs and ELISAs are performed according to standard operating procedures from the CCVTM laboratory. The detailed schedule of blood sampling was different for each study, and the timings can be found in the chapters relating to each trial.

2.6.7 Tertiary Evaluation Criteria: Efficacy

A detailed description of the challenge protocol can be found in chapter 7, but briefly, all volunteers are challenged with P. falciparum 14 days after the final vaccination. The challenges take place at Imperial College, London, as it contains a suitable insectary equipped to safely rear and store infected mosquitoes and to challenge human volunteers. The mosquitoes used for both studies described in this thesis were supplied by Captain Jack Williams of the Walter Reed Army Institute for Research. These were lab reared Anopheles stephensi mosquitoes infected with the 3D7 strain of P. falciparum parasites. Volunteers are taken into the insectary and each is provided with a cardboard carton, covered with netting, containing infectious mosquitoes. The carton is held over the arm of the volunteer, allowing mosquitoes to feed, until each volunteer has received a minimum of five bites from infectious mosquitoes. From the evening of day 6 following the challenge, volunteers attended clinic twice daily for a review of symptoms, vital signs monitoring (pulse, blood pressure and temperature) and withdrawal of 3mL of blood for thick film and PCR analysis. Field's stain films were examined immediately by experienced microscopists for the appearance of parasitized red

blood cells. A total of 200 high power fields were examined before a subject was declared slide negative. Subjects who reached day 15 without blood film evidence of malaria were monitored daily until day 21. All subjects were treated immediately with Riamet (artemether [20 mg], lumefantrine [120 mg]; Novartis) upon diagnosis of malaria. Subjects returned to clinic on two consecutive days for negative blood films post treatment.

Follow-up after the challenge is important. Before volunteers can be challenged with malaria, they are required to provide detailed contact information for themselves and also for a close friend or relative who will know where they are for the duration of the follow up period. Volunteers that do not own mobile phones are issued with them, and all are given emergency alert cards to carry with them at all times. As the studies we conduct are performed on an out-patient basis, we also restrict recruitment to volunteers who live within a specific geographical area, so that any problems can be responded to quickly. A doctor is on call for the volunteers at all times to provide advice or assistance if required.

If a vaccine is effective, volunteers will not develop malaria. A volunteer who has remained blood film negative by day 21 after the challenge is protected from disease. Any protected volunteers are treated with anti-malarial medication at this stage to prevent the theoretical possibility of malaria developing after that time. Provided volunteers attend visits as outlined in the protocol, the risk of developing any serious illness as a result of taking part is minimal. A review of the symptoms experienced by control volunteers taking part in studies such as this may be found in Chapter 7.

These studies have their limitations; they identify vaccines that protect malaria naïve volunteers from Oxford against one particular strain of malaria. There are several reasons why a vaccine that works in these conditions might not be effective in the field. The ethnicity of the volunteers, which will in part determine the types of immune response they make, is different from that of most people living in endemic areas. The strain of malaria used, 3D7, has been found to be relatively dissimilar to strains that are currently causing infection around the world. This strain is used because it has been studied extensively in laboratories around

the world, and is known to be sensitive to all antimalarial medications. If a vaccine is found to demonstrate efficacy in a study of this sort it may then be taken on to further Phase II and phase III studies in endemic areas.

Chapter 3 VAC027

"A Phase I study to assess the safety and immunogenicity of the polyprotein malaria vaccine candidates "FP9-PP and MVA-PP" in healthy adults using a prime-boost delivery schedule" (VAC027.1)

&

"Assessment of protection against malaria by sporozoite challenge of healthy adults vaccinated with the polyprotein malaria vaccines "FP9-PP, MVA-PP" and control non-vaccinated volunteers" (VAC027.2)

3.1 Introduction and Purpose

VAC027 was planned as two clinical trials with separate protocols, VAC027.1 and VAC027.2. These two trials were planned to overlap, with some volunteers from one study going on to participate in the second. The main reason for dividing the vaccination protocol (VAC027.1) from the challenge protocol (VAC027.2) was historical, with previous trials having been carried out in this way, in the past allowing volunteers from several vaccination studies to be challenged at the same time under the same protocol. This is the last study that was divided in this way as,

under the new Clinical Trials Regulations [166] introduced in 2004, it no longer made sense to separate the protocols for vaccination and challenge phases.

Although most vaccine trials of pre-erythrocytic malaria vaccine candidates have included either the CS or TRAP antigen alone these represent but two of a large number of antigens expressed by sporozoites. The hypothesis behind the design of the vaccines to be used in this study was that using multiple antigens would broaden the target antigen range of vaccine induced immunity, increasing the number of potential epitopes available. This approach should also prevent the development of escape mutants by P. falciparum.

The polyprotein vaccine used in this trial expresses six malaria antigens and its insert has previously been described by the acronym L3SEPTL [167]. This reflects the order of the six antigens in this insert: LSA3, STARP, Exp1, Pfs16, TRAP and LSA1.

The amino-terminal three antigens of the polyprotein are clearly expressed by blood-stage parasites as well as sporozoites of P. falciparum. Despite its name, LSA3 (liver stage antigen 3) is expressed by blood-stage schizonts (initially named D260) [181], as well as sporozoites [182]. It is the target of HLA-B*53-restricted CTL in Gambians [88]. Importantly, LSA-3 has been shown to induce protective immunity by vaccination against sporozoite challenge in a chimpanzee model of P. falciparum infection [183] LSA-3 was administered to humans in a phase I plasmid DNA vaccine trial as part of a five plasmid DNA vaccine by the US Navy group (Richie et al. unpublished); there were no serious adverse events associated with vaccination and on sporozoite challenge there was no evidence of protection.

STARP (sporozoite threonine and asparagine-rich protein) was described by Fidock et al. [184, 185], as a relatively conserved sporozoite antigen. Relatively low titres of antibodies against STARP seem to block sporozoite invasion [75]. It is also expressed by early ring-stage blood stage parasites but not by blood-stage schizonts. Gambians naturally exposed to malaria have CTL that target this antigen [82]. STARP has apparently not previously been administered to humans as part of a malaria vaccine candidate.

Exp-1, exported protein-1 [186] is a sporozoite and blood stage antigen that is recognized by CTL in Gambians [82]. A study in Burkina Faso found that a positive antibody response to Exp-1 correlated with significantly lower risk of malaria during the subsequent transmission season [187]. The P. yoelii homologue of this protein induced protective immunity when used as a DNA vaccine in mice [188]. As a subunit protein vaccine, Exp-1 could induce protective immunity against blood-stage parasite challenge in squirrel monkeys [189]. This antigen has previously been administered to humans as part of a fusion protein with the central NANP repeat of the circumsporozoite protein (192 Nigerian school children were vaccinated, and all were protected from clinical malaria for a 12 week period) [190].

Pfs16 is expressed by sexual stage parasites and possibly also by sporozoites. Disruption of this gene in P. falciparum results in reduced gametocyte production [191]. Antibodies induced to this molecule by vaccine constructs were reported to inhibit sporozoite invasion of human hepatoma cells [192]. Pfs16 has not to our knowledge been used previously in humans as part of a human candidate malaria vaccine.

The two antigens at the carboxyl-terminal, LSA1 and TRAP, are expressed only at the pre-erythrocytic stage of malaria infection. LSA1 was a component of the NYVAC-Pf7 candidate vaccine that was administered safely to about 32 subjects in the USA [168]. Thrombospondin-related adhesion protein (TRAP) was selected as it is well characterized and has a protective homologue in rodents [127]. TRAP was originally reported as a blood-stage antigen, but now is generally considered to be expressed on sporozoites only [193]. An antigen of particular interest is LSA-1. This is expressed by liver-stage parasites but not sporozoites. It is the target of HLA-B*53 restricted cytotoxic T cell responses in Gambians [83], a population in which this HLA allele was associated with protection against severe malaria [87].

Prieur et al. [167] have described L3SEPTL vaccines with this insert expressed in plasmid DNA, FP9 and MVA vectors. Immunogenicity of the viral vectors was greater than of the plasmid DNA vector. In heterologous prime-boost immunisation

studies in mice strong CD8 T cell responses were induced. The expression of all 6 antigens from the viral vector constructs was confirmed by immunogenicity studies in various strains of mice. Enhanced T cell responses were demonstrated with both FP9 priming and MVA boosting and the converse immunisation regime.

This study examined the combination of these six malaria antigens in humans in a single vaccine construct. These were new vaccines to be tested for the first time in man, and so the study included a series of dose lead in groups (groups 1 – 5), who received one vaccination only, and were subsequently followed up for safety data collection. This was then followed by two prime-boost vaccination groups (6 & 7), who received 3 doses of vaccine (either FFM, or MMF, depending on group). These volunteers were then invited to participate in the subsequent challenge study VAC027.2 in order to assess efficacy. For brevity the FP9-L3SEPTL and MVA-L3SEPTL constructs are hereafter referred to as FP9-PP and MVA-PP.

3.2 Methods

3.2.1 Objectives

The primary objective of VAC027.1 was to assess the safety of the malaria vaccines FP9-PP and MVA-PP when administered individually and sequentially in a prime-boost strategy. The primary objective of VAC027.2 was to assess if volunteers from groups 6 and 7 of VAC027.1 (who received 2 doses of FP9-PP given 4 weeks apart followed by MVA-PP given another 4 weeks (± 1 week) later, or 2 doses of MVA-PP given 4 weeks apart followed by FP9-PP given 4 weeks (± 1 week) later) were protected wholly or partially against malaria infection in a sporozoite challenge model. The secondary objective for both studies was to assess the cellular immune response generated by FP9-PP and MVA-PP when administered in a prime-boost regimen, and after challenge; and if there was evidence of partial or complete protection by the vaccinations, to explore immunological correlates of protective immunity.

In the context of this thesis, both studies have been combined into one chapter, and therefore for ease of description, the objectives for both studies have been

combined. As this study was testing new vaccines for the first time in man, safety remains the primary concern, and is therefore described as the primary objective throughout. Immunogenicity and efficacy are therefore both secondary objectives.

Exploratory objectives for both studies included assessment of the humoral immune response generated by FP9-PP and MVA-PP when administered in a prime-boost regimen, by measuring anti-sporozoite antibodies; and assessment of the long term efficacy of FP9-PP and MVA-PP in a re-challenge of any volunteers protected at initial malaria challenge.

3.3 Outcome Measures

3.3.1 Primary Objective – Safety

Safety was assessed by the collection of local and systemic adverse events. Each subject was observed for at least 30 minutes after vaccination, and underwent clinical review 2, 7 and 28 days after each vaccination for reporting of solicited and unsolicited adverse events. Subjects completed a diary card each day for the first 7 days after each vaccination.

Safety blood testing, including analysis of full blood count and biochemistry (urea, electrolytes, alanine aminotransferase, bilirubin, alkaline phosphatase, albumin) was also performed at intervals throughout the study (see figures 3.1 – 3.3 for details).

3.3.2 Secondary Objective – Immunogenicity

The immunogenicity of the vaccines was assessed primarily by measurement of ex-vivo and cultured IFN-γ ELISPOT responses to the polyprotein antigens before and after malaria infection.

For the ex vivo IFN-γ ELISPOT freshly isolated PBMC are stimulated with malaria peptides, control peptides, recombinant antigens, or recombinant viruses expressing part or all of the L3SEPTL insert in ELISPOT plate (Millipore) wells

coated with antibody specific for human IFN-γ (Mabtech). After 18-20 hours incubation the PBMC are washed away and biotinylated anti-IFN-γ added, followed by alkaline phosphatase-conjugated streptavidin (both from Mabtech). Capture of IFN-γ can then be visualized by adding chromogenic substrate (Bio-Rad). The coloured spots are calculated with an ELISPOT reader (AID) and the results are expressed as spot forming units (SFU) per $1x10^6$ PBMC. Peptides (Thermoelectron) were 20mers overlapping by 10, designed to cover the entire sequence of the polyprotein vaccine. Details of the sequences of the peptides, and the pools used in this study, may be found in Appendix 3. The sequence of TRAP used in the vaccine was from the T9/96 strain of falciparum malaria. The strain of malaria used in the subsequent challenge in the study VAC027.2 was 3D7 which has a slightly different TRAP sequence. ELISPOT responses to both sequences were therefore tested.

3.3.3 Secondary Objective – Efficacy

Protection against malaria infection was assessed using a P. falciparum sporozoite challenge model. To assess the efficacy of the vaccines all vaccinated subjects and six unvaccinated control subjects underwent experimental challenge with Plasmodium falciparum, fourteen days after the final vaccination. The details of the challenge method can be found in chapter 7. From the evening of day 6 subjects attended clinic twice daily for review of symptoms, vital signs monitoring (pulse, blood pressure and oral temperature) and withdrawal of 3 mL of blood for thick film and PCR analysis for parasite DNA. Field's stain thick films were examined immediately by experienced microscopists for the appearance of viable parasites. A minimum of 200 high power fields were examined before a subject was declared slide negative. Subjects who reached day 15 without blood film evidence of malaria infection were followed up daily until day 21. All subjects were treated immediately with Riamet® (artemether 20mg, lumefantrine 120mg, Novartis) on diagnosis of malaria by the identification of a viable parasite on thick film. Subjects returned to clinic on two consecutive days for negative blood films post treatment. The pre-patent period was defined as the time to diagnosis, and efficacy was assessed by comparing this time period in vaccinated and unvaccinated subjects.

The analysis for the primary objective was based on the number of hours between infectious challenge and blood stage parasitaemia. Each of the groups was compared with the other and with the control group using the Kaplan Meier method. Statistical significance of any differences observed was then assessed by the log rank test.

Blood was taken daily for PCR analysis for parasite DNA. This was performed in real time, although the clinicians assessing the volunteers were blinded to the results. The method used is described in detail in [194]. The results of the PCR analysis were used to estimate parasite growth rates. A description of this method; and the reasons for choosing it over the other available methods for calculating parasite growth rates can be found in chapter 6 of this thesis.

3.4 Investigational Plan

3.4.1 Trial Plan

The following two flow charts show the schedule of visits and procedures from subject screening through to the final visit.

Group 1 to 5: lead-in dose regimen, 6 Visits, 1 Vaccination, Open, 90 Days duration/subject

Visit Number	V1	V2	V3	V4	V5	V6
Trial Timelines (Days,)	S	D0	D2	D7	D28	D90
Time Windows (Days)			[±1 D]	[± 2D]	[±3 D]	[±14 D]
Visit Intervals	-		V2 + 2 days	V2 + 1 Week	V2 + 4 Weeks	V2 + 3 Months
Inclusion & Non Inclusion Criteria	X					
Informed Consent	X					
Medical History	X					
Physical Examination	X					
β-HCG urine test	X	X				
HCV, HIV, HBV tests	6mL					
HLA typing		3mL				
Contra-Indications Review		X				
Vaccination Doses		Vac1				
Immediate surveillance (30 min)		X				
Post-Dose Follow-Up (n Days)			2 days	7 days	28 days	90 days
Vital signs		X	X	X	X	X
Local & Systemic Events/Reactions		X	X	X	X	X
Diary Cards Provided		DC1		DC2		
Diary Cards Collected				DC1	DC2	
Review of Diary Cards			X	X	X	
Blood Sampling (n mL)	11 mL BS1	63 mL BS2		65 mL BS3	5 mL BS4	65 mL BS5
Biochemistry	3mL			3mL	3mL	3mL
Haematology	2mL			2mL	2mL	2mL
Antisporozoite Ab		10mL		10mL		10mL
Exploratory Immunology		50mL		50mL		50mL
Termination Record / Final						X
Serious Adverse Events	to be reported at any time during the trial					

Figure 3.1: Flow chart groups 1 – 5

Summarising trial visits and procedures for groups 1 - 5.

Group 6 and 7: prime-boost regimen, 12 Visits, 3 Vaccinations, Open, 150 Days duration/subject

Visit Number	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
Trial Timelines (Days,)	S	D0	D2	D7	D28	D30	D35	D56	D58	D63	D84	D150
Time Windows (Days)			±1D	±2D	±10 D	±1D	±2D	+4wks/ -2wks	±1D	±2D	±7 D	±14 D
Visit Intervals	-		V2+ 2days	V2+ 1Wk	V2 + 4Wks	V5+ 2days	V5+ 1Wk	V5+ 4Wks	V8+ 2days	V8+ 1Wk	V8+ 4Wks	V8+ 3Mos
Inclusion & Non Inclusion Criteria	X											
Informed Consent	X											
Medical History	X	X										
Phys. Exam.	X											
β-HCG urine test	X	X			X			X				
HCV, HIV, HBV tests	6mL											
HLA typing		3mL										
Review Contra-Indications	X	X		X								
Vaccination		Vac1			Vac2			Vac3				
Immediate surveillance (30 min)		X			X			X				
Post-Dose Follow-Up (Days)			2	7	28	2	7	28	2	7	28	90
Vital signs	X	X	X	X	X	X	X	X	X	X	X	X
Local & Systemic Events/Reactions		X	X	X	X	X	X	X	X	X	X	X
Diary Cards Provided		DC1			DC2			DC3				
Diary Cards Collected				DC1			DC2	DC		DC3		
Review Diary Cards			X	X	X	X	X	X	X	X	X	
Blood Sampling (n mL)	11mL BS1	73mL BS2		55mL BS3	55mL BS4		55mL BS5	55mL BS6		75mL BS7		75mL BS8
Biochemistry	3mL			3mL	3mL		3mL	3mL		3mL		3mL
Haematology	2mL			2mL	2mL		2mL	2mL		2mL		2mL
Antisporozoite Ab		10mL								10mL		10mL
Gene Expression		10mL								10mL		10mL
Exploratory Immunology		50mL		50mL	50mL		50mL	50mL		50mL		50mL
Termination Record												X
SAEs		*to be reported at any time during the trial*										
Trial phase		Primary Series						Booster series				

Figure 3.2: Flow chart groups 6 & 7

Summarising trial visits and procedures (VAC027.1, vaccination study)

Visit Number	S	V1	V2	V3	V4	V5-V18	V19-V27	V28	V29*
Visit Day	D-60 to D-2	D -1	D 0	D6	D7	D7.5-D14	D15-D23	D35	D150
Time Windows (Days)		±1	±7					±10	±14
Sign consent	X								
Eligibility	X								
History	X								
Physical Examination	X								
Challenge			X						
Vital signs	X			X	X	X	X	X	X
Interim history/Clinical assessment				X	X	X	X	X	X
Recording AE				X	X	X	X	X	X
Urine analysis	X								
Pregnancy test	X		X						
Blood in ml needed for:									
HLA typing (controls only)					5				
LFT / UE	3							3	3
FBC incl. platelets	2							2	2
HBV, HCV, HIV serology	10								
thick smear / PCR			1	3	3	3x14	3x9		
ELISPOT			50		50			50	50
anti sporozoite antibodies			10					10	10
Gene expression study			10		10			(10ǂ)	10
Blood drawn each time	15		71	3	73	3x14	3x9	60	75
Cumulative blood taken (controls)	15		86	89	162	165-204	207-231	291	366
Cumulative blood taken (vaccinees)	470		541	544	612	613-654	657-681	741	816

Figure 3.3 Flow chart for challenge study, VAC027.2

Flow chart for challenge study for Groups 6 & 7 and unvaccinated control volunteers

3.4.2 Trial Design

This was a phase I/IIa, prospective, open partially-randomised clinical trial of a prime-boost malaria vaccination regimen using 2 different vaccine candidates. This began with five lead-in dose ranging groups of volunteers (groups 1 – 5) followed by two larger groups undergoing prime-boost vaccination. Subjects in these latter groups were subsequently invited to participate in a sporozoite challenge study (VAC027.2) to examine vaccine efficacy against malaria infection.

Allocation of study subjects to groups 6 and 7 (but not groups 1 – 5) was randomised.

During the dose lead-in phase of the study, interim safety data analyses were carried out before increasing the vaccine dose. This was carried after the day 7 follow up visit had been completed for all volunteers in a given vaccine group. As this study was testing vaccines for the first time in human volunteers, the first doses of each vaccine were given to one volunteer only at a time (i.e. one volunteer in group 1 and one volunteer in group 2), with subsequent volunteers in those groups being vaccinated after a 24 hour period.

Further analyses were carried out after vaccinations for groups 1 – 5 were completed to determine which doses were most appropriate to use during the prime-boost regimes subsequently used in groups 6 and 7. The vaccination and challenge schedule for volunteers in these groups is shown in Figure 3.4.

	Vaccinations						Challenge	
Group 6	F		F		M		C	
Weeks	**0**	**1**	**4**	**5**	**8**	**9**	**10**	**15**
Group 7	M		M		F		C	
Blood Sampling	x	x	x	x	x	x	x	x

Figure 3.4: Vaccination plan for groups 6 & 7

3.4.3 Trial Calendar

First visit first volunteer:	11th April 2006
Last visit last volunteer:	12th January 2007
Last vaccine given:	1st November 2006
Challenge Dates:	14th and 15th November 2006
Statistical Analysis:	Completed July 2007

3.5 Vaccines

3.5.1 Formulation of the MVA-PP Vaccine

The vaccine, MVA-PP containing L3-SEPTL, was manufactured by a contract manufacturer (IDT, Germany) and provided in vials of 300 µL volume for intradermal injection at a concentration of 5×10^8 pfu/mL in 10mM Tris buffer. Vaccines were stored below -18°C in a freezer at the trial centre and allowed to thaw to room temperature by standing in the clinic room or by hand warming prior to administration.

3.5.2 Formulation of the FP9-PP Vaccine

The vaccine, FP9-PP containing L3-SEPTL, also manufactured under contract by IDT, was provided in vials of 300 µL volume for intradermal injection at a concentration of 3×10^8 pfu/mL in 10mM Tris buffer. Vaccines were stored below -18°C in a freezer at the trial centre and allowed to thaw to room temperature by standing in the clinic room or by hand warming prior to administration.

3.5.3 Selection of Doses

Groups 1 – 5 were designed to assess safety and to gather immunogenicity data from escalating doses of the two vaccines FP9-PP and MVA-PP. Doses of up to 1.5×10^8 pfu of similar viral vectored vaccines have been given in previous studies within this group with a good adverse event profile.

The doses of MVA-PP used in this study were:
1×10^8 pfu (200 µL) for group 2,
2×10^8 pfu (400 µL) for group 4,
5×10^8 pfu (1000 µL) for group 5.

The doses of FP9-PP used in this study were:
1×10^8 pfu (330 µL) for group 1,
2×10^8 pfu (660 µL) for group 3.

The doses for groups 6 & 7 for each vaccine were determined by the investigators in conjunction with the Local Safety Monitor (LSM) after considering safety and immunogenicity data for the dose lead-in groups 1 – 5. The dose of MVA-PP used in groups 6 and 7 was 1×10^8 pfu for priming immunisations (i.e. first and second doses) and 2×10^8 pfu for boosting immunisations (i.e. third doses). The dose of FP9-PP used in groups 6 and 7 was 1×10^8 pfu for priming immunisations (i.e. first and second doses) and 2×10^8 pfu for boosting immunisations (i.e. third doses). The lower dose (1×10^8 pfu) was chosen for both vaccines for priming immunisations as no clear increase in post-prime immunogenicity was seen with the higher doses. The intermediate (2×10^8 pfu) was chosen for the boosting dose given that the safety profile was broadly similar to the low dose of each vaccine and that post-boost immunogenicity might be more reliant on dose than post-prime.

3.5.4 Preparation for Use

The vaccines were supplied ready for injection. For groups requiring a larger volume of vaccine, multiple sterile syringes and needles were used to deliver up to six injections of up to 180 µL of vaccine each. All vaccine doses were divided equally between both arms, given as intradermal injections into the skin overlying the deltoid muscle of the upper arm.

3.6 Selection of the Trial Population

3.6.1 Inclusion Criteria

Healthy adults aged 18 to 55 years
Resident in or near Oxford for the duration of the vaccination study
Willingness to allow the investigators to access hospital and General Practitioner medical notes
For females only, willingness to practice continuous effective contraception during the study and if participating, during the subsequent challenge study.
Agreement to refrain from blood donation during the course of the study
Written informed consent
Willingness to undergo an HIV test

3.6.2 Exclusion Criteria

Any deviation from the normal range in biochemistry or haematology blood tests or in urine analysis as defined in Appendix 1 of the protocol.
Prior receipt of an investigational malaria vaccine
Use of any investigational or non-registered drug, vaccine or medical device other than the study vaccine within 30 days preceding dosing of study vaccine, or planned use during the study period
Administration of chronic (defined as more than 14 days) immunosuppressive drugs or other immune modifying drugs within six months of vaccination. (For corticosteroids, this will mean prednisolone, or equivalent, ≥ 0.5 mg/kg/day. Inhaled and topical steroids are allowed.)
History of malaria chemoprophylaxis with chloroquine within 5 months prior to the planned challenge, with Lariam within 6 weeks prior to the challenge, and Riamet within 2 weeks prior to the challenge
Any history of malaria
Travel to a malaria endemic country within the previous 6 months prior to the planned challenge
Planned travel to malarious areas during the study period

Any confirmed or suspected immunosuppressive or immunodeficient condition, including human immunodeficiency virus (HIV) infection and asplenia
History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, e.g. egg products
Evidence of cardiovascular disease
History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ)
History of haemoglobinopathies: Sickle cell disease, thalassaemia, G6PD deficiency
History of diabetes mellitus
Chronic or active neurological disease
Chronic gastrointestinal disease
History of > 2 hospitalisations for invasive bacterial infections (pneumonia, meningitis)
Suspected or known current alcohol abuse as defined by an alcohol intake of greater than 42 units every week
Seropositive for hepatitis B surface antigen (HBsAg)
Seropositive for hepatitis C virus (antibodies to HCV)
Hepatomegaly, right upper quadrant abdominal pain or tenderness
Evidence of serious psychiatric condition
Any other on-going chronic illness requiring hospital specialist supervision
Administration of immunoglobulins and/or any blood products within the three months preceding the planned administration of the vaccine candidate
Pregnant or lactating female
Female who is willing or intends to become pregnant during the study
Any history of anaphylaxis in reaction to vaccination
PI assessment of lack of willingness to participate and comply with all requirements of the protocol (including history or clinical evidence of iv drug abuse)
Any other finding which in the opinion of the investigator would significantly increase the risk of having an adverse outcome from participating in this protocol

3.6.3 Randomisation Procedures

Volunteers in groups 1-5 were not randomised but were enrolled sequentially into groups 1 – 5. Subjects were randomised into groups 6 or 7. To achieve equal numbers of volunteers in each group, restricted randomisation was performed as described in Kirkwood, Essentials of Medical Statistics (1st edition), page 185, using the random number tables in Appendix 10 of this book.

3.7 Statistical Analysis

3.7.1 Sample Size

The primary objective of the study was descriptive – the safety of the vaccines. The numbers of volunteers were therefore chosen primarily for reasons of safety and practicality. The challenge study aims to reveal potentially useful vaccine candidates so that they may be selected for further study, rather than demonstrate the eventual efficacy of a vaccine. The size of the study groups need to be adequate to demonstrate big differences between vaccinated volunteers and unvaccinated control volunteers. In this instance, the number of volunteers included (10 per vaccine group for the challenge study), would have 80% power to demonstrate a vaccine efficacy of 65% or greater.

3.7.2 Statistical Methods

The main objective of this study was assessment of safety, and it was therefore not powered to provide statistical comparisons between groups. Immunogenicity was assessed for each volunteer by summing the ELISPOT responses to each antigen across peptide pools. Immunogenicity was compared between groups using geometric mean and medians, with the significance of any differences observed calculated using Mann Whitney U test.

Efficacy was assessed by calculation of the number of hours between infectious challenge and blood stage parasitaemia. Each of the groups of vaccinated volunteers that took part in the challenge was compared with the other and with

the control group using the Kaplan Meier method. Statistical significance of any differences observed was then assessed by the log rank test.

All data, including adverse event data collected on StudyBuilder™, was imported into and analysed using Microsoft® Excel or SPSS® statistics packages.

3.8 Results

Healthy malaria naïve adult subjects aged 18 – 50 years were recruited in the Oxford area and underwent medical screening as previously described [133]. All volunteers provided fully informed consent to participate in this study by signing a written consent form, prior to any study procedures being initiated. All vaccinations and follow up visits took place in the outpatients unit at the Centre for Clinical Vaccinology and Tropical Medicine, at the Churchill Hospital in Oxford. The malaria challenge was performed in the insectary in the Alexander Fleming Building, Imperial College, London. The study received ethical approval from the Oxfordshire Research Ethics Committee A. VAC027.1 received a favourable opinion on 8/10/2004, (COREC reference 04/Q1604/93), whilst the challenge study, VAC027.2 received a favourable opinion on 24/05/2006 (COREC reference 06/Q1604/55). The study was performed in compliance with the requirements of the MHRA under a Clinical Trial Authorisation. It gained full regulatory approval from the MHRA; for VAC027.1 this was received on 14/3/2006, CTA number 27454/0001/001-0001, Eudra CT Number 2004-002424-17. VAC027.2 was fully approved on 13/04/2006, with the CTA number 2754/0001/002-0001 and EudraCT number 2006-000629-67. An Independent Local Safety Monitor was appointed in Oxford. The trial was conducted according to GCP and in accordance with the current version of the declaration of Helsinki (52nd WMA General Assembly, Edinburgh, Scotland, October 2000). It was externally monitored by Appledown Clinical Research Ltd.

Recruitment for this study began in April 2006, and the final visit of the final subject to the study site took place on 12th January 2007. The participant flow is shown in a CONSORT diagram in Figure 3.5.

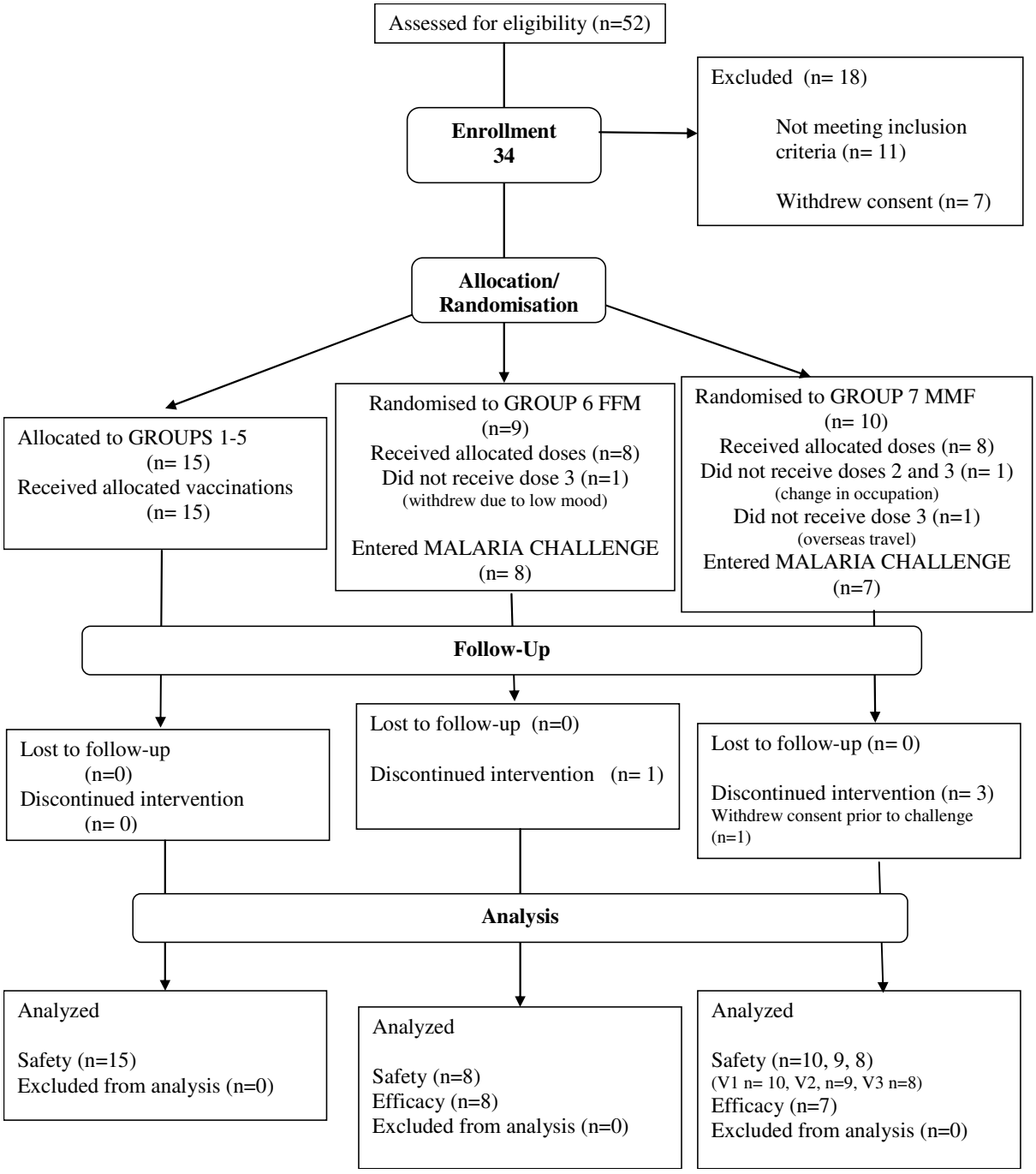

Figure 3.5: CONSORT flow chart

CONSORT chart showing flow of participants through VAC027.

In total, 52 subjects were screened, of whom 18 were excluded, 11 because they were ineligible, and 7 withdrew consent after screening. 34 volunteers were enrolled into the study, 15 into groups 1-5 (3 per group) and 19 into groups 6 and 7. All volunteers in groups 1 to 5 received a single vaccine dose and attended follow up visits as planned in the protocol. 9 volunteers were randomised to group

6 and 10 to group 7. Of these, one subject in group 6 withdrew from the study after the second vaccination. Two subjects in group 7 withdrew from the study. One volunteer withdrew after the first vaccine having taken on new work commitments rendering him unable to attend key clinic visits. A second volunteer withdrew after the second vaccination after deciding to travel abroad for an extended period of time during the follow up period. The volunteer attended for follow up at days 2 and 7 following the second vaccination but declined further follow up. Available data has been included in the analysis.

Of those volunteers who completed the course of vaccinations, all 8 volunteers in group 6 participated in the challenge, whilst only 7 of those from group 7 took part; one volunteer withdrew consent after the vaccination study. 6 volunteers were recruited to act as unvaccinated controls for the challenge phase.

Group	N	Mean age (SD)	Min age	Max age	No. female (%)	No. male (%)
Group 1	3	35.4 (8.7)	27.9	45.0	2 (66.7%)	1 (33.3%)
Group 2	3	22.4 (2.8)	23.3	24.6	3 (100%)	0 (0%)
Group 3	3	34 (6.4)	34.7	40.0	2 (66.7%)	1 (33.3%)
Group 4	3	28.8 (11.5)	21.5	42.1	0 (0%)	3 (100%)
Group 5	3	29.5 (13.8)	18.9	45.1	1 (33.3%)	2 (66.7%)
Group 6	9	32.2 (10.5)	20.6	47.3	6 (66.7%)	3 (33.3%)
Group 7	10	29.8 (7.5)	19.5	44.6	3 (30%)	7 (70%)
TOTAL COHORT	**34**	**30.5 (8.9)**	**18.9**	**47.3**	**17 (50%)**	**17 (50%)**

Table 3.1: Demographics

Table demonstrating the demographic characteristics of volunteers by group.

3.8.1 Protocol Deviations

Details of deviations from the protocol are given by study group:

Group 2
The physical examination was not performed during the screening visit for volunteer M1027554 due to the unforeseen unavailability of a clinician. This was instead carried out 6 days after the screening visit. The examination was normal and the volunteer was enrolled and vaccinated 9 days later.

Volunteer M1027548 received a smaller dose of vaccine than intended (140µL instead of 200µL) due to an under filled vaccine vial.

Group 5
Volunteer M1027574 attended for the M+28 visit 8 days earlier than the window allowed due to travel plans unconnected with the trial. The subject attended the subsequent M+90 visit within window.

Subject M1027572 revealed after receiving the second vaccination a history of low mood which predated the study but had not revealed this at screening and had not sought medical attention. The volunteer admitted finding committing to the trial procedures more difficult than expected and decided to withdraw from the study before being seen again. The volunteer did attend for day 2 and day 7 follow up visits and agreed to medical referral to the General Practitioner. A subsequent request for an update from the General Practitioner received no reply. As far as can be determined this volunteer would not necessarily have met exclusion criteria at screening if the full history had been divulged so data has been included for analysis.

Subject M1027595 was unable to attend the FF+7 visit on time due to family problems but attended 7 days after the visit window. Safety data was collected at this visit, but blood was not taken or analysed for immunogenicity due to this delay.

Group 7
Subject M1027585 failed to attend the MMF+28 visit due to academic commitments but did attend the final visit at MMF+90.

Subjects M1027580 and M1027586 attended clinic on time for the FFM+28 visit but some records and procedures were not fully completed. These were completed subsequently 15 and 6 days after the visit window respectively.

Subject M1027579 withdrew from the study after the first vaccine having taken on new work commitments rendering him unable to attend key clinic visits. The volunteer was able to attend for safety follow-up visits at 2, 7 and 28 days following vaccination but did not have blood collected for immunogenicity. Available data has been included in the analysis.

Subject M1027575 withdrew after the second vaccination after deciding to travel abroad for an extended period of time during follow up. The volunteer attended for follow up at days 2 and 7 following the second vaccination but declined further follow up. Available data has been included in the analysis.

3.8.2 Safety Results

Eleven volunteers (32%) had at least one out of range blood result during the trial but none of these appeared vaccine related and only two (4%) warranted any further investigation. One was a mild iron deficiency anaemia associated with menorrhagia, and the other a persistent mildly raised bilirubin. Both volunteers were referred to their GPs for further investigation with their consent.

Both vaccines used were well tolerated and appeared safe in this small-scale phase I trial. All 15 volunteers (100%) in groups 1 – 5 completed the trial successfully. Of nineteen volunteers in groups 6 & 7, 1 (5%) withdrew following a worsening of a pre-existing problem and two (10%) withdrew from the trial for reasons unrelated to the study.

No serious adverse events (SAEs) occurred during the study. Reactions within 30 minutes of vaccination were predominantly mild (96% mild, 4% moderate) and local (93%). Of 701 adverse events (AEs) recorded in total, 581 were judged probably or definitely related to vaccination and 568 of these (97.7%) were solicited adverse events. Severe AEs represent approximately 0.3% of all AEs and moderate AEs approximately 8%. All others were classed as mild. The following tables describe the local and systemic adverse events reported in the 28 days following each vaccination. No safety concerns were noted with these two novel vaccines during the course of the studies.

	Group 1: FP9-PP low dose No. volunteers (%) (n = 3)			Group 2: MVA-PP low dose No. volunteers (%) (n = 3)			Group 3: FP9-PP mid dose No. volunteers (%) (n = 3)		
Description	Mild	Mod	Severe	Mild	Mod	Severe	Mild	Mod	Severe
Itch	3 (100%)			2 (67%)			3 (100%)		
Pain	3 (100%)			3 (100%)			2 (67%)	1 (33%)	
Redness	2 (67%)	1 (33%)		3 (100%)			3 (100%)		
Scaling	3 (100%)			3 (100%)			3 (100%)		
Swelling	2 (67%)	1 (33%)		2 (67%)	1 (33%)		1 (33%)	2 (67%)	
Warmth	3 (100%)			2 (67%)			3 (100%)		
Total	16	2	0	15	1	0	15	3	0
Total (all grades)	18			16			18		

Table 3.2: Local AEs groups 1 - 3

Description	Group 4: MVA-PP mid dose No. volunteers (%) (n = 3)			Group 5: MVA-PP high dose No. volunteers (%) (n = 3)		
	Mild	Mod	Severe	Mild	Mod	Severe
Itch	2 (67%)			3 (100%)		
Pain	2 (67%)	1 (33%)		2 (67%)		
Redness	3 (100%)			2 (67%)	1 (33%)	
Scaling	3 (100%)			3 (100%)		
Swelling	2 (67%)		1 (33%)	1 (33%)	2 (67%)	
Warmth	2 (67%)			3 (100%)		
Total	14	1	1	14	3	0
Total (all grades)	16			17		

Table 3.3:Local AEs groups 4 & 5

Tables 3.2 & 3.3: Groups 1 – 5, solicited local adverse events up to 28 days post-vaccination

Description	**Group 1:** FP9-PP low dose No. volunteers (%) (n = 3)			**Group 2:** MVA-PP low dose No. volunteers (%) (n = 3)			**Group 3:** FP9-PP mid dose No. volunteers (%) (n = 3)		
	Mild	**Mod**	**Severe**	**Mild**	**Mod**	**Severe**	**Mild**	**Mod**	**Severe**
Arthralgia	1 (33%)						1 (33%)		
Documented fever	1 (33%)						1 (33%)		
Fatigue	3 (100%)								
Feverish	2 (67%)						1 (33%)		
Headache	2 (67%)			1 (33%)			1 (33%)		
Malaise	2 (67%)								
Myalgia	3 (100%)			1 (33%)			1 (33%)		
Total	**14**	**0**	**0**	**2**	**0**	**0**	**5**	**0**	**0**
Total (all grades)	**14**			**2**			**5**		

Table 3.4: Solicited systemic AEs groups 1 – 3

Description	Group 4: MVA-PP mid dose No. volunteers (%) (n = 3)			Group 5: MVA-PP high dose No. volunteers (%) (n = 3)		
	Mild	Mod	Severe	Mild	Mod	Severe
Arthralgia				1 (33%)		
Documented fever				1 (33%)		
Fatigue	2 (67%)			3 (100%)		
Feverish	2 (67%)			3 (100%)		
Headache	3 (100%)					
Malaise	2 (67%)			1 (33%)		
Myalgia	1 (33%)			2 (67%)		
Nausea	1 (33%)			1 (33%)		
Total	11	0	0	12	0	0
Total (all grades)	11			12		

Table 3.5: Solicited systemic AEs groups 4 & 5

Tables 3.4 & 3.5: Groups 1 – 5, solicited systemic adverse events up to 28 days post-vaccination.

Description	Vaccine 1 No. volunteers (%) (n = 9)			Vaccine 2 No. volunteers (%) (n = 9)			Vaccine 3 No. volunteers (%) (n = 9)		
	Mild	Mod	Severe	Mild	Mod	Severe	Mild	Mod	Severe
Itch	7 (78%)			5 (56%)			6 (67%)		
Pain	7 (78%)	1 (11%)		6 (67%)	1 (11%)		8 (89%)	1 (11%)	
Redness	7 (78%)	2 (22%)		4 (44%)	5 (56%)		4 (44%)	4 (44%)	
Scaling	7 (78%)			9 (100%)			6 (67%)		
Swelling	6 (67%)	3 (33%)		6 (67%)	1 (11%)		4 (44%)	4 (44%)	
Warmth	7 (78%)			7 (78%)			8 (89%)		
Total	41	6	0	38	7	0	36	9	0
Total (all grades)	47			45			45		

Table 3.6: Solicited local AEs group 6

Group 6, solicited local adverse events up to 28 days post-vaccination.

Description	Vaccine 1 No. volunteers (%) (n = 10)			Vaccine 2 No. volunteers (%) (n = 9)			Vaccine 3 No. volunteers (%) (n = 9)		
	Mild	Mod	Severe	Mild	Mod	Severe	Mild	Mod	Severe
Itch	9 (90%)			9 (100%)			7 (78%)		
Pain	8 (80%)			7 (78%)			5 (56%)	3 (33%)	
Redness	9 (90%)	1 (10%)		6 (67%)	3 (33%)		4 (44%)	4 (44%)	
Scaling	10 (100%)			8 (89%)			6 (67%)		
Swelling	8 (80%)	2 (20%)		5 (56%)	3 (33%)	1 (11%)	3 (33%)	5 (56%)	
Warmth	7 (70%)			7 (78%)			7 (78%)		
Total	51	3	0	42	6	1	32	12	0
Total (all grades)	54			49			44		

Table 3.7: Solicited local AEs group 7

Group 7, solicited local adverse events up to 28 days post-vaccination.

Description	Vaccine 1 No. volunteers (%) (n = 9)			Vaccine 2 No. volunteers (%) (n = 9)			Vaccine 3 No. volunteers (%) (n = 9)		
	Mild	Mod	Severe	Mild	Mod	Severe	Mild	Mod	Severe
Arthralgia	4 (44%)			4 (44%)			2 (22%)		
Documented fever	2 (22%)			3 (33%)					
Fatigue	3 (33%)			4 (44%)			3 (33%)		
Feverish	2 (22%)			4 (44%)			2 (22%)		
Headache	4 (44%)	1 (11%)		6 (67%)			4 (44%)		
Malaise	2 (22%)			4 (44%)			3 (33%)		
Myalgia	4 (44%)			7 (78%)			4 (44%)		
Nausea				1 (11%)			1 (11%)		
Total	21	1	0	33	0	0	19	0	0
Total (all grades)	22			33			19		

Table 3.8: Solicited systemic AEs group 6

Group 6, solicited systemic adverse events up to 28 days post-vaccination

Description	Vaccine 1 No. volunteers (%) (n = 10)			Vaccine 2 No. volunteers (%) (n = 9)			Vaccine 3 No. volunteers (%) (n = 9)		
	Mild	Mod	Severe	Mild	Mod	Severe	Mild	Mod	Severe
Arthralgia	3 (30%)			1 (11%)			4 (44%)		
Documented fever	2 (20%)						1 (11%)	2 (22%)	
Fatigue	5 (50%)			2 (22%)			4 (44%)		
Feverish	5 (50%)			1 (11%)			6 (67%)		
Headache	6 (60%)			3 (33%)			6 (67%)		
Malaise	6 (60%)			1 (11%)			5 (56%)		
Myalgia	6 (60%)			2 (22%)			5 (56%)		
Nausea	1 (10%)			1 (11%)			3 (33%)		
Total	34	0	0	11	0	0	34	2	0
Total (all grades)	34			11			36		

Table 3.9: Solicited systemic AEs group 7

Group 7, solicited systemic adverse events up to 28 days post-vaccination.

3.8.3 Immunogenicity

Immunogenicity was measured by assessing cellular immune responses in an ELISPOT. Peripheral blood mononuclear cells (PBMC) were isolated from fresh ex-vivo human blood samples collected during the trial and either frozen and stored or assessed using the ex-vivo IFN-γ ELISPOT assay (figure 3.6). Responses to each antigen were calculated by summing responses to peptide pools across each antigen.

3.8.3.1 Groups 1 – 5

The following graphs show the responses in groups 1-5. These responses are low, as expected. Previous experience with these vaccines demonstrates that a single dose rarely results in a significant response [146].

The study was not powered to provide statistical comparison of the response between groups, and the data is from three individuals per group only. Geometric mean responses to the whole insert L3SEPTL one week after vaccination were as follows:

Group 1: 62.1 sfu/million PBMCs
Group 2: 186.1 sfu/million PBMCs
Group 3: 30.5 sfu/million PBMCs
Group 4: 135.5 sfu/million PBMCs
Group 5: 18.7 sfu/million PBMCs

However, comparison with the Mann-Whitney test using STATA™ software showed no significant difference in responses to the whole L3SEPTL insert at day 7 in the following cases: when comparing FP9 and MVA vectors at low ($p = 0.13$) and medium dose ($p = 0.28$); when comparing responses by vector regardless of dose (FP9 groups 1 & 3 v MVA groups 2, 4 and 5, $p = 0.35$); and when comparing different doses with the same vector ($p = 0.13$ to 0.83).

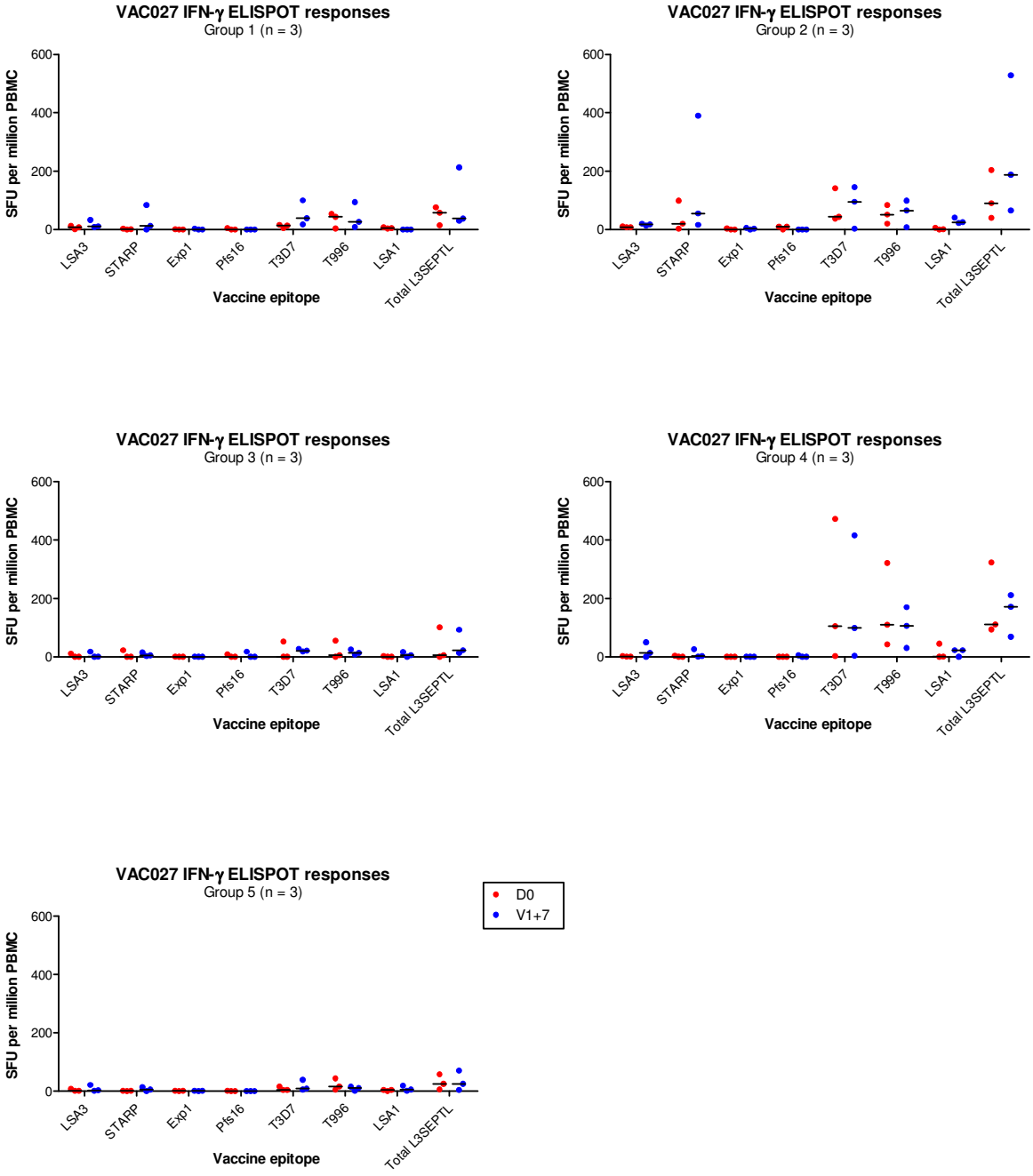

Figure 3.6: IFN-γ ELISPOT responses groups 1-5

ELISPOT responses to vaccine epitopes in groups 1 – 5 at baseline (D0) and 7 days after vaccination (V1+7). Horizontal lines show median response. Individual vaccine components are displayed along the x axis and response against the y axis, summed from multiple pools where applicable. The final data column represents the summed responses against the entire vaccine insert.

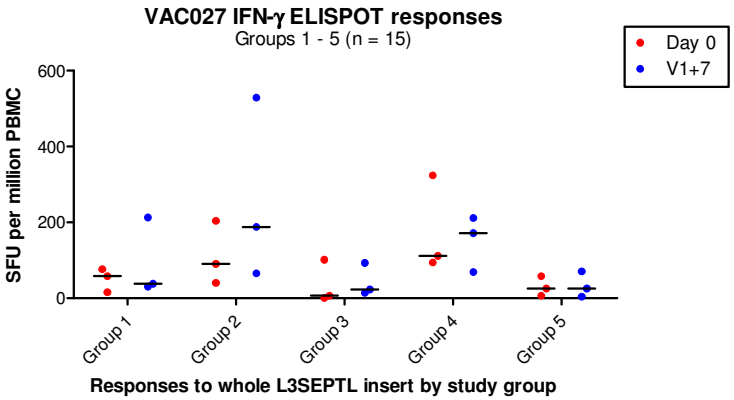

Figure 3.7: IFN-γ ELISPOT responses to whole L3SEPTL insert groups 1–5
Summed ELISPOT responses to whole L3SEPTL insert at baseline (D0) and 7 days after vaccination (V1+7). Horizontal lines show the median response. Responses are summed from multiple peptide pools.

There is no clear relationship between dose of vaccine and the measured immune response in groups 1 to 5. There appears to be a trend towards an increased response following MVA at low or intermediate dose (groups 2 & 4) compared to FP9 at the equivalent doses (groups 1 & 3, see figure 3.7), however the high dose of MVA generates a response equivalent to the low or intermediate doses of FP9.

3.8.3.2 Groups 6 & 7

Figure 3.8 contains graphs showing the immune responses for groups 6 and 7. MVA induces a statistically significant priming response to the whole L3SEPTL insert in group 7 ($p = 0.008$) where FP9 fails to do so in group 6 ($p = 0.68$) when comparing responses at D0 with those at V1+7 (Wilcoxon signed rank test in STATA™). There is no significant rise in responses after the second vaccination ($p = 0.31$ for MVA and $p = 0.67$ for FP9 at V2+7 compared to V1+28). However MVA again induces a significant rise in responses to L3SEPTL at the final (boosting) dose ($p = 0.04$ for MVA in group 6, $p = 0.67$ for FP9 in group 7, comparing V3+7 with V2+7 in each case).

The peak response (that obtained 7 days after the third vaccination) in these two groups was again low. Geometric mean response at V3+7 in group 6 was 100.8 sfu/million PBMCs, and group 7 was 92.0 sfu/million PBMCs.

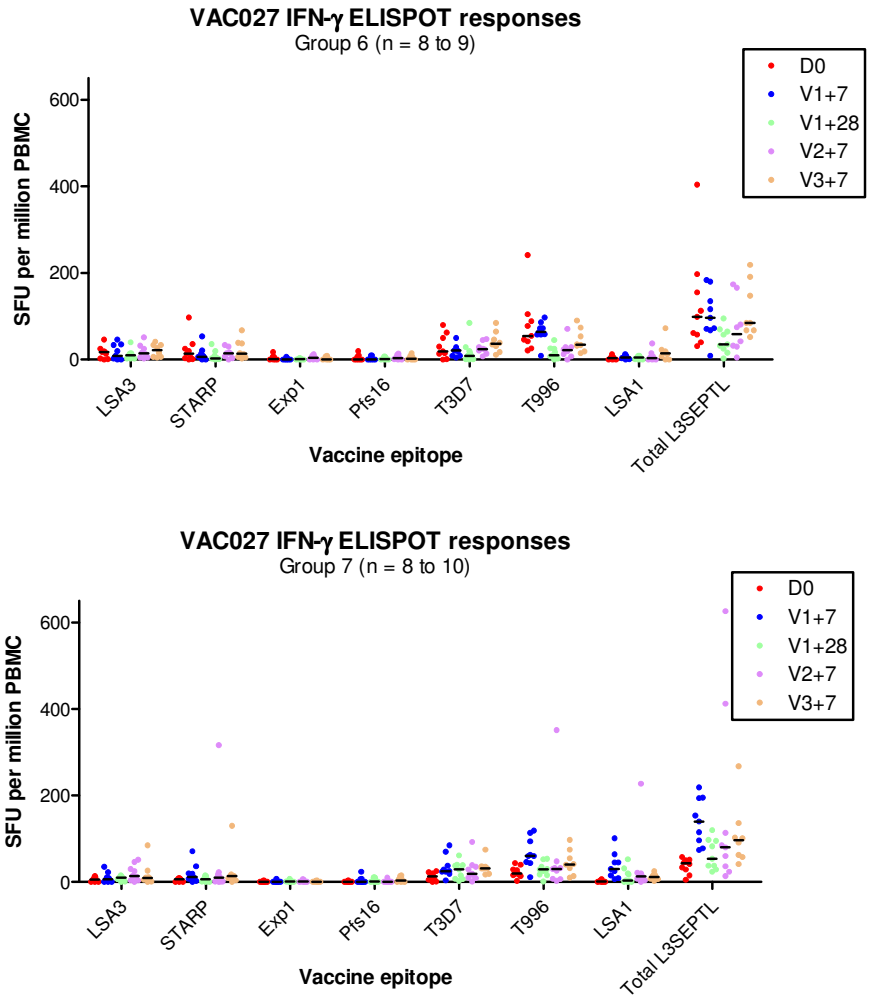

Figure 3.8: IFN-γ ELISPOT responses groups 6 & 7

ELISPOT responses to vaccine epitopes in groups 6 and 7 at baseline and following vaccination. Horizontal lines show the median response. Individual vaccine components are displayed along the x axis and response against the y axis, summed from multiple pools where applicable. The final data column represents the summed responses against the entire vaccine insert. For group 6, n=9 for time points D0, V1+7 and V1+28 but n=8 for time points V2+7 and V3+7. For group 7, n=10 for D0, n=9 for V1+7, V1+28 and V2+7 and n=8 for V3+7.

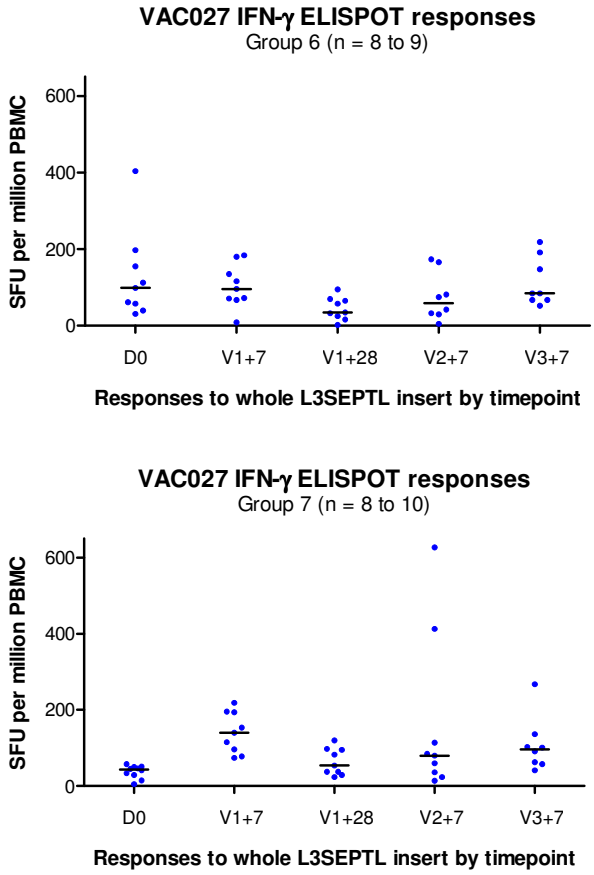

Figure 3.9: IFN-γ ELISPOT responses to whole L3SEPTL insert groups 6 & 7
Summed ELISPOT responses to whole L3SEPTL at baseline (D0) and following vaccination. Horizontal lines show the median response. The data shown represents the summed responses against the entire vaccine insert. For group 6, n=9 for time points D0, V1+7 and V1+28 but n=8 for time points V2+7 and V3+7. For group 7, n=10 for D0, n=9 for V1+7, V1+28 and V2+7 and n=8 for V3+7.

Analysis of responses 7 days after the first vaccine for volunteers in all groups (1 – 7) by epitope shows that levels are significantly above baseline for LSA1 (p = 0.001) but not for any other epitopes or the total L3SEPTL insert (p = 0.053). By the end of the trial (at V3+7), responses to the whole L3SEPTL insert were significantly higher than at baseline in group 7 (p = 0.01) but not in group 6 (p = 0.94). This may in part be due to unexpectedly high baseline responses to the vaccine strain TRAP epitope (T996) in group 6 compared to group 7 (p = 0.003).

The cellular immune response to vaccination was disappointingly poor. There was no clear relationship between vaccine dose and response in the dose-lead in

groups or between vaccine vector and response at prime or boost in the prime-boost groups. Group 7 achieved a modest but significant increase in overall responses by 7 days following final vaccination (p = 0.01 using Wilcoxon signed rank test). Disappointingly, however, group 6 responses at this time point were not significantly different from the relatively high baseline.

3.8.4 Efficacy

Mean time to parasitaemia for controls (n = 6) was 12.8 days (S.D. 1.4 days), compared to 11.6 days for group 6 (n = 8, S.D. 1.5 days), and 12.4 days for group 7 (n = 7, S.D. 1.0 days). A Kaplan Meier plot of survival is shown in figure 3.10. All volunteers developed malaria during the follow up period. There is no significant difference in time to parasitaemia for volunteers in either vaccination group compared to controls (Log rank = 3.2, p = 0.2).

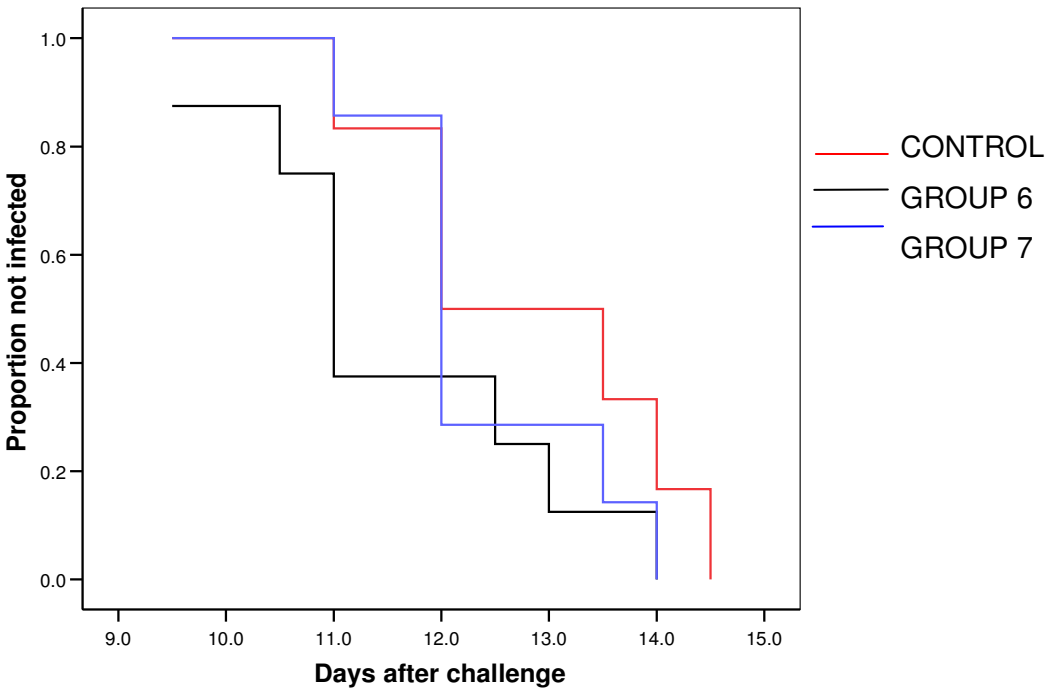

Figure 3.10: Kaplan meier plot

Kaplan Meier plot showing time to diagnosis of malaria after challenge for all subjects by group.

The mean rate of blood stage parasite growth were as follows: in group 6, 7.5 parasites per ml per cycle (95% confidence intervals 3.9 – 11; standard deviation 4.2), for group 7 this was 7.4 parasites per ml per cycle (95% CI 4.1 – 10.7; SD 3.5) and for controls it was 9.1 parasites per ml per cycle (95% CI 7.4 - 10.8; SD 1.6). There was no significant difference in growth rates between different groups of vaccinees, (Group 6 vs. Group 7, Mann Whitney U test, two tailed p = 0.95) or between control volunteers and those who received either vaccine regimen (Group 6 plus Group 7 vs. Controls, Mann Whitney U, two tailed p = 0.19).

As the vaccines used have both a liver stage and a blood stage component, as well as looking at the blood stage parasite growth rates, the numbers of infected hepatocytes are of interest. In group 6, the mean estimate of infected hepatocytes was 146, (95% CI, 60-232, SD 103), for group 7, the mean was 133 (95% CI, -3-270, SD 148), whilst for control volunteers the mean was 109, (95% CI, 33-183, SD 71). There was no significant difference, either between group 6 and group 7 (Mann Whitney U, two tailed p = 0.56), or between all vaccinated volunteers together and controls (Group 6 + Group 7 vs. Controls, Mann Whitney U, two tailed p = 0.76).

3.9 Discussion

This trial examined the safety and immunogenicity of a novel candidate malaria vaccine encoding a synthetic ‘polyprotein’ string of six pre-erythrocytic Plasmodium falciparum antigens [167]. In theory a broad response against multiple parasite epitopes might enhance the immune response to provide immune protection and also reduce the risk of parasite escape mutants arising.

Preclinical work in mice suggested that a heterologous prime-boost immunisation regime employing poxvirus vaccines provided better immunogenicity than a homologous prime-boost regime or a heterologous regime incorporating a DNA vaccine, however it was not clear in which order the poxviruses should be employed as prime and boost. Strong responses were seen in splenocytes after intravenous vaccination when measured by the IFN-γ ELISPOT assay and by assessing the proportion of antigen-specific CD8+ T-cells by tetramer-staining.

The immunogenicity following vaccination in all study groups was disappointing. Previous human trials of poxviruses encoding P. falciparum antigens have produced specific T cell responses of the order of 400 spot-forming units per million PBMC. The more effective prime-boost group in this trial (Group 7) achieved an increase in the summed response to the whole insert of just under 100 spots per million PBMC at the peak time point (V1+7).

The reasons for the poor immune responses generated by this vaccine in humans are not clear. The vaccine had been stored as recommended by the manufacturer and remained infectious when tested prior to use. The intradermal route has proved the most immunogenic in previous trials of poxvirus vaccines encoding malaria antigens in humans, but it is possible that this is not the optimal route for this vaccine in humans. The HLA type of the volunteers participating determines their capacity to generate responses to a specific antigen, however there was a good spread of different class I and class II types in volunteers in this study. In keeping with the low immunogenicity observed in these vaccines, no efficacy was demonstrated in the sporozoite challenge study. All volunteers succumbed to malaria infection by day 14.5 after the challenge.

The clinical safety profile of this vaccine was good. 34 volunteers enrolled and 31 (91%) completed the trial successfully. Only one of these withdrew due to an adverse event, which was judged to be unrelated to either vaccine or challenge. A total of 68 doses of vaccine were administered. Of 701 adverse events recorded, 581 were judged probably or definitely related to vaccination and 568 of these (97.7%) were solicited adverse events. Only two adverse events were classed as ‘severe’ – one related and solicited (swelling around the vaccine site) and one unrelated and unsolicited (a fractured coccyx). Severe adverse events represent approximately 0.3% of the total number, whilst approximately 8% were moderate. All others were classed as mild. No suspected unexpected serious adverse reactions (SUSARs) or serious adverse events (SAEs) occurred during this study.

The safety profile of this vaccine provides further support for the ongoing program to develop effective vaccines against malaria, tuberculosis and HIV employing this

approach and for exploring the use of other recombinant viral vectors to enhance immunogenicity.

Further work is needed to determine whether other viral vectors or other P. falciparum antigens can deliver enhanced immunogenicity. This work is ongoing within the laboratory and clinic. If alternative vectors such as adenoviruses are found to be more immunogenic than the poxviruses used here, the multi-antigen approach used here would be worth reconsidering. However, there are no further plans for use of the polyprotein construct at present.

Chapter 4 VAC030

'Assessment of protection against malaria by sporozoite challenge of healthy adults vaccinated with the virosomal vaccine PEV3A and FP9-MVA ME-TRAP'

4.1 Introduction and Purpose

This trial was the first to evaluate clinically the combined administration of two promising malaria vaccines targeting different life-cycle stages: FP9/MVA ME-TRAP and PEV3A. Recent studies in murine malaria, assessing combinations of an anti-sporozoite vaccine and an anti-liver-stage vaccine [195], suggested that the observed outcome of combining these vaccines may be synergy. In this work, mice were vaccinated with recombinant viral vaccines FP9 and MVA expressing the P. berghei circumsporozoite protein, or a protein vaccine (CV-1866) consisting of hepatitis B core antigen particle containing two copies of the P. berghei CS B cell epitope DPPPPNPN (DP4), or a combination of the two. Mice were then challenged with P. berghei sporozoites to determine the protective efficacy of different vaccine regimens. The protein vaccine CV-1866 induced strong humoral immune responses that protected 12% animals from infection. The viral vectored vaccines, FP9 followed by MVA, induced strong T cell immunity that was partially protective (37% of animals) against the liver-stage of malaria. A mixture of the two vaccine types administered as a combination powerfully induced both types of immunity to the malarial antigen and afforded substantially higher levels of protection (combination; 90%) than either vaccine alone. Analysis of T cells and antibodies induced by the combination of vaccines revealed that both contributed to the enhanced levels of protection conferred by this regime.

For diseases such as malaria in which different potent immune responses are required to protect against different stages, using combinations of partially effective vaccines may offer a more rapid route to achieving deployable levels of efficacy than further development of individual vaccine strategies. This clinical trial was devised to test two currently available vaccine regimens in combination, with the hope that greater efficacy might be achieved by combining humoral and cellular immune responses.

The viral vectors Fowl pox strain FP9 (FP9) and Modified vaccinia Virus Ankara (MVA) expressing the pre-erythrocytic antigen thrombospondin-related adhesion protein (TRAP), fused to a multi-epitope (ME) string, were developed in Oxford. When used in a prime boost regimen in malaria naïve individuals in Oxford, these vaccines induced good CD4 and CD8 T cell responses, and protected two individuals out of five completely from malaria in a sporozoite challenge. This protection persisted in one individual on two further challenges at 14 and 20 months after vaccination [132].

These encouraging data have led to the assessment of FP9-MVA ME-TRAP in a series of phase I studies in adults and in children in Gambia [148] and in Kenya. Good safety and strong T cell immunogenicity led to the initiation of a phase IIb efficacy trial of this vaccine against clinical malaria in 406 Kenyan children in March 2005. These studies have failed to replicate the results seen in Oxford. In both studies, immune responses in semi-immune adults in the Gambia, and particularly in children in Kenya, were lower than those seen in Oxford, and no efficacy against febrile malaria was observed in the Kenyan study of children living in an area of hyper-endemic malaria [150].

The ME string is a multiple epitope string including fourteen CD8+ T cell epitopes, one CD4+ T cell epitope, and two B cell epitopes from six pre-erythrocytic P. falciparum antigens. See table 4.1 for further details. It also contains two non-malarial CD4+ T cell epitopes [145]. The ME string is fused in frame to the entire T9/96 strain of P. falciparum TRAP [127, 193, 196]. Of particular importance for this study, one of the B cell epitopes present in the ME string includes copies of the NANP sequence derived from the circumsporozoite protein, also used in UK39

in PEV3A. In addition, it contains three CD8+ and one CD4+ T cell epitopes also derived from the circumsporozoite protein, but no AMA-1 derived epitopes.

Epitope	Antigen	Type	HLA restriction	
ls8	LSA1	CTL	B35	
cp26	CSP	CTL	B35	
ls6	LSA1	CTL	B53	
tr42/43	TRAP	CTL	B8	
tr39	TRAP	CTL	A2.1	
cp6	CS	CTL	B7	
st8	STARP	CTL	A2.2	
ls50	LSA1	CTL	B17	
pb9	PbCS	CTL	mouse H2	- K d
tr26	TRAP	CTL	A2.1	
ls53	LSA1	CTL	B58	
tr29	TRAP	CTL	A2.2	
NANP	CS	B cell		
TRAP AM	TRAP	Heparin binding		
cp39	CS	CTL	A2.1	
la72	LSA3	CTL	B8	
ex23	Exp1	CTL	B58	
CSP	CS	T helper	Universal epitopes	
BCG	BCG	T helper		
TT	TT	T helper		

Table 4.1: Epitopes included in the ME-TRAP vaccine

PEV3A was developed by Pevion Biotech in collaboration with the Swiss Tropical Institute. This vaccine uses virosomal technology; initially developed as a delivery system for hepatitis A vaccine [197]. Virosomes (immunopotentiating reconstituted influenza virosomes; or IRIVs) are small spherical vesicles, prepared by detergent removal from a mixture of natural and synthetic phospholipids and influenza virus surface glycoproteins. On their surface, they carry the influenza surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). They have been shown to act as an efficient and highly effective means of enhancing the immune response to a variety of antigens [122]. The haemagglutinin membrane glycoprotein of influenza virus plays a key role in the mode of action of virosomes. As a major influenza viral antigen, it is a fusion-inducing component which facilitates antigen delivery to immunocompetent cells. In this vaccine, two synthetic P. falciparum peptide-PE conjugates are incorporated into the virosomes. These are derived from the circumsporozoite protein (CS) and the apical membrane antigen-1 protein (AMA-1) of P. falciparum. The peptide from the CS protein (UK39) is the major B cell epitope NANP in a specific conformation [198]. The

peptide from AMA-1 (AMA-49) mimics the semi-conserved loop I of domain III and has been found capable of inducing antibodies that impairs the growth of P. falciparum blood-stage parasites [199]. These peptides are linked to a phospholipid (phosphatidylethanolamine) and this conjugate is then integrated into the virosomal membrane during the reconstitution process [198, 200]. A key feature of the synthetic peptides used here is that they are cyclised, and therefore they are displayed in a native-like state on the surface of the virosome. The sequences of both peptides used in this vaccine were derived from the K1 isolate of P. falciparum. The two components of this vaccine have been used individually and in combination in a Phase I study in Switzerland previously and found to be safe and immunogenic [201].

4.2 Methods

4.2.1 Objectives

The primary objective for this study was to assess protection against P. falciparum malaria infection following the virosomal vaccine PEV3A alone or in combination with FP9-MVA ME-TRAP vaccination. The secondary objective was to confirm the immunogenicity of PEV3A vaccination alone or in combination with FP9-MVA ME-TRAP with measures of anti parasite immunity. These included assessment of the induced cellular and antibody immune responses. The third objective was to assess safety of the vaccines when administered alone and in combination.

4.3 Outcome Measures

4.3.1 Primary Objective - Efficacy

Protection against malaria infection was the primary objective, and this was assessed using a P. falciparum sporozoite challenge model. To assess the efficacy of the vaccines all vaccinated subjects and six unvaccinated control subjects underwent experimental challenge with Plasmodium falciparum, fourteen days after the final vaccination. The details of the challenge method can be found in Chapter 7 of this thesis, but briefly, laboratory-reared Anopheles stephensi

mosquitoes were infected with the 3D7 strain of P. falciparum parasites in an adapted model [202] as described before [146]. From the evening of day 6 subjects attended clinic twice daily for review of symptoms, vital signs monitoring (pulse, blood pressure and oral temperature) and withdrawal of 3 mL of blood for thick film and PCR analysis for parasite DNA. Field's stain thick films were examined immediately by experienced microscopists for the appearance of viable parasites. A minimum of 200 high power fields were examined before a subject was declared slide negative. Subjects who reached day 15 without blood film evidence of malaria infection were followed up daily until day 21. All subjects were treated immediately with Riamet® (artemether 20mg, lumefantrine 120mg, Novartis) on diagnosis of malaria by the identification of a viable parasite on thick film. Subjects returned to clinic on two consecutive days for negative blood films post treatment. The pre-patent period was defined as the time to diagnosis, and efficacy was assessed by comparing this time period in vaccinated and unvaccinated subjects.

The analysis for the primary objective was based on the number of hours between infectious challenge and blood stage parasitaemia. Each of the groups was compared with the other and with the control group using the Kaplan Meier method. Statistical significance of any differences observed was then assessed by the log rank test.

Blood was taken daily for PCR analysis for parasite DNA. This was performed in real time, although the clinicians assessing the volunteers were blinded to the results. The method used is described in detail in [194]. The results of the PCR analysis were used to estimate parasite growth rates. A description of this method; and the reasons for choosing it over the other available methods for calculating parasite growth rates can be found in Chapter 6 of this thesis. Briefly, it is based on a statistical model of parasite distribution, using a convolution of two probability density functions to estimate the numbers of parasites present in the blood at any time [203]. The model was coded into an Excel ™ spreadsheet, and the solver minimization routine was used to estimate the best solution by minimization of the squared difference between calculated and predicted values. Further analysis of

parasite growth rates was undertaken using Mann Whitney U tests to compare groups.

4.3.2 Secondary Objective - Immunogenicity

The secondary objective was to assess the immunogenicity of the vaccines, when used in this way in combination. In order to quantify this, blood samples were taken from each volunteer at various time points during the study. A variety of methods were used to assess the magnitude and specificity of the induced immune response. Cellular immunity in the form of T cell responses to the vaccines were assessed using IFN-γ ELISPOT. ELISA was used to look for vaccine specific antibodies.

ELISPOT was performed on PBMCs obtained from each volunteer at various time points (described in Section 4.3 Investigational Plan) as reported previously [204]. Anti-UK-39 and anti-AMA49-C1 antibodies were measured by ELISA. Briefly, ELISA polysorp microtiter plates (Nunc, Dr. Grogg, Stetten-Deiswill, Switzerland) were coated at 4℃ overnight with 10 µg/ml AMA49-C1 (for PEV301) or UK-39 (for PEV302) in PBS, pH 7.4. Wells were then blocked with 5% milk powder in PBS for 2 h at 37℃ followed by three washes with PBS containing 0.05% Tween-20. Plates were then incubated with two-fold serial dilutions of human serum starting with 1:50 in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37℃. After washing, the plates were incubated with horseradish-peroxidase-conjugated goat anti-human IgG antibodies (KPL, Socochim, Lausanne, Switzerland) (1:2000 in PBS containing 0.05% Tween-20) for 1 h at 37℃ and then washed. 1, 2-Diaminobezene substrate (OPD) (20 mg/tablet (Fluka, Sigma, Buchs, Switzerland)) in citrate-buffer (4 mg/ml OPD) + 0.01% H_2O_2 was added and incubated at room temperature. After 10 minutes the reaction was stopped by addition of sulphuric acid (final concentration 0.5M (Merck, Darmstadt, Germany)). The optical density (OD) of the reaction product was recorded at 492 nm using a microplate reader (SpectraMax plus, Bucher Biotech, Basel, Switzerland). Titration curves were registered using Softmax PRO software. Endpoint titres were calculated by comparing the ELISA OD of the test serum with the ELISA OD of a

negative serum pool. The endpoint titre is the last serum dilution where the $OD_{test\ sera} \geq 2 \times OD_{negative\ serum}$.

Avidity index (concentration of thiocyanate leading to the dissociation of 50% of the bound IgG in ELISA) was measured by adding serial dilutions of ammonium-thiocyanate after serum incubation (triplicates at halfmax saturation), leading to partial dissociation of bound antibodies [205]. Western blotting with a lysate of P. falciparum (strain NF54) infected A. stephensi salivary glands, or a lysate of in vitro cultivated P. falciparum (strain K1; schizont stage) blood stage parasites, was performed to measure anti-CS protein or anti-AMA-1 IgG seroconversion at a serum dilution of 1:100. Immunofluorescent antibody assays (IFA) were used to measure anti-parasite IgG endpoint titres (defined as last serum dilution where a staining of the parasite is visible). IFA with a suspension of P. falciparum (strain NF54) infected A. stephensi salivary glands and IFA with a suspension of synchronised P. falciparum (strain K1; schizont stage) infected red blood cells, was used to assess anti-sporozoite and anti-blood stage endpoint titres, respectively. Western blotting and IFA are described in more detail in [199] (Okitsu et al., accepted). Positive and negative sera from a Phase 1 trial with PEV301 and PEV302 [123] were used as controls for ELISA, IFA and Western blotting.

4.3.3 Tertiary Objective - Safety

The third objective, safety and tolerability, is descriptive. It was assessed by collection of local and systemic adverse events. Each subject was observed for at least 30 minutes after vaccination, and underwent clinical review 2, 7 and 28 days after each vaccination for reporting of solicited and unsolicited adverse events. Subjects also completed a diary card every day for the first 7 days after each vaccination. Full blood count and biochemistry (urea, electrolytes, alanine aminotransferase, bilirubin, alkaline phosphatase, albumin) was performed at day 0, 7, 28, 35, 56, 63, day of challenge (day 77), challenge + 7 days (day 84), challenge + 35 days (day 112) and challenge + 90 days (day 167).

4.3.4 Methods for Ancillary Analyses – Growth Inhibition Assay

In order to quantify the ability of the induced antibodies to affect or modify parasite growth, Growth Inhibition Assays (GIA) were performed on plasma samples from each volunteer. Samples were shipped to Carole Long at the NIH for parasite Growth Inhibition Assay (GIA) analysis as described elsewhere [206]. Briefly, polyclonal IgG was purified from plasma, adjusted to a concentration of 30.0mg/mL in incomplete RPMI 1640, and tested for biological activity against both the 3D7 and FVO strains of Plasmodium falciparum. Both strains are heterologous to those used in the construction of the vaccines – T9/96 in the ME TRAP construct, and K1 for PEV3A. A standardized GIA assay with samples from all volunteers on the day of challenge was performed and was compared with activity in samples from day 0.

4.3.5 Methods for Ancillary Analyses – Parasite Sequencing

One volunteer, M1030525, remained undiagnosed until day 20 after the challenge and then succumbed to malaria infection. The possibility that this could be an example of parasite immune escape was considered. Vaccination induced mutations in another Plasmodium antigen have been described in monkeys, after relatively short periods of infection [207]. Parasite DNA was extracted from a blood sample from volunteer 525 on day 20 as described [194]. Samples were prepared using the following method.

Leukocytes were removed from the blood by filtration. Samples were centrifuged (5 minutes, 2,500 rpm, Beckman GS-6K centrifuge, Beckman Coulter, Inc., Fullerton, CA) and the sample volume marked on each tube. Plasma was removed and the cells were then resuspended with 1 mL sterile PBS and mixed by inversion. The 24-well filter plate containing two layers of a glass-fibre based material (Whatman International, Clifton, NJ; product code 7700-9902) was positioned above a 24-well collection plate in a UniVac3 vacuum manifold (both from Whatman International) and samples added to each well. The vacutainer tubes were washed with 1 mL PBS, and this was poured into the corresponding sample well before drawing the sample through the filter by vacuum. Vacutainer

tubes were washed again with 1 mL PBS and blood solutions filtered in the appropriate well. Filter wells were washed with 0.5 mL PBS, and this was filtered through. Samples were transferred back to their original vacutainer tubes, centrifuged (5 minutes, 2,500 rpm, (Beckmann GS-6K), and returned to the original blood sample volumes by removing excess supernatant. The blood samples were mixed by inversion, 0.5 mL was taken for immediate DNA extraction, and the remainder stored at –80°C.

DNA extraction was then performed using the QIAamp DNA Blood Mini kit (QIAGEN, Crawley, UK) with some modifications to the standard protocol. The sample volume was 0.5 mL filtered blood. Volumes of protease, lysis buffer, and ethanol were 40 µL, 400 µL, and 400 µL, respectively, and wash buffer volumes increased to 750 µL. The second wash buffer allowed to soak on the DNA purification columns for 2 minutes before the vacuum was applied. Columns were transferred to collection tubes and centrifuged at 13,000 rpm for 4 minutes. DNA was eluted with 50 µL sterile 10 mM Tris, pH 8.0, incubating for 1 minute at room temperature before centrifugation (1 minute, 8,000 rpm) to collect the DNA sample.

Primers were designed using primer design software (PrimerSelect, part of LaserGene from DNAStar, Madison, Wisconsin, USA) to produce a PCR product of 517 base pairs, from the AMA-1 gene of P. falciparum, 3D7 strain, from 998 – 1515 bp. This sequence includes the region coding for the AMA-1 peptide in PEV3A.

Primers
1: 517 bp product including PEV3A peptide
Upper primer: 21 mer 5’ GAGTGCTTCGGATCAACCTAA 3’
Lower primer: 24mer 5’ TACTTCTGCCCTTCTTTCTACACA 3’

After optimization experiments, the PCR conditions were selected, and the Expand High Fidelity PCR system (Roche Applied Science) was chosen to produce PCR fragments for cloning. This system contains proof reading polymerase enzymes to correct transcriptional errors, minimizing the chance that any changes in sequence

detected were as a result of the PCR itself. Two mixes were prepared containing the following components (volumes given are for one PCR reaction).

	Volume (µL)
PCR grade water	17.25
Nucleotide mix	1.25
Forward primer (10 µM)	0.75
Reverse primer (10 µM)	0.75
Final reaction volume	20

Table 4.2: PCR mix 1

	Volume (µL)
PCR grade Water	16.25
$MgCl_2$ (25 µM stock)	2.5
Buffer	5
Expand enzyme	0.75
Final reaction volume	25

Table 4.3: PCR mix 2

One volume of each of mix 1 and 2 was added to each well for PCR reaction, and 5 µL of template DNA was added. A positive control of a sample of 3D7 parasite DNA was included, and a negative control of water instead of sample DNA. Each reaction was overlaid with 25 µL mineral oil (Sigma Aldrich) to prevent evaporation. The PCR reactions were carried out on a Tetrad thermocycler (MJ Scientific) in 96 well microtitre plates. The PCR programme used was 2 minutes at 94°C, followed by 35 cycles of 15 seconds at 94°C, 30 seconds at the specific annealing temperature (54°C) and 1 minute at 72°C.

Agarose gel electrophoresis confirmed the bands were of the correct size. The band was cut from the gel, and DNA was isolated from using a MinElute Gel Extraction kit (Qiagen, Crawley, West Sussex, UK). Briefly, the gel section is placed in a 1.7 mL tube containing 750 µL solubilisation buffer, and heated in a

water bath at 50℃ until the gel had dissolved completely. The solution is then added to a MinElute column, and centrifuged at 13,000 rpm for one minute, the flow through is discarded. Any remaining solution is added to the column and again spun through. The column is then washed with 750 µL PE wash buffer, and again centrifuged at 13,000 rpm for 1 minute. This spin is repeated to ensure all wash buffer is removed from the column. To elute the PCR fragment, 10 µL of water is then added to the column, and left to soak for 1 minute before another spin at 13000 rpm for 1 minute.

The PCR fragments were then cloned using the pGEM-T Easy vector system (Promega) The following mix was prepared on ice, and then left at room temperature for two hours to ligate.

	Volume (µL)
Purified PCR product	2
Water	5
Buffer	1
DNA Ligase	1
pGEM-T Easy vector	1
Final volume	10

Table 4.4: Cloning mix

LB agar plates containing Ampicillin (100µg/mL), X-gal in DMF (60µg/mL) and 0.1mM isopropyl-β-D-thiogalactopyranoside (IPTG) dissolved in 50% EtOH (Sigma Aldrich) were prepared. The ligated vector was transformed into MAX efficiency® DH5α™ chemically competent Escherichia coli cells (Invitrogen Ltd.) by adding 10µL ligation mix to 100µL cells and incubating on ice for 30 minutes for the vector to bind to the surface. The cells were then placed in a water bath at 42℃ for 45 seconds to heat-shock the vector into the cells. The samples were placed immediately back on ice for 10 minutes before plating 20mL volumes onto the agar plates and incubating overnight at 37℃.

White colonies were picked from these plates and then purified using a QIAGEN Plasmid Mini kit (Qiagen Crawley, West Sussex, UK). Six colonies were selected and each was inoculated into a starter culture of 5mL LB medium containing Ampicillin (100µg/mL). These were incubated at 37℃ with vigorous shaking for 16 hours. Bacterial cells were harvested from 3 mL for each colony by centrifugation at 13200 rpm for 10 minutes. The bacterial pellet was then resuspended in 150 µL P1 buffer by vortexing. 300 µL P2 buffer was then added and mixed vigorously, samples were then incubated for 5 minutes at room temperature. 300 µL P3 buffer is then added to each tube, and again vigorously mixed, and incubated immediately on ice for 5 minutes. Samples were then centrifuged at 13200 rpm for 10 minutes, the supernatant was then added to a pre-equilibrated Qiagen-Tip 20 column, and allowed to drip through by gravity. Columns were washed with Buffer QC, and the DNA was then eluted from the column with 0.8mL of Buffer QF. DNA was then precipitated with 0.56mL of isopropanol and centrifuged at 13000 rpm for 30minutes. Pellets were washed with 1mL 70% Ethanol and left to air dry.

Restriction digests were then performed to confirm the plasmid identity (to check all selected clones contain an inserted sequence of the correct size). The restriction enzyme EcoRI was used (New England Biolabs) and a mix prepared as follows:

	Volume (µL)
EcoRI	1
Restriction Buffer	1
Sterile water	11
DNA	7
Final volume	20

Table 4.5: Restriction digestion mix

The digestion mix was incubated at 37℃ for 2 hours, Loading dye was added to the digestion and samples were run on agarose gel to visualise the inserted sequence. Five out of six clones contained the inserted sequence, and the DNA was ethanol precipitated. DNA pellets were left to air dry, and were then sent to

MWG-Biotech (Ebersberg, Germany) for sequencing with universal M13 forward and reverse primers.

Subsequently, primers were also designed to cover the upstream region, including the promoter of AMA-1.

2: 762 bp product
Upper primer: 22mer 5' TGCTTTTCCTCCAACAGAACCT 3'
Lower primer: 22mer 5' ATGGGATGGGACAAAGCAGTAG 3'

3: 656bp product
Upper primer: 26mer 5' CGTATTATTATTGAGCGCCTTTGAGT 3'
Lower primer: 25mer 5' TCATATTTCCTGCATGTCTTGAACA 3'

4: 636bp product
Upper primer: 31mer 5' GTGCTTCTTTTTTATTTCACTTTTGTTAGAG 3'
Lower primer: 28 mer 5' ATGCGTGTTGTAATGTATTTTCGTCTTC 3'

These primers were used with both the Expand PCR high fidelity system and the Phusion High Fidelity DNA polymerase system (New England Biolabs, Ipswich, Massachusetts, USA). Multiple conditions and PCR programmes were tried with each system, it was not possible to amplify any PCR product from the sample from M1030525 using these primers. The positive controls worked, indicating that the primers themselves were binding to parasite DNA, however it is possible that there was insufficient good quality DNA remaining in the sample by this stage.

4.3.6 Methods for Ancillary Analyses – Crisis Forms

During the challenge phase of the study, microscopists detected the presence of morphologically abnormal parasites in films from volunteers pre-diagnosis. These differ from normal parasites in the staining of the nuclear material. While live parasites have nuclei that stain red/blue, these abnormal parasites stained only

blue (see figure 4.1). We hypothesised that these were ‘crisis forms’ and that they might be an effect of vaccine-induced blood stage immunity.

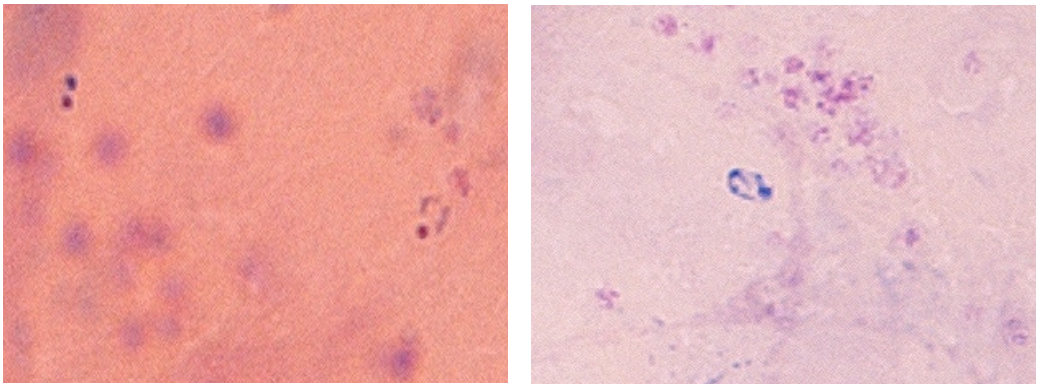

Figure 4.1: Crisis forms
Morphologically normal parasite (left) and Crisis form (right). Note the difference in the parasite nuclear staining.

Crisis forms were first described in 1944 by Taliaferro, WH and Taliaferro LG [208]. They are parasites whose intra-erythrocytic development has been retarded, resulting in morphologically abnormal parasites. These were initially found in Plasmodium brasilianum infection of Cebus monkeys undergoing a severe immunologic crisis. The crisis parasites appeared within erythrocytes at the same time as a rapid decrease in parasitaemia. They have since been described in mouse [209] and human malarias [210]. A study by Jensen et al. [211] demonstrated induction of crisis forms in cultured P. falciparum with human immune serum from Sudan. Intra-erythrocytic development was retarded, leading to the formation of crisis forms and parasite deterioration to some extent, by all sera that were tested in this study. They proposed two models to explain this intra-erythrocytic, anti-parasitic action: 1) the presence in immune sera of a toxic cytokine, crisis form factor, 2) crisis forms result from oxidative stress generated during respiratory bursts associated with phagocyte activation, or indirectly by toxic products of lipid peroxidation produced by reactive oxygen species associated with phagocyte respiratory bursts. It has subsequently been shown that TNF alpha (a prime candidate for ‘crisis form factor’) does not induce crisis forms in cultured Plasmodium falciparum [212]. The exact mechanism for crisis form

induction remains unknown; however their presence indicates an immunological intervention by the host in the course of infection. Thus, in these malaria naïve volunteers, they may represent evidence of blood stage immunity. Historically, these abnormal parasites have never been observed in studies at this centre before.

In order to examine this phenomenon in more detail, blood films from volunteers taking part in this study were re-examined. Six slides were identified from each volunteer, the slide from the time of diagnosis, and those from the 5 time points preceding diagnosis. Films were also selected from some historical studies for the same analysis. Challenge studies VAC021.2 and VAC023.2 were chosen; and 6 volunteers from each study – 3 vaccinees and 3 controls were included. The same 6 slides were identified for each volunteer – the slide from the time of diagnosis, and those from the 5 time points preceding diagnosis.

Each film was re-examined by one of the microscopists involved in the study, who were blinded to the group allocation of the volunteers. The whole film (1000 high power fields) from each time point was re-examined, and the number of normal and abnormal parasites was recorded.

4.4 Investigational Plan

4.4.1 Trial Plan

Flow charts showing the schedules of visits and procedures to be performed at each visit follow. The first chart shows the procedures for the vaccination phase, whist the second shows the challenge phase.

Attendance number	1	2	3	4	5	6	7	8	9	10	11
Timeline (days)*	S	D0	D2	D7	D28	D30	D35	D56	D58	D63	D77
Time windows (days)			1	3	7	1	3	+28/ -14†	1	3	10
Vaccinations (groups 1 AND 2) (P=PEV3A)		P			P			P			
Vaccinations (group 2 ONLY) (F=FP9, M=MVA)		F			F			M			
Attendance ref.	S	P1	P1A	P1B	P2	P2A	P2B	P3	P3A	P3B	P3C
Inclusion/Exclusion criteria	X										
Informed consent	X										
Medical history	X										
Phys. exam.	X										
Urinalysis	X										
b-HCG urine test	X	X			X			X			
Review contra-indications	X	X			X			X			
Post-dose follow-up (days)			2	7	28	2	7	28	2	7	21
Vital signs	X	X	X	X	X	X	X	X	X	X	X
Local & systemic events/reactions		X	X	X	X	X	X	X	X	X	X
Diary cards provided		1			2			3			
Diary cards collected				1			2			3	
HLA typing (mL)		3									
HBV, HCV, HIV (mL)	5										
Biochemistry (mL)	4			4	4		4	4		4	4
Haematology (mL)	2			2	2		2	2		2	2
Antibody assays (mL)		10			10			10			10
Gene expression (mL)		10								10	10
Exploratory immunology (incl ELISPOT) (mL)		50		50	50		50	50		50	50
Blood volume (mL)	11	73		56	66		56	66		66	76
Cumulative blood volume (mL)	11	84		140	206		262	328		394	470

Figure 4.2: Flow chart, vaccinations

Challenge attendance no.	1	2†	3	4	5 – 19	20 – 28	29	30
Trial timeline (days)*	D77	D77	D83.5	D84	D84.5 – D91	D92 – D100	D112	D167
Time window (days)	7	7					10	14
Attendance reference		C	C+6.5	C+7	C+7 – C+14.5	C+15 – C+23	C+35	C+90
Malaria challenge		X						
Physical examination								
b-HCG urine test		X						X
Review contraindications		X						
Vital signs	X		X	X	X	X	X	X
Local & systemic events/reactions	X		X	X	X	X	X	X
Thick smear / PCR (mL)		2	2	2	2 × 15	2 × 9		
Biochemistry (mL)	4						4	4
Haematology (mL)	2						2	2
Antibody assays (mL)	10							10
Gene expression (mL)	10			10				10
Exploratory immunology (incl ELISPOT) (mL)	50	50†		50			50	50
Blood sampling (mL)	76	52	2	62	30	18	56	76
Cumulative blood taken – groups 1 & 2 (mL)	470	522	524	586	616	634	690	766
Cumulative blood taken – group 3 (mL)	488	540	542	604	634	652	708	784

Figure 4.3: Flow chart, challenge phase

4.4.2 Trial Design

This was a phase IIa prospective open partially-randomised clinical trial of a combination malaria vaccination regimen using 2 different vaccine regimens. This included 2 main study groups, receiving either the PEV3A vaccine alone, or the PEV3A vaccine in combination with the FFM ME-TRAP regimen. All vaccinations were given 4 weeks apart, and all volunteers were challenged with malaria 2 weeks after the final vaccination. Figure 4.4 represents the trial layout.

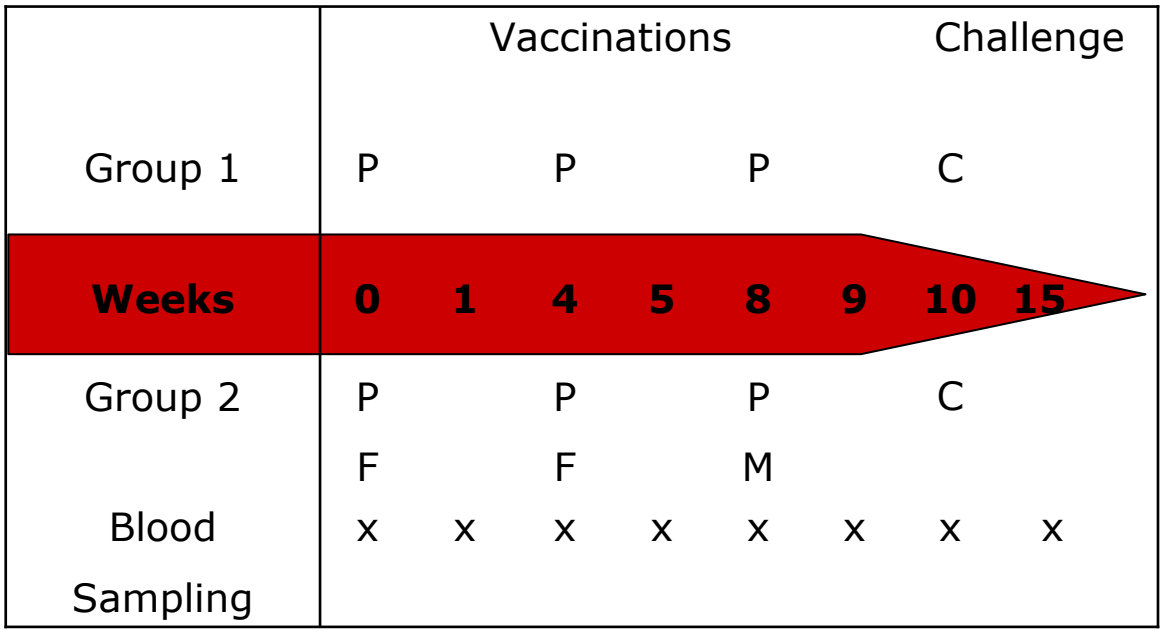

Figure 4.4: Vaccination and challenge plan

4.4.3 Trial Calendar

First visit first volunteer:	22nd August 2005
Last visit last volunteer:	22nd February 2006
Last vaccine given:	9th November 2005
Challenge dates:	22nd & 23rd November 2005

4.5 Vaccines

4.5.1 Formulation of FP9 and MVA ME-TRAP

FP9 and MVA ME-TRAP were developed by Oxford University; and manufactured by a contract manufacturer (IDT, Germany). MVA and FP9 ME-TRAP were stored at –20°C and allowed to thaw prior to administration. The batch of FP9 ME-TRAP used in this study was 05 12 04, which was provided in vials of 225 µL volume at a concentration of 1.8×10^9 pfu/mL in 10 mM Tris buffer. The batch of MVA ME-TRAP used was 05 12 04, which was provided in vials of 300 µL volume at a concentration of 5×10^8 pfu/mL in 10 mM Tris buffer.

The potency of these vaccines was tested prior to the trial in a standardised assay on Balb/c mice, 14 days following administration of the vaccine; peptide specific ELISPOT responses were measured in splenic lymphocytes. Both vaccines were tested and found to be potent.

4.5.2 Formulation of PEV3A

PEV3A was manufactured by Pevion Biotech Ltd., Switzerland, and provided in vials of 0.5 mL containing 50 µg of PEV301 and 10 µg of PEV302 in phosphate buffered saline (pH 7.4) and stored at +2 to +8 °C. The batch of PEV3A used was 05PEV01.

4.5.3 Selection of Doses

The dose of FP9 ME-TRAP used in this study was 1.0×10^8 pfu, given intradermally in a volume of 60 µL. All doses of FP9 ME-TRAP were given into the right arm. The dose of MVA ME-TRAP used in this study was 1.5×10^8 pfu, given intradermally in a volume of 300 µL. All doses of MVA ME-TRAP were given into the right arm. These doses are the same as those used in previous studies using these vaccines, when they have been found to be safe and immunogenic.

The dose of PEV3A used in this study was 0.5 mL (PEV301 containing 50 µg AMA49, PEV302 containing 10µg UK39), given intramuscularly. All doses of PEV3A were administered into the left arm. This dose was selected following a preliminary phase I study, [123] in which it was shown to be most immunogenic for both antigens.

4.6 Selection of the Trial Population

4.6.1 Inclusion Criteria

Healthy adults aged 18 to 50 years
Written informed consent
Resident in or near Oxford for the duration of the vaccination study
For women only, willingness to practice continuous effective contraception during the study and (if participating) during the subsequent challenge study.
Agreement to refrain from blood donation during the course of the study
Willingness to undergo an HIV test

4.6.2 Exclusion Criteria

Any deviation from the normal range in biochemistry or haematology blood tests or in urine analysis as defined in Appendix A of the protocol
Prior receipt of an investigational malaria vaccine
Use of any investigational or non-registered drug, vaccine or medical device other than the study vaccine within 30 days preceding dosing of study vaccine, or planned use during the study period
Administration of chronic (defined as more than 14 days) immunosuppressive drugs or other immune modifying drugs within six months of vaccination. (For corticosteroids, this will mean prednisolone, or equivalent, ≥ 0.5 mg/kg/day. Inhaled and topical steroids are allowed.)
History of malaria chemoprophylaxis with chloroquine within 5 months prior to the planned challenge, with Lariam within 6 weeks prior to the challenge, and Riamet® within 2 weeks prior to the challenge
Any history of malaria

Travel to a malaria endemic area within the previous 6 months
Planned travel to malarious areas during the study period
Any confirmed or suspected immunosuppressive or immunodeficient condition, including human immunodeficiency virus (HIV) infection and asplenia
History of allergic disease or reactions likely to be exacerbated by any component of the vaccine, e.g. egg products
History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ)
History of haemoglobinopathies: Sickle cell disease, thalassaemia, G6PD deficiency
History of diabetes mellitus
Chronic or active neurological disease including seizures
History of > 2 hospitalisations for invasive bacterial infections (pneumonia, meningitis)
Suspected or known current alcohol abuse as defined by an alcohol intake of greater than 42 units every week
Seropositive for hepatitis B surface antigen (HBsAg) or hepatitis C virus (antibodies to HCV)
Hepatomegaly, right upper quadrant abdominal pain or tenderness
Evidence of serious psychiatric condition
Any on-going chronic illness requiring hospital specialist supervision
Administration of immunoglobulins and/or any blood products within the three months preceding the planned administration of the vaccine candidate
Pregnant or lactating woman
Any woman who is willing or intends to become pregnant during the study
Any history of anaphylaxis in reaction to vaccination
Principal Investigator assessment of lack of willingness to participate and comply with all requirements of the protocol
History or clinical evidence of intravenous drug abuse
Any other finding which in the opinion of the Investigator would significantly increase the risk of having an adverse outcome from participating in this protocol.

4.7 Statistical Analysis

4.7.1 Sample Size

This study aimed to provide both an initial estimate of the efficacy of the vaccines used in combination and to compare this with the single vaccine PEV3A. Prior to this trial, it was difficult to estimate the potential size of any beneficial effect of PEV3A. The analysis of overall efficacy in the 24 vaccinated volunteers had 80% power to detect a significant difference from the six controls in rate of sterile protection if 60% efficacy was achieved. Volunteers were allocated to groups by the investigators. Neither volunteers nor the investigators performing the study were blinded to the intervention given.

4.7.2 Statistical Methods

The analysis for the primary objective was based on the number of hours between infectious challenge and blood stage parasitaemia. Each of the groups was compared with the other and with the control group using the Kaplan Meier method. Statistical significance of any differences observed was then assessed by the log rank test.

Immunogenicity was assessed by comparing the sum of the interferon-γ ELISPOT responses to malaria peptides, the geometric mean and 95% confidence intervals of the antibody titres determined by ELISA and IFA in the two different vaccination groups. The statistical significance of the effect of combined vaccine delivery was determined using a Mann-Whitney U test to compare ELISA and IFA titres between the two vaccination groups. The impact of co-administration of vaccines on antibody avidity was calculated with a two-tailed unpaired t test comparing avidities after first, second, and third immunization. The statistical significance of the difference in number of responders after three vaccinations in the two groups was calculated with a Fisher's exact test. To assess the statistical significance of titre increases (ELISA, IFA) after sporozoite challenge we used a Wilcoxon signed-rank test to compare titres before and after sporozoite challenge. Results were analysed for correlation to any delay in parasitaemia. All data, including adverse

event data collected on StudyBuilder, was imported into and analysed using Microsoft Excel or SPSS statistics packages. All statistical analyses and graphs of ELISA, IFA and avidity results were made using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA.

4.8 Results

Healthy malaria naïve adult subjects aged 18 – 50 years were recruited in the Oxford area and underwent medical screening as previously described [133]. All volunteers provided fully informed consent to participate in this study by signing a written consent form, prior to any study procedures. All vaccinations and follow up visits took place in the outpatients unit at the Centre for Clinical Vaccinology and Tropical Medicine, at the Churchill Hospital in Oxford. The malaria challenge was performed in the insectary in the Alexander Fleming Building, Imperial College, London. The study received ethical approval from the Oxfordshire Research Ethics Committee A on 10/6/2005, (COREC reference 05/Q1604/69). The study was performed in compliance with the requirements of the MHRA under a Clinical Trial Authorisation. It gained full regulatory approval from the MHRA on 23/5/2005, Eudra CT number 2005-001041-42. An Independent Local Safety Monitor was appointed in Oxford. The trial was conducted according to GCP and in accordance with the current version of the declaration of Helsinki (52nd WMA General Assembly, Edinburgh, Scotland, October 2000). It was externally monitored by Appledown Clinical Research Ltd.

Recruitment for this study began in August 2005, and the final visit of the final subject to the study site took place on 22nd February 2006. In total, 44 subjects were screened, of whom 13 were excluded, 7 because they were ineligible (previous history of intravenous drug use (IVDU), history of psychiatric illness, a new finding of heart murmur, recent travel to endemic areas), and 6 withdrew consent after screening. 30 volunteers were initially enrolled into the study. One of those enrolled into group 2 had an undisclosed history of IVDU; this subject was withdrawn as soon as this was revealed to the investigator, shortly after the first vaccination. Data concerning the safety of one dose of vaccine was collected from this volunteer, but they were otherwise excluded from the analysis. One extra

volunteer who had been screened but initially not enrolled was then allocated to group 2 to replace this subject. In total, 24 volunteers received all three vaccinations in groups 1 and 2. Demographic characteristics of volunteers in each group are provided in Table 4.6. All of these volunteers subsequently took part in the malaria challenge, and completed all follow up. 6 of the enrolled volunteers were recruited to act as unvaccinated controls for the challenge phase. One of these subjects was withdrawn from the challenge early and treated; this was a result of an unforeseen change in personal circumstances unrelated to the trial. This volunteer subsequently completed all follow up. These two volunteers accounted for the two protocol deviations that were recorded during the trial. The participant flow is shown in a CONSORT diagram in figure 4.5.

Group	N	Mean AGE (SD)	Min AGE	Max AGE	No. FEMALE (%)	No. MALE (%)
Group 1	12	25.8 (4.3)	21	33	6 (50)	6 (50)
Group 2	13	29.5 (5.3)	23	44	5 (38)	8 (62)
Controls	6	30 (7.5)	23	40	3 (50)	3 (50)
TOTAL COHORT	31		21	44	14 (45)	17 (54)

Table 4.6: Demographics

Demographic characteristics of volunteers by group

24 volunteers were enrolled into the study, with an additional 6 unvaccinated controls for the malaria challenge. Group 1 volunteers received three doses of PEV3A 0.5mL (P) given intramuscularly at baseline, 4 weeks later and 8 weeks, while those in Group 2 received a combination of PEV3A 0.5mL given intramuscularly (P) and FP9 ME-TRAP 1 x 10^8 plaque forming units (pfu) given intradermally (F), at baseline; (P) and (F) 4 weeks later; and (P) and MVA ME-TRAP 1.5 x 10^8 pfu given intradermally (M) at 8 weeks. Intramuscular injections were given into the left deltoid; intradermal injections were administered into the skin over the right deltoid. Up to 80 mL of blood was drawn at day 0, 7, 28, 35, 56, 63, day of challenge (day 70), challenge + 7 days (day 77), challenge + 35 days (day 105) and challenge + 90 days (day 160) for safety assessment and measurement of immunogenicity.

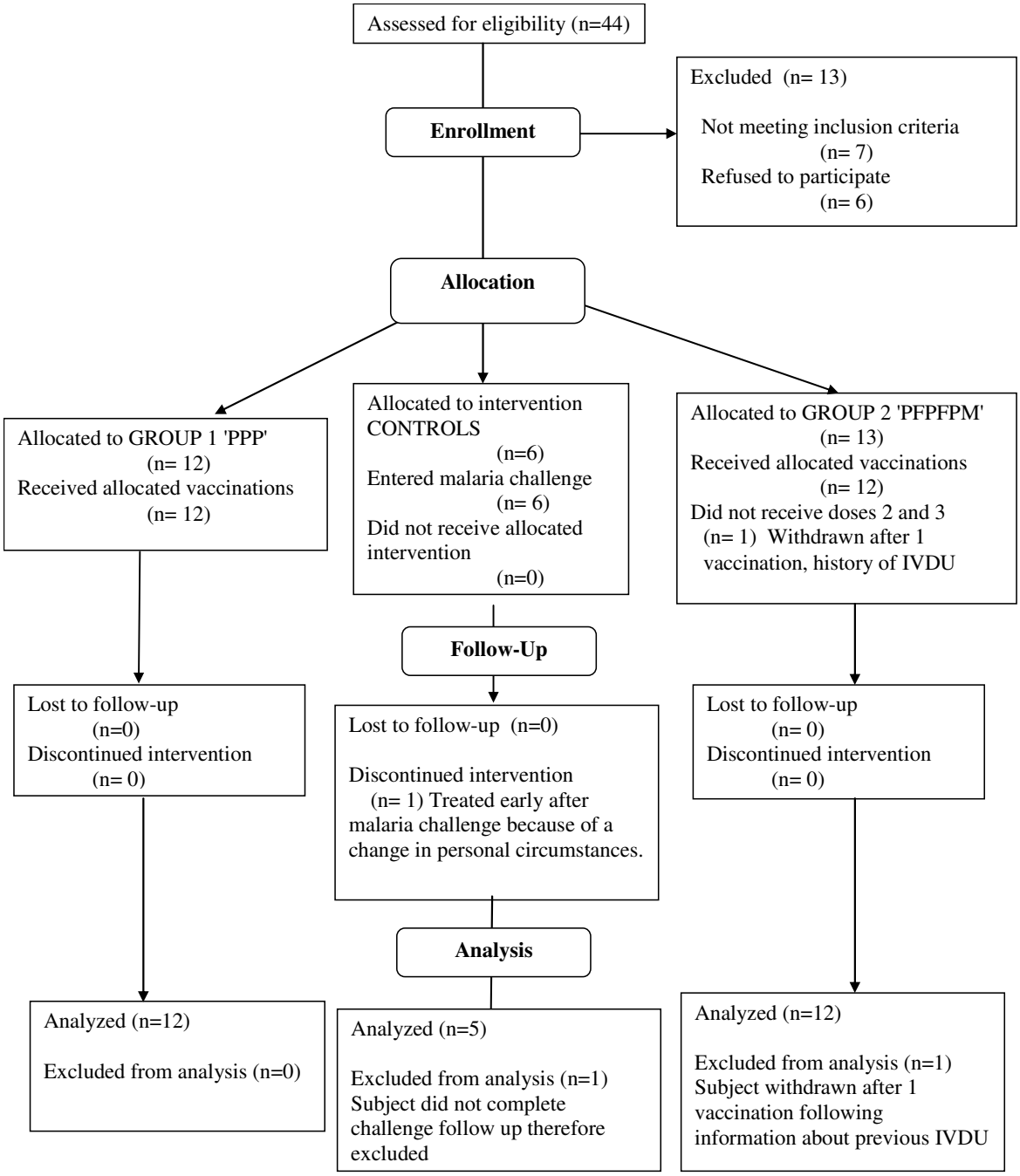

Figure 4.5: CONSORT flow chart

CONSORT flow chart demonstrating participant flow through VAC030.

4.8.1 Efficacy

Mean time to parasitaemia for controls (n = 5) was 11.8 days (S.D. 1.6 days), compared to 12.8 days for group 1 (n = 12, S.D. 2.68 days), and 12.1 days for group 2 (n = 12, (S.D. 0.96 days). A Kaplan Meier plot of survival is shown in figure 4.6. One volunteer in group 1 was not diagnosed until day 20, however, there is no significant difference in time to parasitaemia for volunteers in either vaccination group compared to controls (Log rank = 0.87, p = 0.65).

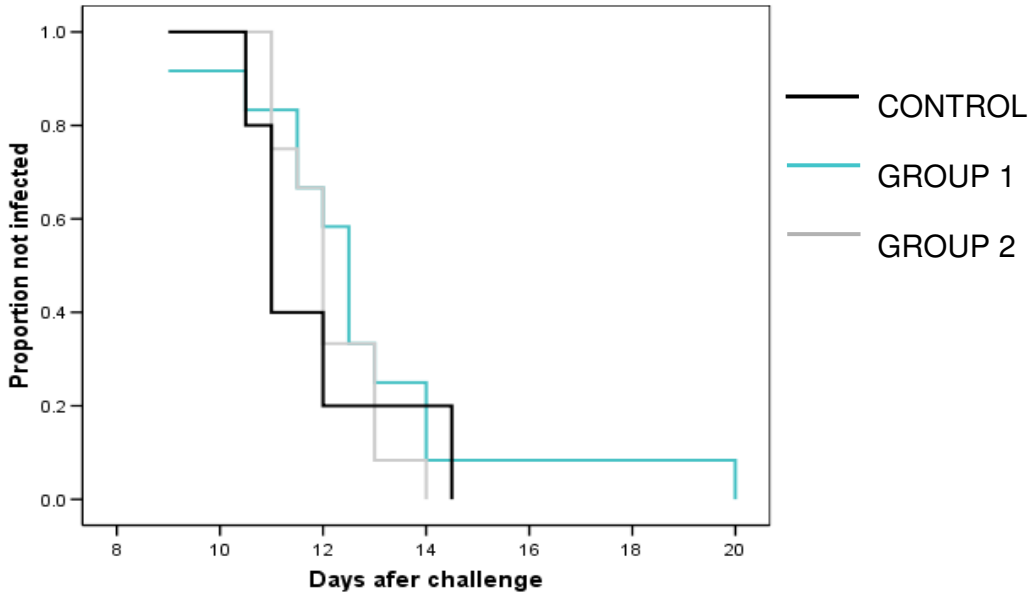

Figure 4.6: Kaplan meier plot

A survival plot showing time to diagnosis of malaria infection for each group of volunteers.

Although no sterile protection was seen following the sporozoite challenge, we observed some evidence of blood stage protection for the first time in humans in a challenge study. Examples of estimated parasite densities based on PCR data are shown in figures 4.7 and 4.8. Most volunteers showed an exponential increase in parasite densities starting at day 7 after challenge (Figure 4.7). However, a number of subjects (525 and 529 in group 1 and 513 and 532 in group 2), had unusual PCR results (Figure 4.8). In these volunteers PCR for parasite DNA was positive at low levels known to be undetectable by microscopy and then became

negative before becoming positive again, at least once, before rising up to the point of diagnosis.

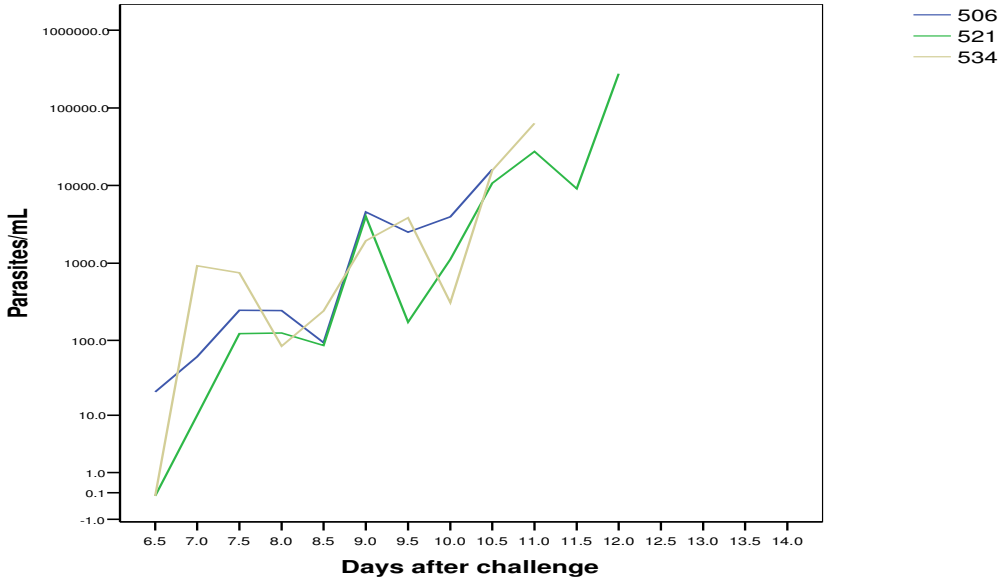

Figure 4.7: PCR results

Three volunteers selected to demonstrate expected pattern of PCR results: an exponential increase in the numbers of parasites over time, with some cycling seen as parasites are sequestered and released.

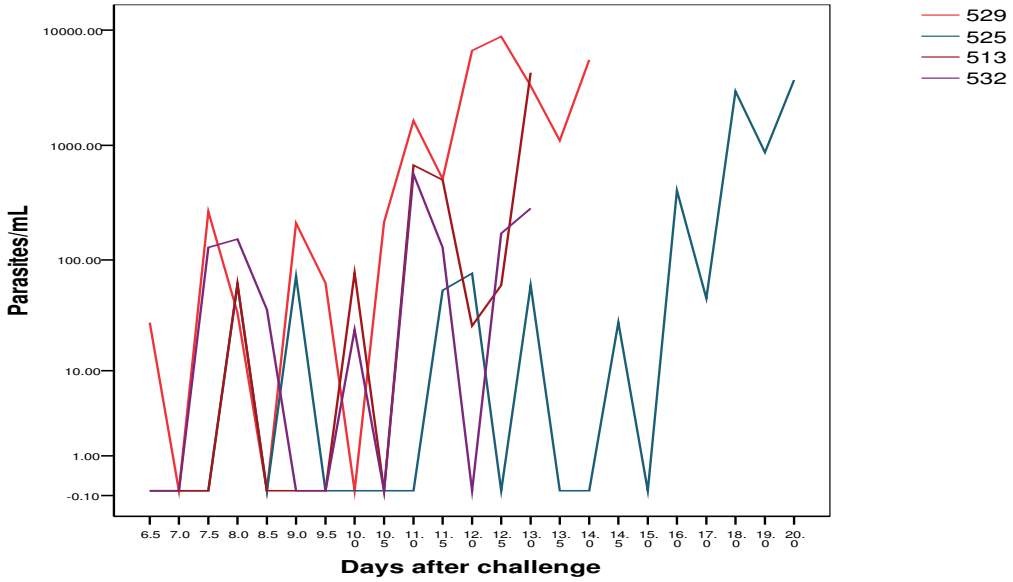

Figure 4.8: PCR results

This figure shows data from all 4 volunteers who had unusual PCR results. Up to 150 parasites/mL are detected, and are subsequently undetectable for one or more time points, before they are measured again. This pattern occurs several times, up to a maximum of 5 times in volunteer 525.

Parasite growth rates were calculated. The mean rate of blood stage parasite growth in volunteers in group 1 was 5.7 parasites per ml per cycle (95% confidence intervals 4.1 - 7.3; standard deviation 2.6), for group 2 was 6.3 (95% CI 4.0 - 8.5; SD 3.5) and for controls it was 8.7 (95% CI 7.2 - 10.2; SD 1.2). Comparing the growth rates of vaccinated volunteers, those in group 1 are not significantly different to those of volunteers in group 2 (Mann Whitney U test; two tailed p=0.63). Comparing group 1 to controls, growth rates are significantly lower in group 1 volunteers than in controls (Mann Whitney U; two tailed p = 0.02). For group 2 versus controls, there is again a significant difference (Mann Whitney U; two tailed p = 0.02).
Grouping all vaccinated volunteers together, and comparing them to controls, there is again a significant reduction in parasite growth rates; (Mann Whitney U; two tailed p = 0.012). More detailed analysis of these results can be found in Chapter 6 of this thesis.

Compared to the control group the estimated number of infected hepatocytes was 1.8 times lower in the PEV3A group and 2.3 times lower in the combination group respectively. Statistical analysis of these estimates showed no significant difference between group 1 and group 2 (Mann Whitney U test, p = 0.6), or between either group of vaccinated volunteers and controls (group 1 versus controls, Mann Whitney U test, p = 0.3, group 2 versus controls, p = 0.4; all vaccinated volunteers versus controls, Mann Whitney U, p = 0.3). The details of this analysis can be found in Chapter 6 of this thesis.

4.8.2 Immunogenicity

Immunogenicity was assessed by measurement of vaccine induced T cell responses and antibodies at multiple points during the trial.

4.8.2.1 Antibody Responses

Anti-AMA49-C1 antibodies were induced at high levels in all volunteers following immunization with PEV3A (See figure 4.9A). One immunization was sufficient to produce 100% seroconversion in both groups. No increase in anti-peptide titres was observed after sporozoite infection. Unvaccinated controls did not show any increase in anti-AMA49-C1 antibody titres. Co-administration of FFM ME-TRAP led to an increase of anti-peptide IgG titres, which became significant after the third immunization ($p = 0.03$). The avidity of this anti-peptide response increased following every vaccination (Figure 4.9B). The mean avidity index did not differ between the two vaccination groups at any time point (data not shown) but a stronger increase of the mean avidity observed in the PEV3A group became statistically significant after the third immunization (two-tailed unpaired t test $p = 0.04$). Sporozoite challenge led to a decrease of avidity in group 1 (two-tailed paired t test $p = 0.004$), whereas no significant change was observed in the group receiving a combination of the two vaccines (two-tailed paired t test $p = 0.6$). Antibody cross-reactivity with blood-stage parasites was assessed by IFA with P. falciparum (strain K1) blood stage parasites (figure 4.10). Three immunizations with PEV3A led to an increase of IgG titres in 4 out of 12 volunteers in group 1 and in 3 out of 12 volunteers in group 2. Mean IgG titres in IFA after three immunizations did not significantly differ between the two groups (Mann-Whitney U two-tailed $p = 0.49$). There was no evidence of a difference in number of responders after three vaccinations between group 1 and 2 (Fisher's exact test $p = 0.64$). Although not statistically significant, there appeared to be an increase in parasite-binding antibodies in IFA after sporozoite challenge. 78% (7/9) of all volunteers who were IFA positive after challenge (both groups) had an increased endpoint titre after sporozoite infection. Unvaccinated controls did not produce detectable levels of blood-stage parasite-binding antibodies (data not shown). Generally, interpretation of IFA results with blood stage parasites was very difficult due to non-specific background staining. As only clearly positive results were included in the analysis, parasite-binding antibody responses in some volunteers may have been missed. Western blot analysis with sera from immunized volunteers (after three vaccinations) showed specific recognition of parasite-derived AMA-1 in 16 out of 24 volunteers at a serum dilution of 1:100 (data not

shown) supporting the notion that some parasite cross-reactive responses in IFA were missed.

High levels of anti-UK-39 antibodies were detected after vaccination with PEV3A (Figure 4.9A). Two immunizations were required to achieve 100% seroconversion in both vaccination groups. Co-administration of FFM ME-TRAP had no impact on the magnitude of mean anti-UK-39 titres at any time-point (Mann-Whitney U two-tailed p = 0.95 after 3rd vaccination). Sporozoite challenge led to an increase in anti-peptide titres in the combination group (Wilcoxon sign-rank p = 0.03), whereas no change in titre was seen in the PEV3A group after infection (Wilcoxon sign-rank p = 0.64), (Figure 4.9A). No anti-UK-39 IgG was detected in unvaccinated controls. The avidity of anti-UK-39 IgG increased following every immunization (Figure 4.9B). Although there was a higher mean avidity index in group 1 compared to group 2 after second and third immunization (two-tailed unpaired t test p = 0.001 after second and p = 0.008 after third vaccination) (data not shown) we did not observe any difference in avidity increase between the two vaccination groups or a significant change in avidity after sporozoite challenge (two-tailed paired t test group 1 p = 0.64; group 2 p = 0.44). Immunization with PEV3A alone induced parasite cross-reactive antibodies in all volunteers after two vaccinations, as observed in IFA with P. falciparum sporozoites (Figure 4.10). The number of volunteers with parasite-binding antibodies in the combination group increased with every immunization reaching 82% (9/11) after the third immunization. There was no evidence for a difference in number of responders after three vaccinations between group 1 and 2 (Fisher's exact test p = 0.22). Higher mean IFA titres induced by immunization with PEV3A alone compared to combined vaccination became significant after three vaccinations (Mann-Whitney U two tailed p = 0.02). A boost of the vaccine-induced response after infection was observed in 33% (4/12; group 1) and 75% (9/12; group 2) of the volunteers as detected by increased IFA titres after sporozoite challenge. The observed increase of IFA titres after infection was significant only for the group receiving both vaccines (Wilcoxon sign-rank p=0.04). No sporozoite-binding antibodies were detected in unvaccinated controls after sporozoite challenge (data not shown). Staining of a CSP characteristic double band was found in Western blot with 13 out of 23 sera

after three vaccinations at a dilution of 1:100 (data not shown). There was no correlation in levels of any induced antibodies with time to infection.

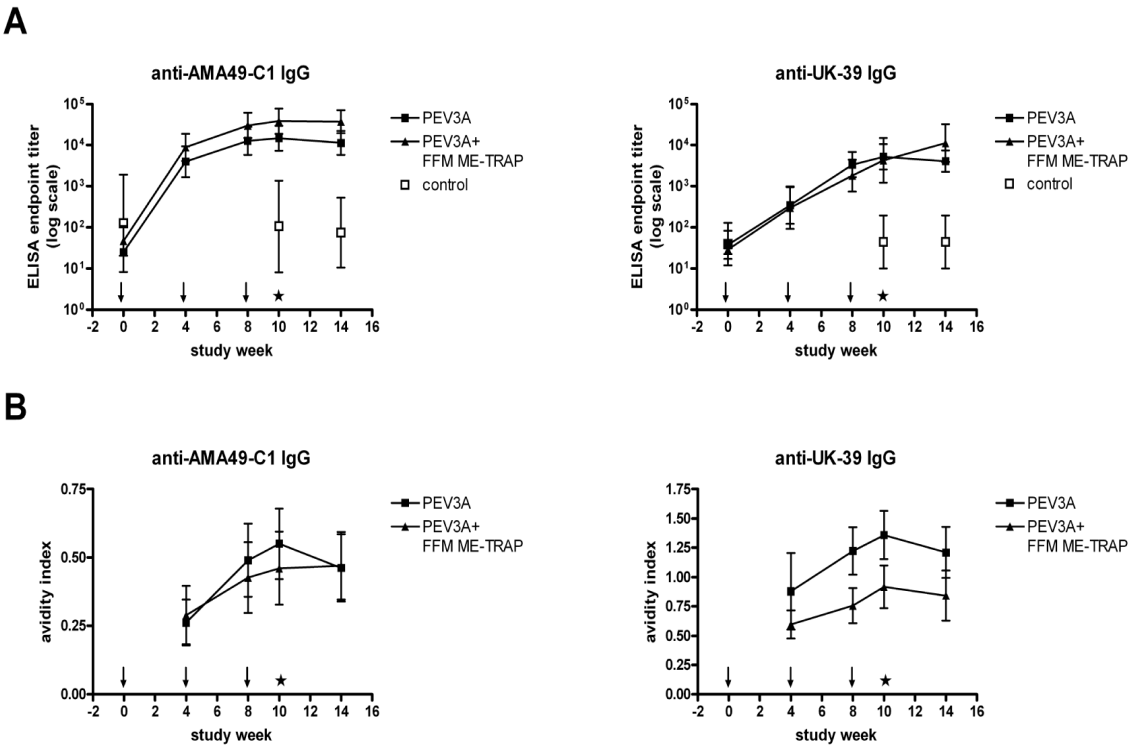

Figure 4.9: Anti-peptide responses

A: (Upper panel) Geometric mean anti-AMA49-C1 (PEV301) and anti UK-39 (PEV302) endpoint-titres (log10) with 95% confidence intervals for group 1 (PEV3A), group 2 (PEV3A + ME-TRAP) and unvaccinated controls.

B: (Lower panel) Mean avidity increase (relative to avidity index after first immunization) with 95% confidence intervals.

Arrows along the x axis represent the timing of each vaccination, and the asterisk denotes the challenge.

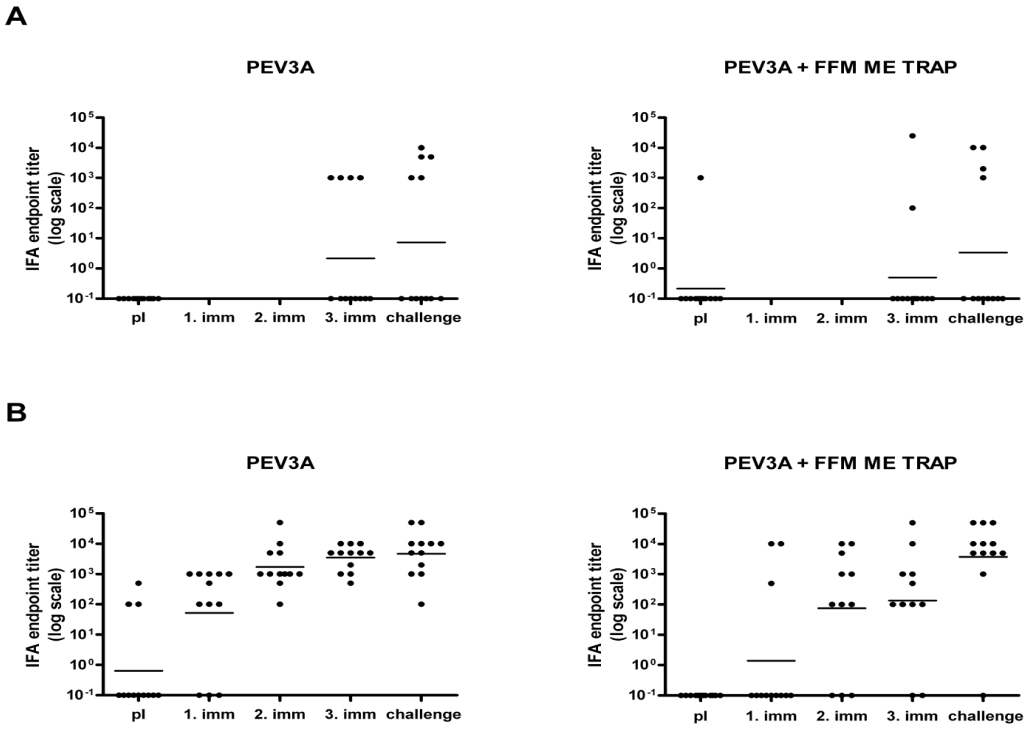

Figure 4.10: Antibody reactivity with P. falciparum

A: (Upper panel) IgG endpoint titres measured in IFA with P. falciparum (K1) blood stage parasites

B: (Lower panel) IgG endpoint titres measured in IFA with P. falciparum (NF54) sporozoites.

Individual titres are shown, with the geometric mean for every time point on a log scale.

4.8.2.2 T cell Responses

There were low level background responses to ME-TRAP in group 1 (summed response at peak time point was 13.3 SFU/10^6 PBMC). Responses in group 2 were significantly higher than those in group 1- the geometric mean summed responses to ME and T996 TRAP pools at peak time point was 49.8 SFU/10^6 PBMC (two tailed t test on log converted data p = 0.001). However these responses were low compared to those seen in previous trials with this vaccine (previously, a geometric mean summed response to ME-TRAP of 454 SFU/10^6 PBMC was observed at the peak time point). Figure 4.11 illustrates the median responses by group, before vaccination, at the peak time point (third vaccination +7) and on the day of challenge.

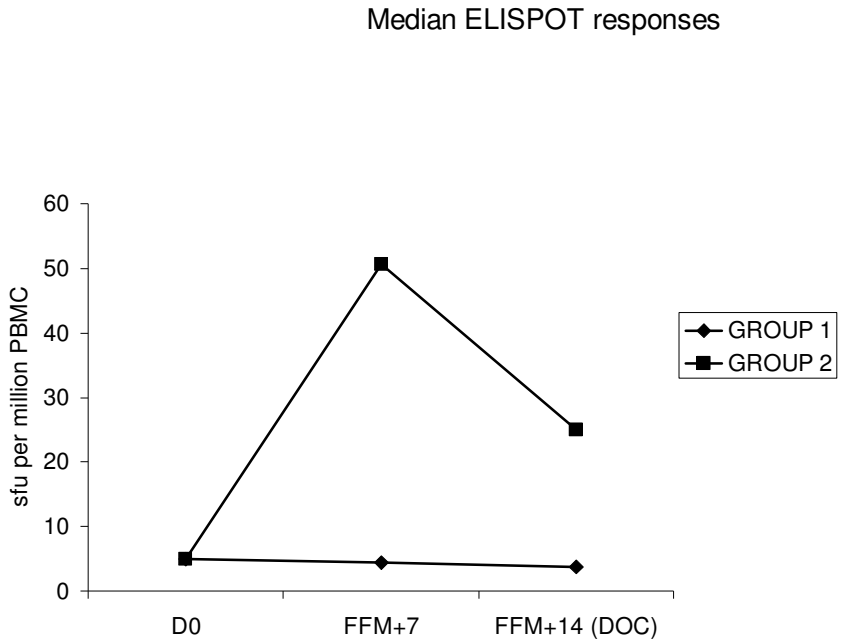

Figure 4.11: Median ELISPOT response by group

4.8.3 Safety

There were no clinically significant changes in haematological (full blood count) or biochemical (sodium, potassium, urea, creatinine, ALT, alkaline phosphatase, albumin, bilirubin) parameters throughout the study. No serious adverse events occurred during this study. One volunteer developed an enlarged right supraclavicular lymph node following the first vaccination with FP9 ME-TRAP. The maximum recorded size of this lymph node was 1.3cm. It increased in size again following subsequent vaccinations in the right arm. This node was still present at study close out. The volunteer was referred to their General Practitioner for assessment and investigation, who has indicated no further follow up is required. Adverse events following vaccination followed a profile similar to that seen before with these vaccines, [123], [133]. The frequency of vaccination site pain, swelling, redness, warmth, itch and scaling was, as expected, higher following FP9 and MVA ME-TRAP than PEV3A. One volunteer (514) reported severe pain following the third vaccination with PEV3A. This was recorded as starting on the evening of

the third vaccination, and lasting 6 days. It was recorded as intensity 3 (severe pain at rest) for two days, and then subsided to intensity 2 (pain on movement) for a further two days. For the final two days it was recorded with a score of 1 (pain on touch). After this period it had completely resolved, with no long term effects. The frequency of general symptoms in group 1 was also lower than that in group 2. In group 1, 50% of volunteers experienced at least one systemic side effect (5/12 = 41% after dose 1, 1/12 = 8% after dose 2, and 2/12 = 17% after dose 3), whilst 83% (10/12) in group 2 experienced at least one (6/13 = 46% after dose 1, 7/12 =58% after dose 2, 9/12 = 75% after dose 3). This is slightly different to previously reported studies using similar virally vectored vaccines, where general symptoms seemed to be attenuated following second and subsequent doses [133]. See the following tables for details of adverse events considered related to vaccination.

VACCINE DOSE 1	Group 1, n = 12		Group 2, n = 13			
	PEV3A i.m.		PEV3A i.m.		FP9 ME-TRAP i.d.	
	n	%	n	%	n	%
Pain	3	25	3	23	6	46
Redness	2	17	2	15	13	100
Swelling	2	17	1	8	11	85
Warmth	0	0	0	0	3	23
Itch	0	0	1	8	4	31
Scaling	0	0	0	0	7	54
VACCINE DOSE 2	**Group 1, n = 12**		**Group 2, n = 12**			
	PEV3A i.m.		PEV3A i.m.		FP9 ME-TRAP i.d.	
	n	%	n	%	n	%
Pain	5	42	7	58	9	75
Redness	2	17	0	0	12	100
Swelling	1	8	0	0	11	92
Warmth	0	0	0	0	1	8
Itch	0	0	0	0	5	42
Scaling	0	0	0	0	7	58
VACCINE DOSE 3	**Group 1, n = 12**		**Group 2, n = 12**			
	PEV3A i.m.		PEV3A i.m.		MVA ME-TRAP i.d.	
	n	%	n	%	n	%
Pain	8	67	8	67	12	100
Redness	2	17	2	17	12	100
Swelling	1	8	0	-	12	100
Warmth	0	-	0	-	10	83
Itch	0	-	0	-	8	67
Scaling	0	-	0	-	12	100

Table 4.7: Frequency of solicited local symptoms

VACCINE DOSE 1	Group 1, n=12		Group 2, n=13	
	n	%	n	%
Documented Fever >37.5	1	8	0	-
Symptoms of feverish	2	17	4	31
Malaise	0	-	4	31
Arthralgia	2	17	2	15
Headache	0	-	4	31
Myalgia	5	83	5	38
Nausea/vomiting	0	-	0	-
VACCINE DOSE 2	**Group 1, n=12**		**Group 2, n=12**	
	n	**%**	**n**	**%**
Document. Fever >37.5	0	-	0	-
Symptoms of feverish	1	8	4	33
Malaise	1	8	5	42
Arthralgia	1	8	1	8
Headache	0	-	3	25
Myalgia	3	25	6	50
Nausea/vomiting	0	-	1	8
VACCINE DOSE 3	**Group 1, n=12**		**Group 2, n=12**	
	n	**%**	**n**	**%**
Documented Fever >37.5	1	8	1	8
Symptoms of feverish	1	8	4	33
Malaise	1	8	5	42
Arthralgia	1	8	3	25
Headache	0	-	3	25
Myalgia	2	17	5	42
Nausea/vomiting	0	-	1	8

Table 4.8: Frequency of solicited general symptoms

4.8.4 Ancillary Analyses – Growth Inhibition

No significant GIA activity was seen in any sample relative to day 0 activity.

4.8.5 Ancillary Analyses – Sequencing

No changes were identified in the DNA sequence of DNA extracted from parasites from the sample on day 20. Unfortunately we failed to amplify DNA from the upstream region, so are unable to exclude another mutation for example in the promoter, which might have resulted in a shift in the open reading frame. This failure is likely to have been due to a problem with the quality of the sample DNA. This could have been caused by multiple freeze-thaws leading to degradation of the DNA, but it may just reflect the difficulty of designing suitable primers for use with the relatively A and C rich parasite DNA. The sample was amplified using a whole genome amplification technique called GenomiPhi™ (GE Healthcare Life Sciences), however, using this amplified DNA still did not result in any PCR product.

4.8.6 Ancillary Analyses – Crisis Forms

Morphologically abnormal parasites were seen in films from every vaccinated volunteer (n = 24) in this study. They were identified in the films of significantly fewer (two out of the six) controls (Fisher's exact test, p value=0.001). The proportion of crisis forms as a percentage of total number of parasites for each participant was been calculated. For volunteers in group one, the average proportion of crisis forms was 66% (95% CI, 54-78, SD 19), for those volunteers in group two, the proportion was 55% (95% CI, 41-69, SD 22) and for controls the proportion was 13% (95% CI -11-37, SD 23). There was a significant difference between the proportion of crisis forms among all detected parasites in vaccinated volunteers and controls (Mann Whitney U, p = 0.001). No crisis forms were detected in the blood films that were reviewed from previous challenge studies.

4.9 Discussion

This trial is the first to provide evidence of vaccine-induced blood stage parasite activity and partial protection in a healthy volunteer challenge study. Two observations are indicative of a parasite inhibitory effect, the first (I) of which may be divided into three components. (I.i) One volunteer was not diagnosed until day 20 after infection, this being a substantial delay in time to diagnosis. In 52 non-vaccinated control volunteers involved in identical challenge studies at the centre over the last six years, none have remained parasite free on microscopy beyond day 14 post challenge. (I.ii) The same volunteer, along with three others, had fluctuations in PCR-measured parasite densities that are consistent with a blood stage inhibitory effect on parasite growth. (I.iii) There was a significant difference in the parasite growth rates in vaccinated volunteers versus controls. This is observed in both groups of vaccinated volunteers, and is therefore likely to be related to vaccination with PEV3A. (II) The presence of morphologically abnormal parasites suggests a vaccine-induced immune response against the parasites. However, as they were also observed in the blood of two of six control volunteers, their presence is not necessarily vaccine related.

The volunteer in whom diagnosis was delayed until day 20 developed high levels of anti AMA49 and UK39 antibodies (peak AMA49 response end point titre 33779, UK39 end point titre 18102). Positive IFA results were obtained with sporozoites, but not with blood stage parasites. ELISPOT responses were negligible, with AMA-1 and NANP responses all remaining <10 sfu/million PBMCs throughout the study. No detectable GIA activity was measured. The mechanism of partial protection in this volunteer, therefore, appears to be something other than that which we have directly measured.

Taken together these results suggest that PEV3A has induced a protective immune response against blood stage parasites. However this was not enough to prevent patent infection in malaria naïve individuals, in the stringent challenge model used that involves high numbers of sporozoites for infection. Blood stage protection might be enhanced by targeting several blood stage antigens with a

multivalent subunit vaccine and this is technically feasible with the virosomal vaccine approach.

This study was designed to search for a possible synergistic effect of combining two potentially complimentary vaccine strategies. The idea that combining induction of anti-sporozoite antibodies by the CS component of PEV3A and anti-liver-stage T cell responses by recombinant viruses would lead to a synergistic enhancement of protection was suggested by experiments in a mouse model [195]. In this model, viral vectors appeared to act as an adjuvant to a protein vaccine, enhancing the magnitude of the antibody response. The best results (i.e. highest levels of protection from a subsequent malaria challenge) were obtained when the vaccines were mixed physically in the same syringe and administered at the same site. Both T cells and antibodies were shown to be important in mediating this protection. In this first phase I/IIa trial, we elected to administer the vaccines at the same time, but at separate sites rather than mixed in the same syringe. This was in order to reduce the potential risk of interference of the two vaccines. The vaccines used were safe when administered concurrently, in opposite arms, and the PEV3A virosomes were immunogenic. High levels of anti AMA49-C1 and anti UK-39 antibodies were measured post vaccination in all volunteers. A high proportion of these antibodies were able to bind to the native parasite proteins in vitro in IFA suggesting they are likely to be functional.

There is a clear discrepancy between the number of vaccinees who were IFA positive (only 25%), while AMA-1 was recognized by a much higher percentage of vaccinees in Western blot analysis. This discrepancy appears to be related to the fact that the background staining in the IFA with blood stage parasites was already high at day 0 in many volunteers. It is most likely that this background staining is caused by cross-reactive antibodies induced by other pathogens such as toxoplasma. While pre-immune sera (from day 0) showed no staining of the 83/66KDa AMA-1 bands in Western blot analysis, these bands were shining up with most immune sera (from after the third vaccination - see Figure 4.12). Many pre-immune sera yielded background staining with different sets of unrelated proteins; these antibody titres were not boosted by the vaccination. However,

these antibodies are apparently responsible for high and variable IFA background staining, impeding detection of the development of an AMA-1 specific staining.

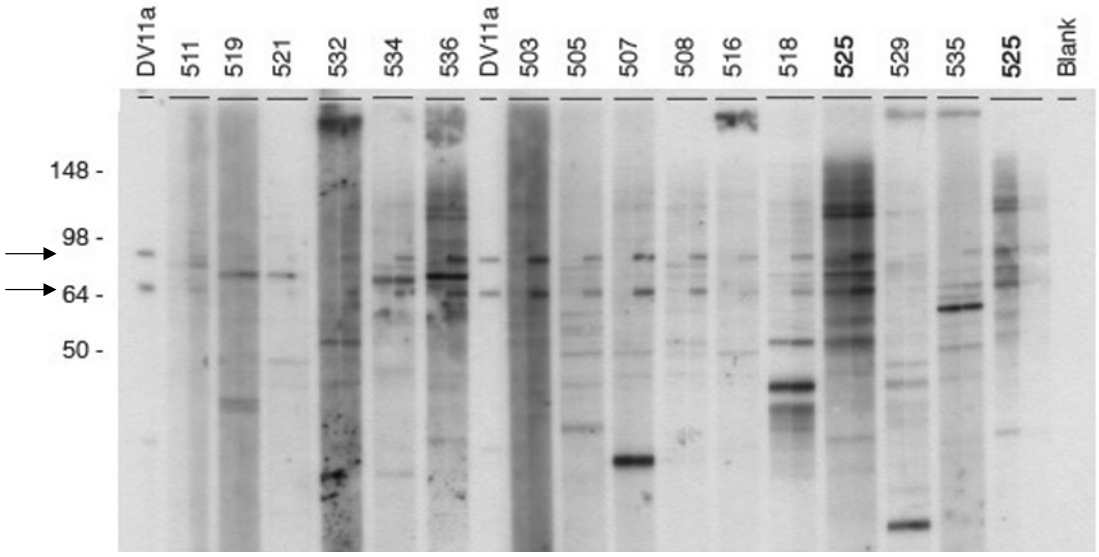

Figure 4.12: Western blot analysis.
Sera of the vaccinees were used to analyse blood-stage-lysates of P. falciparum as indicated as numbers above the lanes. For all probands the Western blot was developed using the pre-immune (left lanes) serum and the serum obtained after the 3rd immunization (right lanes for each proband). As controls for AMA-1-staining the monoclonal antibody DV11a was used. The arrows mark the size of the two AMA-1-processing products.

The criteria for IFA were fairly stringent: a serum was only considered IFA positive when a vaccination-associated emergence of an AMA-1 characteristic punctuate staining pattern was observed.

AMA49 has been designed and optimized to induce antibodies binding to the parasite. This has been proven by immunization of mice that had no parasite cross-reactive pre-immune antibodies [199]. The analysis clearly showed the importance of a critical length of the peptide to induce parasite interacting antibodies and parasite-binding mAbs generated after immunization with the virosomes primarily recognized discontinues epitopes of the loop. Furthermore, virosomes have proven to present peptide epitope in a native like three dimensional structure compared to the same structures adsorbed to alum[198].

The high background staining caused by cross-reactive antibodies in combination with the stringency of the interpretation of IFA results are likely to be responsible for the observed discrepancy between IFA and Western blot positivity.

The induced ELISA titres were comparable with those from a previous Phase I study using this vaccine [123]; Okitsu et al. submitted). Co-administration of FFM ME-TRAP did boost the magnitude of the anti-AMA49-C1 antibody response but not the anti-UK-39 response. However, whereas in the animal model, good T cell induction was achieved by the viral vectored vaccines this was reduced about 10-fold in this study compared to previous trials. Co-administration of vaccines could have led to antigenic competition or interference, which might account for this low immunogenicity. Both FP9 and MVA ME-TRAP were tested for potency in mice prior to the study, and were found to be potent, and if anything more immunogenic than previously used batches of this vaccine (data not shown). Estimated numbers of P. falciparum infected hepatocytes were similar between both groups of vaccinated volunteers, leading to the conclusion that there was no clear evidence of any liver stage effect by FFM ME-TRAP, in keeping with the low T cell immunogenicity observed.

Another important observation in this study was an increase in parasite reactive antibodies after sporozoite challenge. While anti-peptide titres did not markedly change, titres measured in IFA with P. falciparum blood stage parasites and sporozoites were boosted by infection. This indicates that PEV3A-induced immune responses can be boosted and skewed towards parasite-binding antibody populations by sporozoite infection. Results from in vitro sporozoite inhibition assays in a phase 1 trial with the same vaccine have shown inhibition of sporozoite migration and invasion in the presence of anti-UK-39 IgG (Okitsu et al., submitted). Although statistically not significant, we found a trend towards reduced numbers of infected hepatocytes in both vaccination groups compared to unvaccinated controls, possibly suggesting a role for anti-UK39 antibodies in the reduction of infected hepatocytes, but there was little power in this inter-group comparison.

Reduced parasite growth rates and the presence of crisis forms in the blood of all vaccinated volunteers who took part in this trial provide evidence that the immune response to the vaccine PEV3A is exerting an anti-blood stage protective effect. Murine monoclonal antibodies against AMA49-C1 are capable of inhibiting blood stage parasite growth in vitro [199], which is at least in part due to inhibition of intra-erythrocytic parasite development (unpublished observation).

The interpretation of these results is complicated by the fact that AMA-1 is expressed by both sporozoites and merozoites [213], and so it is possible that antibodies to this antigen induced by the virosome might have contributed to any pre-erythrocytic effect. The non significant trend to reduced numbers of parasites emerging from the liver of all vaccinated volunteers may reflect this effect.

It might be argued that some of these observations of blood stage immunity (reduction in growth rates, presence of crisis forms etc.) may rather be related to the potential pre-erythrocytic action of PEV3A. However, in previous challenge studies, the number of parasites emerging from the liver of unvaccinated control volunteers has been shown to vary as much as five fold [214]. Despite this variation, rates of parasite growth in these volunteers were similar. Equally, crisis forms have never been observed historically in our studies of pre-erythrocytic vaccine candidates. Indeed, following their observation in this study, the same slide reader went back to examine a selection of blood films from vaccinated and control volunteers in two previous studies where some evidence of pre-erythrocytic efficacy has been observed (VAC021 [215] and VAC023 [216]). The slide reader was blinded to the group allocation of the volunteers. In total 72 slides were selected for re-examination (6 slides each from 6 volunteers from each study, 3 vaccinees plus 3 controls) and no crisis forms were observed. It seems likely therefore, that the differences observed here are indeed related to vaccine induced blood stage immunity.

The current challenge was a heterologous one with 3D7 parasites, rather than the K1, or T9/96 strains, used in the generation of PEV3A or the ME-TRAP vaccines respectively. PEV301 targets the semi-conserved sequence AMA-1446-490, which contains only three dimorphic positions (D/N448, M/K451 and K/I485) at its C and

N terminus, respectively. The elicited antibody responses are focussed on the conserved central portion of this sequence stretch and all murine vaccine-induced monoclonal antibodies tested were cross-reactive with P. falciparum strains expressing natural sequence variants of AMA-1446-490 [199]. In contrast to results with monoclonal antibodies, growth inhibition assays performed with sera of the volunteers immunized in this trial were negative. Further data will be required to assess the utility and sensitivity of this assay for predicting blood stage vaccine efficacy, but these trial results suggest caution in the exclusive use of this assay as an in vitro predictor of blood-stage vaccine efficacy.

An effective malaria vaccine may need to target multiple parasite antigens from different stages of the life cycle. Several vaccine candidates have previously provided evidence of pre-erythrocytic stage efficacy in Phase IIa trials [113, 116, 132, 217]. Here we have shown evidence for a vaccine-induced blood stage protection for the first time in a challenge study. This study also shows that protective efficacy at the blood-stage level can be observed using sporozoites rather than blood stage parasites [109] for challenge studies. Moreover, 100% seroconversion induced by virosomally-formulated peptides and the observed boost of this response by sporozoite infection support the potential of further development of the virosome system as a malaria vaccine.

Chapter 5 Lab work

5.1 Introduction and Literature Review

The multiple stages in the life cycle of P. falciparum make it a difficult target for vaccine developers, as largely different sets of antigens are expressed in each of the various stages. The observation, described in Chapter 1, that protection against malaria infection can be induced by immunisation with radiation attenuated sporozoites has led to a focus on the liver stage of P. falciparum, in the hope of identifying the antigens involved. A variety of liver stage antigens have been investigated; indeed, the vaccine which is currently the most well developed, RTS,S, is derived from the circumsporozoite protein.

Of the nearly one million people who die each year from malaria, over 75% are children under 5 [4]. While the majority of the burden of malaria morbidity and mortality falls on young children, adults living in malaria endemic areas develop immunity to infection. Even with repeated infections, this protective immunity is incomplete; individuals are protected from clinical disease, without being protected from parasitaemia. This appears to be age dependent and results in a lower incidence of clinical malaria in these individuals [60]. There is evidence for a genetic basis for antibody and cellular responses to malaria proteins [30].

Identification of specific immunologic determinants of protection will lead to development of the most promising vaccine candidates, including multistage vaccines. Thus examining the antigen specific immune responses to the liver stages of individuals in endemic areas may help in the development of new malaria vaccines.

This study set out to examine the responses to various liver stage antigens in individuals from endemic areas. The antigens chosen were selected on the basis of previous immunological studies in malaria endemic areas, and the studies of volunteers immunized with radiation-attenuated sporozoites [85,88,218]. They

were selected for inclusion in a new candidate malaria vaccine, which showed promising results in preclinical work, described by Prieur [167]. The clinical trial that was planned using this vaccine is described in Chapter 3. A detailed description of the individual antigens included may be found there. In the order that they are expressed in the insert, they are:

LSA - 3, Liver stage antigen 3 is expressed by blood-stage schizonts as well as sporozoites [181].
STARP, Sporozoite threonine and asparagine-rich protein was described by Fidock et al. as a relatively conserved sporozoite antigen [185].
Exp-1, Exported protein-1 was originally described by Hope et al. [186] as a sporozoite and blood stage antigen.
Pfs 16 is an antigen found in sexual stage parasites [192] and possibly also in sporozoites.
TRAP, Thrombospondin-related adhesion protein is well characterized and has a protective homologue in rodents.
LSA-1, Liver Stage Antigen 1 is expressed solely in the pre-erythrocytic stage, first described in 1987 by Guerin-Marchand et al. [219].

5.2 Methods

The primary objective of this work was to examine immune responses to the pre-erythrocytic antigens described, in adults from endemic areas. Both humoral and cellular responses to these antigens were examined, using ELISA and ELISPOT techniques. These assays are sensitive methods for the detection of cytokines. The data obtained was then analysed to see if there was any link between then magnitude of any individual immune response, and whether that individual developed malaria infection or not. As described in reference [60], this is a crude method to identify immune responses that may be important in protecting from malaria infection.

Two malaria vaccine studies that took place in The Gambia (VAC020 [220] and VAC029 [221]) were selected. VAC020 was a Phase IIb study testing the heterologous prime-boost vaccination regimen of two doses of DNA ME-TRAP

followed by one dose of MVA ME-TRAP. The main objectives of this study were to evaluate vaccine safety and efficacy in semi-immune adults in the Gambia. VAC029 was a study designed to evaluate the use of daily real time PCR to detect parasites, in the field, for evaluation of pre-erythrocytic malaria vaccines. Volunteers were vaccinated with the candidate malaria vaccines MVA and FP9 ME-TRAP in the course of this study. Control volunteers were included in both of these studies, and they received a rabies vaccine rather than a candidate malaria vaccine. Importantly, a follow up period to detect malaria infection was included in each study. Samples from these volunteers were identified for further analysis.

These malaria vaccine studies were performed in The Gambia between 2002 and 2004, and written consent was obtained from all participating volunteers prior to their inclusion. Although this consent included permission to store samples taken during the study, specific consent to use these stored samples was not sought at the time. Permission was therefore obtained from the Oxfordshire Research Ethics Committee (reference number 061/Q1606/123) for these and other samples to be used for further analysis of immune responses to malaria infection.

Cellular samples were obtained from VAC020. In this study volunteers were vaccinated with either a candidate malaria vaccine or rabies vaccine, and subsequently followed for 11 weeks; samples of peripheral blood mononuclear cells were obtained at 12 weeks. During the follow up period blood smears were taken weekly, or if volunteers developed symptoms of malaria, to detect parasites. The primary endpoint was time to first infection with asexual P. falciparum parasites (i.e. first seen on blood smear – not time to clinical malaria). All volunteers were treated with effective antimalarial medication as soon as parasites were detected.

Serum samples were from VAC029. Again, volunteers received either a malaria vaccine or a rabies vaccine, and one week after the final vaccination (of three) serum samples were obtained. From 14 days after the final vaccination, volunteers were followed for a 28 day period, with daily finger prick samples of blood taken for parasite PCR. Blood smears were performed only if volunteers developed symptoms of malaria. Only 12 volunteers out of a total of 103 developed malaria

during the follow up period, so for this analysis of a subsection of control volunteers, PCR positivity was used as a surrogate for clinical malaria.

Immune responses to the malaria antigens described were measured using ELISPOT and ELISA techniques. These were chosen to identify both cellular and antibody mediated immune responses in these individuals.

5.2.1 ELISPOT

The ELISPOT (enzyme-linked immunospot) technique detects T cells that secrete a given cytokine (e.g., gamma interferon [IFN-γ]) in response to an antigenic stimulation. T cells and antigen presenting cells (present in the isolated PBMC) are cultured in wells which have been coated with anti-IFN-γ antibodies. The secreted IFN-γ is captured by the coated antibody and then revealed with a second antibody coupled to a chromogenic substrate. Thus, locally secreted cytokine molecules form spots, with each spot corresponding to one IFN-γ-secreting cell. The number of spots allows one to determine the frequency of IFN-γ-secreting cells specific for a given antigen in the analyzed sample. Cells secreting as few as 100 molecules can be detected by taking advantage of the high concentration of cytokines in the immediate environment of the activated T cells. However, the ELISPOT assay detects preferentially effector T cells. The amount of secreted cytokine is not determined in ELISPOT assays.

Interferon gamma ELISPOTs were performed on frozen PBMCs collected during the study (VAC020). These cells were isolated from heparinised blood samples using Lymphprep (Nycomed) gradient centrifugation, frozen in 10% DMSO-FCS (Sigma-Aldrich) and stored in liquid nitrogen.

Cells were thawed and re-suspended in RPMI 1640 (Sigma-Aldrich) containing 1000U/mL penicillin-streptomycin (Invitrogen Life Technologies), 20mM L-Glutamine (Sigma-Aldrich), and 10% heat-inactivated fetal calf serum (Sigma-Aldrich) (R10) at a concentration of 8×10^6 cells/mL. In the thawing procedure, 25 U/mL Benzonase nuclease (Novagen) was added to avoid cell clumping. Cells were counted with an automated cell counter (CasyCounter TT Schärfe System).

The ELISPOT assay was performed using MultiScreen Immobilon-P 96-well filtration plates (MAIP S45; Millipore), IFN-γ ELISPOT kits (Mabtech) and alkaline phosphatase substrate (Bio-Rad). Cells were stimulated (in duplicate wells) with 10 µg/ml/peptide of pools of 20-mer peptides overlapping by 10 aa, for a 18-20 hours at +37℃, 5%CO_2. Spots were counted with an ELISPOT reader (AID). The peptides used in these assays were ordered from Thermoelectron, and were 20mers overlapping by 10 amino acids. They covered each of the antigens outlined in the introduction, and are listed in Appendix 3. Antigen specific cells per 10^6 PBMC were calculated by subtracting spot numbers in media only wells from spot numbers in peptide-containing wells. Each pool of peptides contained approximately 20 peptides, and so the total response for each antigen was calculated by summing the response for each peptide pool.

5.2.2 ELISA

ELISA (Enzyme-Linked Immuno Sorbent Assay) is a specific and highly sensitive method for quantitative measurements of antibodies, or cytokines in solutions. For detection of antibodies to specific antigens, the antigen of interest is coated on a micro titre plate. An anti-human IgG monoclonal antibody (mAb) is used for detection of any antibody that has bound to antigen on the plate. The detection mAb is labelled with biotin, which allows subsequent binding of a streptavidin-conjugated enzyme. Any unbound reagents are washed away. When substrate is added, a colour reaction will develop that is proportional to the amount of bound antibodies. The concentration of antibody can then be determined by comparison with a standard curve with known concentrations of antibody. Unfortunately in this case, no solutions of antibody of known concentration were available. A sample of serum from a malaria naïve individual was used as a negative control. Arbitrarily, an endpoint titre cut off optical density of >0.2 was chosen as a positive result.

Antibodies against five of the six antigens of interest were examined, it was not possible to source recombinant protein for Pfs 16, and so this antigen was not included. Protein for these ELISAs was obtained from various sources, as shown in table 5.1.

Antigen	Source	Stock Concentration
LSA3 GST-NN	Pierre Druihle, Institut Pasteur, Paris.	1.5mg/mL
LSA3 GST PC		550mcg/mL
LSA3 GST 729		600mcg/mL
STARP	Pierre Druihle, Institut Pasteur, Paris.	500mcg/mL
Exp1	David Lanar, WRAIR, Maryland, USA.	4.22mg/mL
TRAP	Joe Cohen at Glaxo Smith Kline, Rixensart, Belgium.	160mcg/mL
LSA1	David Lanar, WRAIR, Maryland, USA.	824mcg/mL

Table 5.1: ELISA antigens

The recombinant proteins LSA3 GST 729, GST NN and GST PC were designed to cover 95% of the LSA-3 antigen. GST 729: GST (glutathione-S-transferase)-fused protein encoded by the DG729 insert (aa 1'-150'), GST NN: GST-fused protein (aa 369-447), and GST PC: GST-fused protein (aa 869-1786) (see reference [183] for details). The following figure demonstrates the gene for LSA3 and the relative positions of recombinant proteins used.

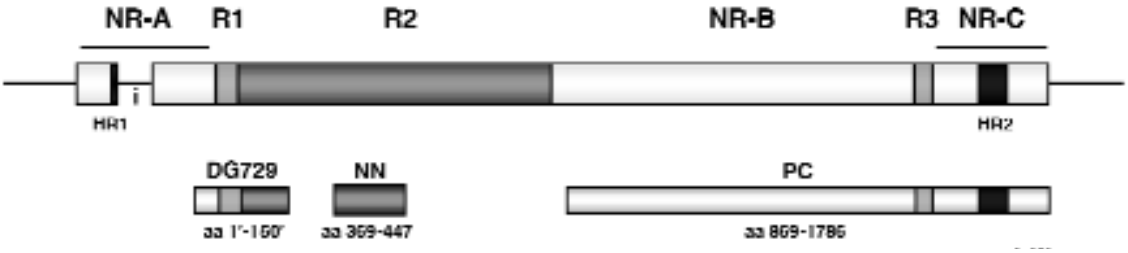

Figure 5.1: LSA3 gene map

LSA3 map showing the positions of the three separate antigens used for the detection LSA3 antibodies.

All proteins were resuspended in carbonate bicarbonate buffer, 0.05M, pH 9.6 (Sigma Aldrich) at a concentration of 1 µg/mL.

NUNC-Immuno 96 well plates (Nunc) were coated at 4℃ overnight with these antigens. Wells were then blocked with 1% Bovine Albumin in PBS containing 0.05 % Tween-20 (Sigma Aldrich) for 2 h at 37℃ followed by three washes with PBS – Tween. Plates were then incubated with serial dilutions of human serum, starting with 1:50, down to 1:6400, in PBS containing 0.05% Tween-20 and 0.2 % Bovine albumin, at 37℃ for 2 h. Samples were performed in duplicate. After washing, plates were then incubated with anti-human IgG at 0.25µgml (Promega) for 30 mins at 25℃ and then washed again. 100 µL of p-Nitrophenyl phosphate substrate (pNPP) (20mg/ tablet, Sigma Aldrich) dissolved in 5mL 0.2M TRIS (pH 9.8) was added and incubated at 25℃ for 25 minutes. The optical density (OD) of the reaction product was recorded at 405 nm using a microplate reader (Dynex Technologies MLX 96 Well Plate Luminometer and Dynex Revelation 4.22 software). A cut off OD of 0.2 was used to determine positive samples, and the end point titre was calculated based on the serial dilutions. Data was analysed using Microsoft Excel to determine the endpoint titre for each sample.

5.3 Results

5.3.1 ELISPOT Results

Individuals from the Gambia had evidence of T cell responses to peptides from LSA3 STARP, Exp1, TRAP and LSA1 detectable by interferon gamma ELISPOT assay. A total of 33 volunteers were selected for analysis. Volunteers were divided into those who developed malaria during the follow up period (n=20) and those who did not (n=13), and mean numbers of spot forming units for each antigen were calculated. The results are shown in the following graphs.

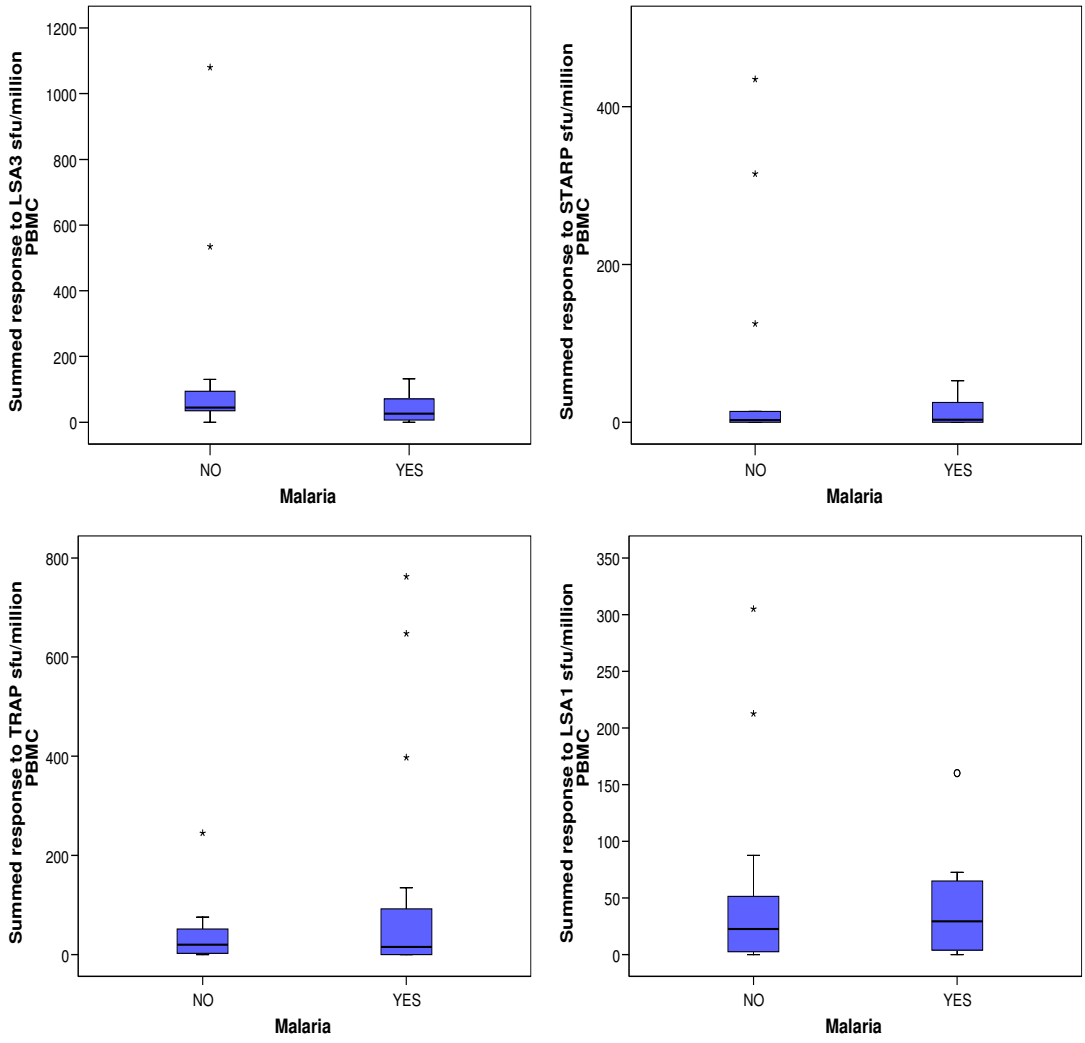

Figure 5.2: ELISPOT results for each antigen by malaria outcome.
Summed number of spot forming units per 10^6 PBMCs. YES = positive diagnosis of malaria during follow up period. NO = not diagnosed with malaria. Two tailed t tests were used compare ELISPOT responses in those who did or did not develop malaria, and the following p values were calculated: LSA3, p = 0.08; STARP, p = 0.09; TRAP, p = 0.34; LSA1, p = 0.49.

Those that did not develop malaria in the subsequent follow up period had a trend to higher T cell responses to LSA3 and STARP (as measured by IFN gamma ELISPOT) than those who succumbed to infection during that period. There was a correlation between the magnitude of the ELISPOT response and the time of diagnosis for LSA 3 (Spearman's Correlation, p = 0.03), and LSA 1 (Spearman's correlation coefficient, p = 0.006), but not for responses to STARP (Spearman's correlation coefficient, p = 0.9) or TRAP (Spearman's correlation coefficient, p = 0.9). This is demonstrated in the following graphs.

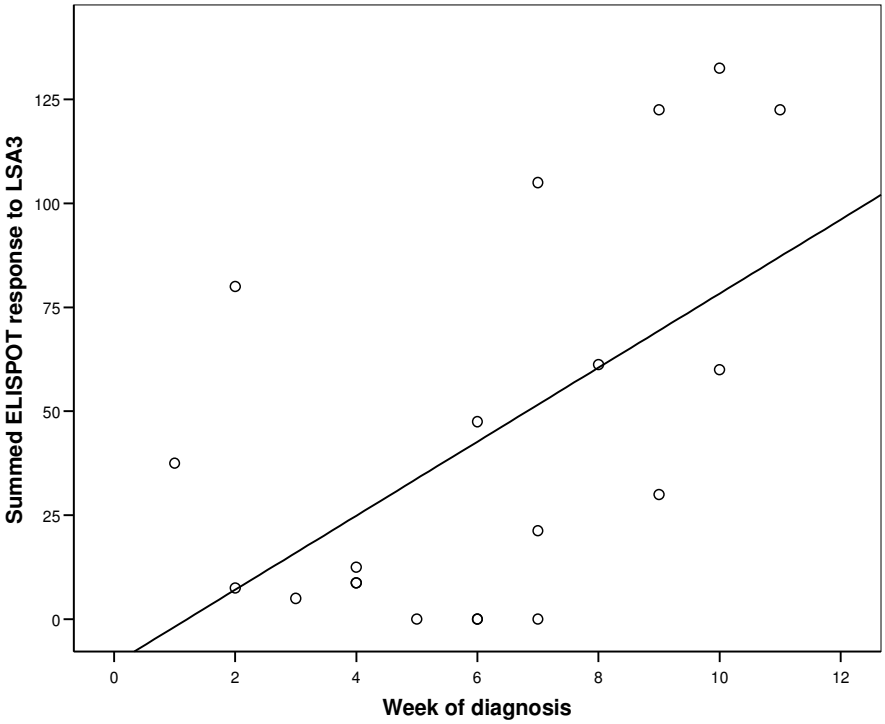

Figure 5.3: ELISPOT response to LSA3 vs. time to diagnosis.
A scatter plot of ELISPOT response to LSA3 against week of diagnosis demonstrates a positive correlation. Spearman's correlation coefficient p = 0.03

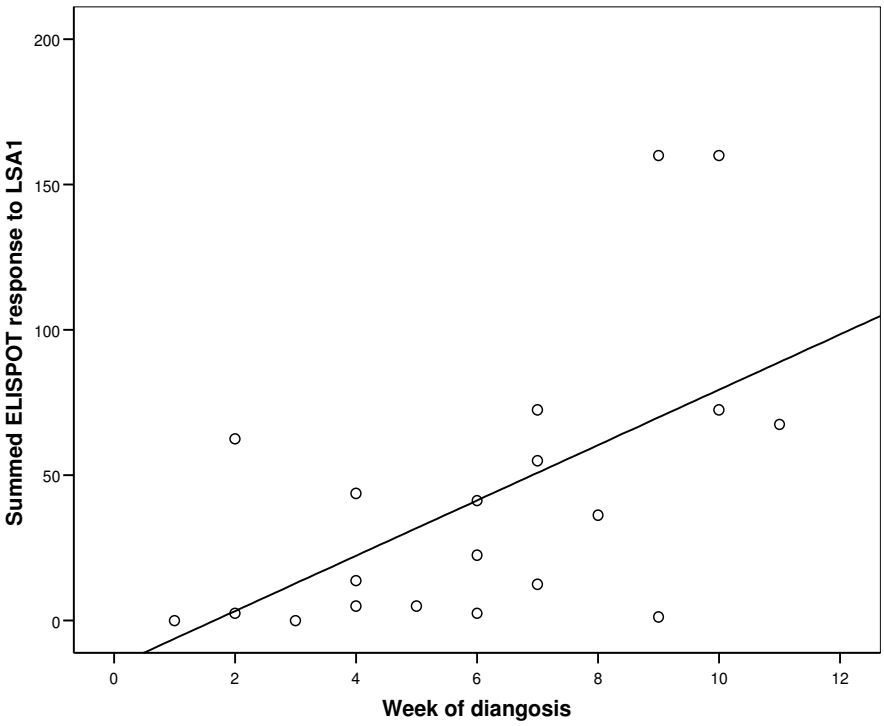

Figure 5.4: ELISPOT response to LSA1 vs. time to diagnosis.
A positive correlation is again demonstrated. Spearman's correlation coefficient p = 0.006

The time to malaria infection was significantly longer in those with higher T cell responses to all antigens apart from STARP (Kaplan Meier survival analysis of summed LSA3 response, Log rank $p < 0.0005$; for TRAP, $p < 0.0005$ and LSA 1, $p = 0.001$, for STARP log rank $p = 0.17$) data not shown.

5.3.2 ELISA Results

The protein antigens provided were initially checked for purity by gel electrophoresis. This showed fragments of the expected molecular weight for each antigen. See figure 5.5.

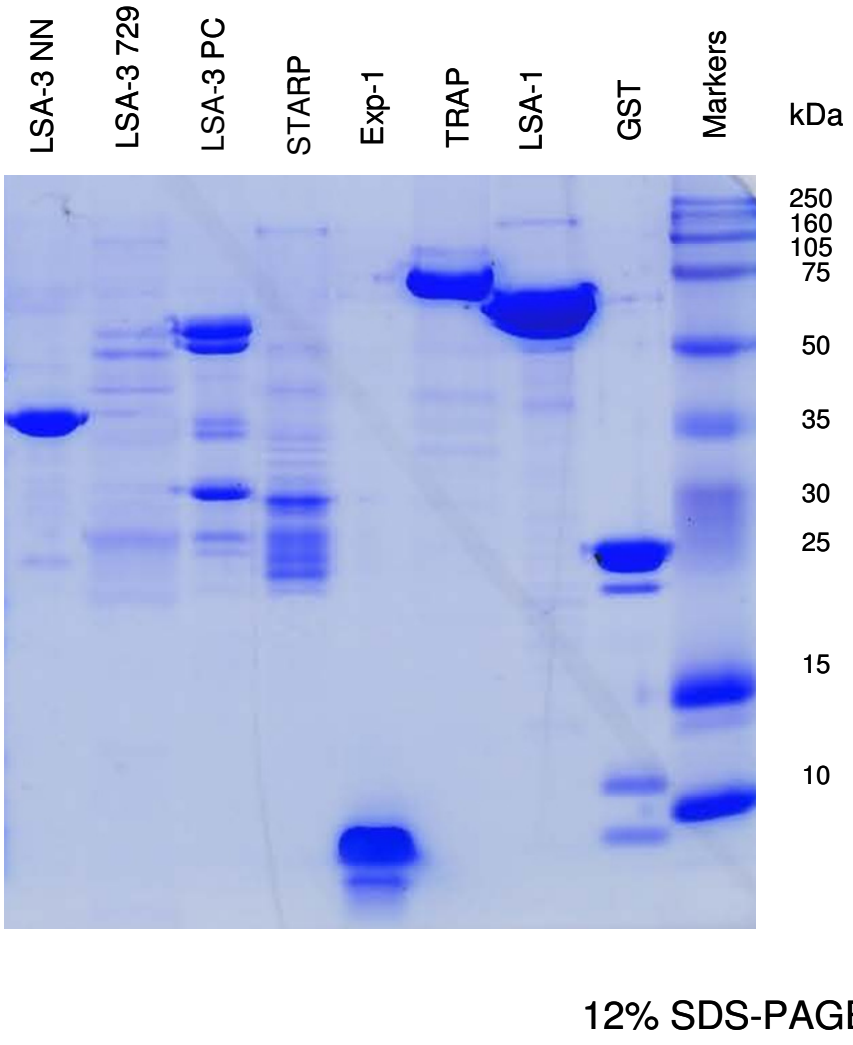

Figure 5.5: Gel electrophoresis

Gel showing the recombinant proteins used in the ELISA experiments.

Samples were chosen from 23 volunteers who participated as control volunteers in the malaria vaccine study, and follow up data was not made available until after all ELISA assays had been performed. Unfortunately, 5 of the volunteers chosen were lost to follow up during the subsequent period, and so information concerning malaria infection was not collected.

Antibodies to all the antigens selected were detected at varying levels in the samples chosen. Antibodies to Exp 1 were detected at only very low levels in a few samples, and this antigen was therefore not used in any subsequent analysis. The endpoint titres for each antigen for all 23 volunteers are shown in the following table.

VOLUNTEER NO.	LSA 3 729	LSA 3 PC	LSA 3 NN	LSA 3 TOTAL	STARP	TRAP	LSA 1
115	209	156	429	795	571	114	70
83	695	98	236	1029	826	149	196
84	541	120	246	907	314	0	70
85	144	0	0	144	0	0	0
86	224	314	120	658	16800	120	85
91	910	1394	990	3294	1635	135	100
94	436	2720	693	3850	369	92	76
101	696	36800	1328	38824	14629	354	127
107	575	2936	347	3858	1538	141	184
110	168	3938	162	4269	92	0	66
63	1584	1507	2667	5758	8457	1294	140
18	223	357	1067	1646	2473	91	114
21	8000	2607	5881	16488	7400	2629	436
32	1010	2624	5967	9600	6784	173	95
35	7362	4509	9452	21323	7529	183	336
39	424	2732	2318	5473	3200	286	166
45	1160	1581	4577	7319	9143	13867	182
48	8743	6600	7270	22613	18880	468	655
66	173	0	357	530	104	0	81
67	691	300	2469	3459	1414	2923	198
71	100	0	203	303	218	59	0
74	150	140	224	514	7893	93	89
51	1782	0	265	2046	1396	266	0

Table 5.2: Endpoint titres

The endpoint titre for each antigen is shown by volunteer. LSA 3 Total is the sum of the titres for the 3 individual recombinant proteins covering LSA 3.

Combining this data with the information concerning malaria infection, it can be seen that rather than there being a correlation between high antibody levels and protection from infection, rather the converse appears to be true. The graphs in figure 5.6 describe this data in more detail.

When volunteers are divided into those who did became PCR positive for malaria during the follow up period (n=10) and those who did not, (n = 8) it can be seen that there is no significant difference in endpoint titre between the two groups. The data is summarised in the table 5.3, and figure 5.6.

	Mean endpoint titre by antigen			
	LSA 3 (95% CI)	**STARP (95% CI)**	**TRAP (95% CI)**	**LSA 1(95% CI)**
PCR +	6716 (791 – 12639)	5946 (955 – 10937)	1805 (1291 – 4901)	182 (46 – 318)
PCR –	9108 (2016 - 20233)	5163 (981 – 9344)	475 (258 – 1208)	147 (38 – 257)

Table 5.3: Mean endpoint titres
Subjects grouped into those who developed malaria (PCR +) and those who did not (PCR -), the PCR method used has a sensitivity of 20 parasites/mL blood. The mean endpoint titre for each antigen in these groups is shown.

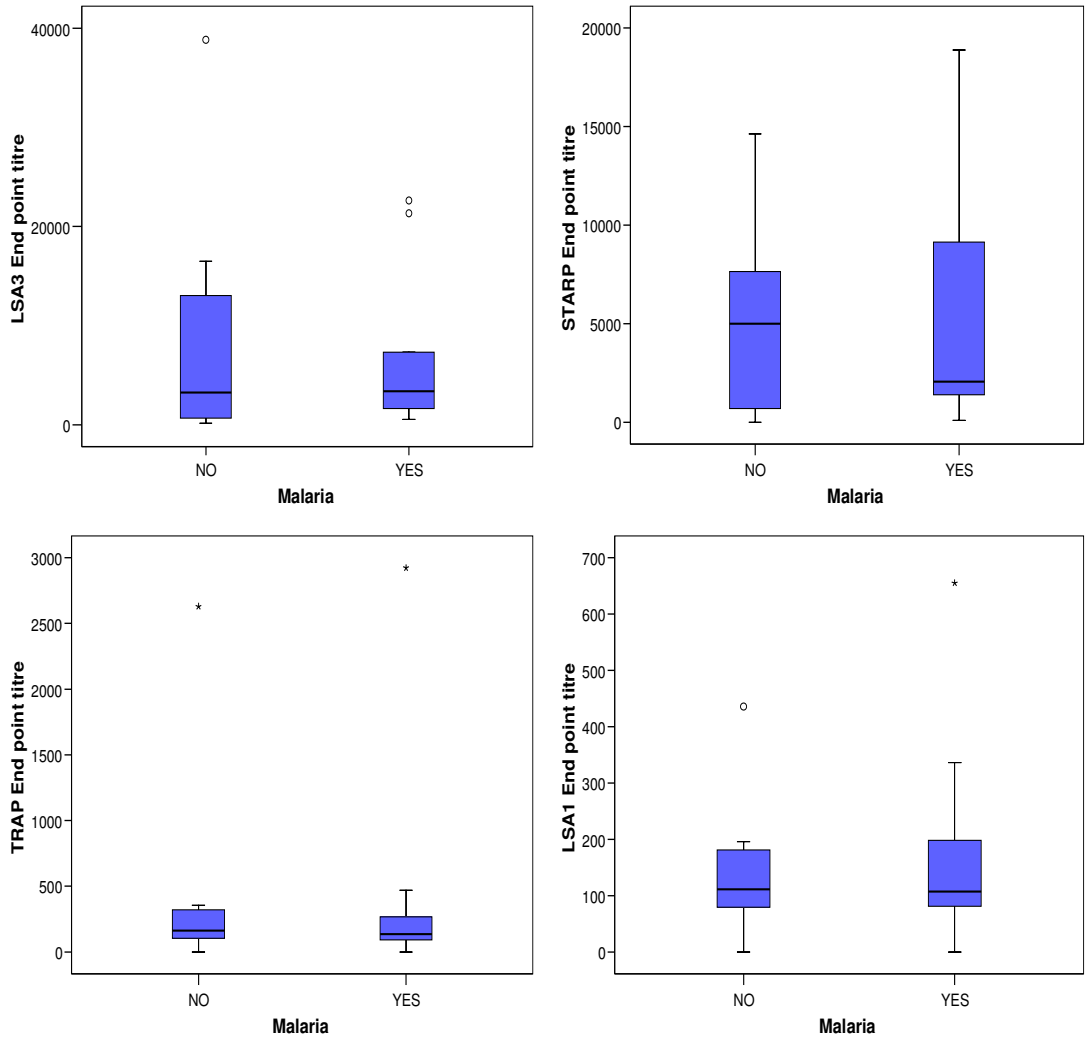

Figure 5.6: Endpoint titres by malaria outcome
(PCR positive = Yes, PCR negative = No). Mann Whitney U test was used to calculate the following p values: LSA3 p = 0.64, STARP p = 0.79, TRAP p = 0.41, LSA1 p = 0.67.

5.4 Discussion

The data presented here support the view that T cell responses to the antigens selected may play a role in protection from malaria infection. While the presence of antibodies to the selected antigens was confirmed, sometimes at low levels, in this study, there was no clear link to protection from malaria infection. However, this may be a result of the particular study from which the samples were taken. There were insufficient diagnoses of clinical malaria during the study VAC029 to allow comparison of antibody response with time to malaria infection, and so for the

purpose of this analysis time to PCR positivity has been used instead. This is a poor surrogate for malaria infection, as demonstrated by this particular trial – some volunteers in the trial received a dose of sulfadoxine-pyrimethamine (SP) to prevent new infections of malaria during the follow up period. Although none of the developed clinical malaria, many of these volunteers developed low level parasitaemia detected by PCR measurement [221]. Thus PCR positivity is not an ideal measure of malaria infection, and this effect may well have influenced the results of this analysis. In order to examine this in more detail further samples from a different study, in which higher levels of clinical malaria were observed would need to be analysed.

The T cell responses measured here do appear to be important in protection from malaria infection. However, care does need to be taken when interpreting these results. The samples used for this study were obtained after the follow up period during which data on malaria infection were collected. There is evidence that malaria infection itself modulates the immune response, leading to immunosuppression [60]. The results obtained here may just reflect this exposure to infection during the follow up period, with those who developed malaria infection remaining immunosuppressed at the time of follow up.

The difficulty with performing studies of this sort with the aim of elucidating responses that may be important in protecting individuals from malaria infection is that the immune response to malaria is extremely dynamic. Both studies here take samples from one time point, and attempt to relate them to what happens (or has happened) to that individual over a period of time. It is clear that individuals become parasitaemic all the time, and that infection is sub clinical in many. These repeated low level infections are likely to alter the immune response of that individual [109]. The ‘snapshot’ approach used here, and in many other studies of this sort, will give some guidance, but it is difficult to extrapolate from these results. In order to really evaluate the importance of particular responses in protection from infection, it will be necessary to measure these responses over a period of time.

Chapter 6 Parasite Life Cycle Modelling

6.1 Introduction

Mathematical models of parasite growth have been developed for several reasons. They have been used, by making biologically plausible assumptions, to explain observed phenomena – for example parasite diversity or density regulation mechanisms – or to predict the impact of interventions e.g. use of anti-malarial medication. Other models were intended to provide a method for estimating total parasite burden, as it became clear disease severity was related to parasite burden, but that sequestration of infected erythrocytes meant estimations of parasitaemia from peripheral blood smear were underestimating this. In the context of clinical trials of a malaria vaccine, however, models of parasite growth have other uses as well. The primary outcome measure of a Phase IIa clinical trial is protection from malaria; however, it is also useful to be able to estimate rates of parasite growth, particularly in the assessment of a vaccine that is designed to induce blood stage immunity, as a useful impact on parasite growth rate may not lead to protection in each individual tested. An accurate model of parasite growth can significantly improve the capacity for evaluation of both pre-erythrocytic and blood stage vaccines in phase IIa studies.

This Chapter contains a brief description of some of the published models in this field, and a comparison is made. Three categories of model have been used:

1. Simple models based on the observation of a sinusoidal oscillating parasitaemia with an exponential growth rate,
2. Time evolution models, which can be divided into continuous time or discrete time, and describe the rate of change of parasite density at the various stages of the parasite life cycle,

3. A sum of probability density functions describing the timings of various stages in the cycle.

The third type of model is chosen as the most pertinent to the data collected during malaria challenge studies. This model is then used to fit data from the studies already described in this thesis (VAC030 and VAC027), and estimates for parasite growth rates, and the original number of infected hepatocytes for each individual obtained.

6.2 Comparison of Models

The simplest method of calculating parasite growth rates is to measure parasite density at two or more times during the course of infection, and draw a best fit straight line through these points. Calculating the gradient of this line will give an estimate of parasite growth rate over the measured period. There are a number of more sophisticated models for estimating parasite growth and burden, the most important of which are described below, where they have been grouped into the three broad categories described. It is not just parasite growth rate that is of interest, but, particularly in trials of pre-erythrocytic vaccines, the liver parasite burden.

Some of these models have been used to predict the effect of an intervention, but never actually compared with data obtained from malaria infected individuals. Other studies have used data from the widespread use of malaria therapy for the treatment of neurosyphilis, both in the US and the UK, up to the early 1960s [222, 223] or from other sources (for example, early clinical trials of anti-malarial treatment [224]) to validate their assumptions.

6.2.1 Simple Models

Simpson et al. [222] aimed to calculate the parasite multiplication rate and the periodicity in the first week of patent parasitaemia in the absence of anti-malarial treatment, and to compare this between different strains of falciparum. They chose a simple “non-linear mixed effects model” which consisted of two terms – one to

describe the exponential parasite growth, which is linear in log10(parasitaemia), log10(p), and an oscillating sine wave function to model the observed periodicity. The equation of their model is as follows:

$$\log10(p) = a + b*t + c \sin(d*t + e) \qquad \text{(Equation 1)}$$

Where t is the time after parasites emerge from the liver, in days. Constants b, c and d have clear physical meaning:
b = ½ $\log_{10}$ (2-day parasite multiplication rate)
c = amplitude of the sine wave (the magnitude of the oscillation of $\log_{10}(p)$)
d = 2π/period of oscillation in days (the frequency of the oscillation of $\log_{10}(p)$)
The physical meaning of e, the phase (time) shift of the sine wave, is unclear.

When e = 0, and t = 0 (time of release)
a = $\log_{10}$ (initial number of parasites)

However, this is not necessarily true when e ≠ 0.

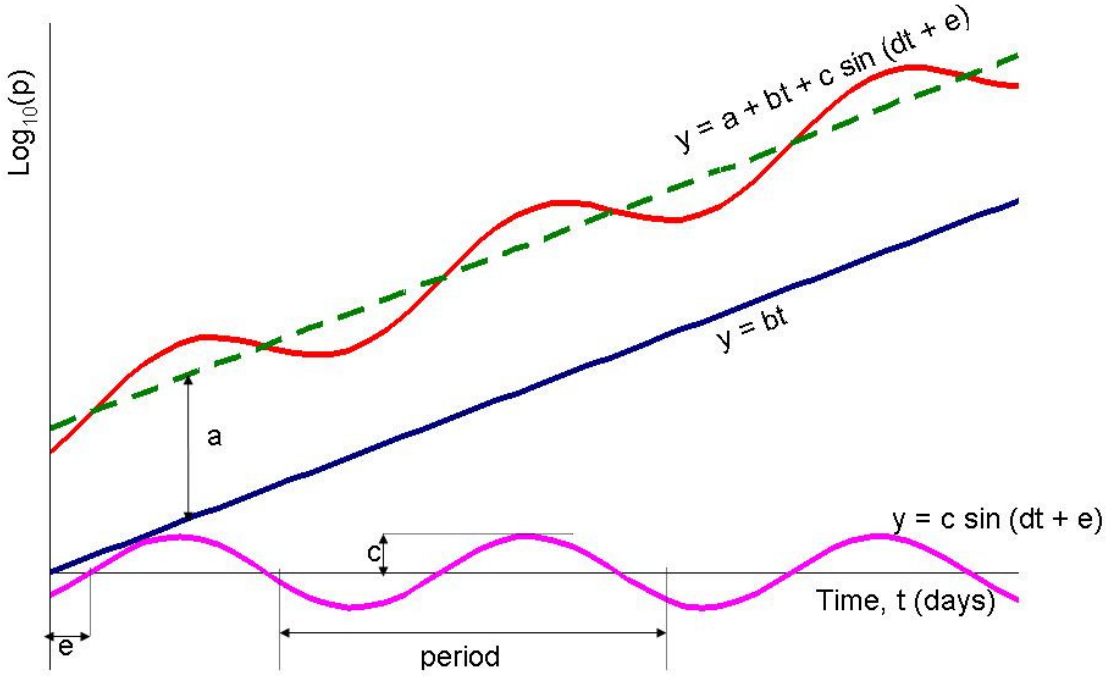

Figure 6.1: Diagram: the Simpson model.
In blue, a linear function representing parasite growth, in pink a sine wave providing periodicity and in red, the sum of the two.

Simpson et al. used this model to fit to the data from neurosyphilis patients treated with different strains of falciparum. The data was from the first week of parasitaemia in order to represent the 'expansion phase' of infection, before any induced control mechanisms could come into play. Ten different strains of falciparum were used in these treatments, and thick and thin films were made daily, and the number of parasitized red blood cells per microlitre of blood was recorded. They found a wide variation in the parasite multiplication rate, this variation was shown to be the result of both host and parasite factors. However, they admit a 'poor fit of the model' for the data from a significant number of patients - as many as 35% (93 out of 245) and they exclude those from over 50% (128 out of 245).

Another paper using this method was published by Bejon et al. [214]. Again using sine wave and logarithmic functions to provide the observed fluctuations in parasite density and growth, it assumes all parasites emerge from liver at day 7 (t = 0 at day 7); and also that they have a 48 hour life cycle (d = 2π / 2 days = π). The parasite growth rate is calculated as the gradient of straight line drawn through the sine wave (green dotted line in figure 6.1). A constant, a, for initial parasite density is included in the equations, allowing back calculation of original numbers of parasites released from the liver, and hence estimation of the number of infected hepatocytes, although this is problematic, as discussed below. This method is essentially the same as that described above [222]. The model was used to fit data from sporozoite challenges of volunteers participating in malaria vaccine studies such as the ones already described in this thesis.

Although adding a sine wave function and a logarithmic function appear to fit the data available (or at least some of it, in Simpson's case), it doesn't take into account what is understood about the biology of the malaria parasite. For example, it seems unlikely that all parasites are released from the liver at the same time in each volunteer, in the same way as asynchronous populations of parasites are observed in the blood stages. So assuming that all parasites are released by day 7 may not be valid. The assumption that the cycle time for each parasite is exactly 48 hours is also not based on good evidence. Simpson et al. [222] observed that the length of the parasite life cycle was generally greater than this, quoting a population mean of 55 hours (2.3 days). Interestingly, they also observed that there was negligible inter-patient variability in this parameter. It is not clear why this observation is not more widely accepted. Using this model to estimate the original numbers of parasites released from the liver, and hence extrapolating the number of infected hepatocytes is problematic. When the phase, e = 0, the original number of parasites released at time t=0, is calculated by the formula:

$$p(t=0) = 10^{a} \qquad \text{(Equation 2)}$$

However, if, in order to fit the data better, e has to be adjusted (perhaps because of variability in the release time) by half a day, then the estimate of parasites released from the liver according to this model is

$\log(p) = a + c \sin(e)$ (Equation 3)

$p(t=0) = 10^{a + c \sin(e)} = 10^{a}\ 10^{c \sin(e)}$

Taking 10^{a} as the estimate for the original number of parasites could, therefore be wrong by a factor as large as $10^{\pm c}$.

If Equation 3 is used to estimate the original number of parasites, it implies that this number depends on the error in the estimation of release time, which is implausible. Because the release time is fixed at day 7, the phase parameter e has been introduced to account for any variability in this in practice. Unfortunately, this affects the estimate of the initial number of parasites p(t=0), where the line crosses the y axis. Any difference in the release time from day 7 therefore translates into an error in the estimate of the initial number of infected hepatocytes. This would not be a problem if the uncertainty in the release date were small, but for a variability of +/- ½ day (1/4 cycle), p (t=0) varies between 10^{a-c} to 10^{a+c}.

This introduces substantial uncertainty into the method for the calculation of estimates for numbers of infected hepatocytes. In practice, this error is significant. As one of the principal reasons for developing such a model is to allow back calculation of numbers of infected hepatocytes, this is a major drawback of this technique.

Even if the preset variables (release time, period) were allowed to vary, this model is unrealistic because it is not derived from an understanding of the biology of malaria.

6.2.2 Time Evolution Models

6.2.2.1 Continuous Time Models – Partial Differential Equations

Anderson 1989 [225] proposed a set of three coupled differential equations representing the rates of change in numbers of uninfected erythrocytes, parasitized erythrocytes, and merozoites over time. Their purpose was to compare the effectiveness of immune response against merozoites and parasitized red blood cells, although the terms relating to immune responses can be excluded from the model if desired. A shortcoming of this model is the inherent lack of any synchronicity or periodicity emerging from the model. Even if one assumes that all merozoites emerge from the liver during a limited time-window, no synchrony of the erythrocytic phase would be expected by this model, contrary to observed data. They use numerical simulation alone to test the model, and make no comparison at all with observed data.

Another example of this kind of model is that proposed by White et al. [224]. This model makes two specific assumptions about the life cycle of falciparum parasites: that the period of the cycle is 48 hours exactly, and that all parasites are sequestered once they have reached an age of 26 hours. The partial differential equations were formulated to calculate the distribution of parasite stages, and assume that these form a Gaussian distribution. They were fitted by inspection to the parasite counts obtained from 4 patients in an unsuccessful trial of Ciprofloxacin for the treatment of falciparum malaria infection. Parasite density was assessed 6-12 hourly in these patients, by thick and thin films stained with Giemsa, and following the failure of Ciprofloxacin all patients were treated successfully with quinine. The parasite multiplication factor was estimated from the ratio of parasitaemia at the time of quinine treatment to that 48 h previously (equivalent to the best fit straight line approach).

These first two models (by Anderson and White) have been taken up by Hoshen et al. [226] and combined to form a theory which can explain the periodicity of malaria solely through the 3-compartment (RBC, IRBC and merozoites) approach. This model again uses partial differential equations to describe the process of

erythrocyte invasion, parasite survival and killing induced by immune responses. A 48 hour parasite life cycle is again assumed, and the parasite multiplication rate is also preset at r = 16. However, they do fit their model to data obtained from one malaria therapy patient, and it seems to fit this data reasonably well.

In 1991 Kwiatowski and Nowak [227] published a model that investigated the role of fever in inhibiting parasite growth and promoting parasite synchronisation in vivo. This was based on the observations that febrile temperatures are related to parasite growth cycle in erythrocytes, and, at the same time, damaging to P. falciparum parasites, particularly during the second half of their cycle. In this model, all parasitized red blood cells are divided into 4 ‘ages’, through which they progress until schizont rupture. The mathematical model uses partial differential equations to predict the survival of parasitized erythrocytes, and the passage of parasites from one cycle to the next. It behaves in a qualitatively realistic way (see also Molineaux and Dietz [228]) with numbers of PRBC rising rapidly to a plateau and then oscillating, a situation which generates a periodic fever, as observed in vivo. It also predicts irregular parasite population dynamics at high multiplication rates, consistent with the classical observation that P. falciparum causes less regular fever than other species of parasite. Gravenor et al. [229] describe a continuous time model based on that of Kwiatowski. This model is backed up by in vitro data obtained from parasite cultures, maintained at varying temperatures to model the situation in vivo. The parasite multiplication rate is included in the model; however, it is preset by the investigators depending on the situation they wish to model e.g. parasite multiplication rate (r) of 4 per cycle – slow parasite growth, r = 16 per cycle – rapid parasite growth. Their suggestion of a ‘pyogenic threshold’, a threshold parasite density above which the host will develop fever, is plausible. It is likely that this threshold is host dependent, indeed Jeffery et al. 1959 [230] observed that patients receiving malaria therapy differed substantially in the parasite density associated with fever. However, when large variation in pyogenic threshold (observed in vivo) is incorporated into this model, it produces a large variation in the periodicity of parasite infections, which does not fit with in vivo experience. There is no attempt to fit either model to measured data from individuals with malaria infections.

6.2.2.2 Discrete Time Model

Molineaux et al. [223] formulated a mathematical model for falciparum asexual parasitaemia. They selected data from 35 Afro-American neurosyphilis patients who were classified as 'spontaneous cures' from malaria treatment. They had no history of exposure to malaria, and were inoculated either by infected blood, or by infectious mosquito bite. Nearly half of them (16/35) received at least one dose of anti malarial medication during their therapy, and data was collected over a period of up to 250 days, all of them were slide negative for a period of at least 5 months before they were finally given 'curative treatment' and discharged. The model assumes a period cycle of 2 days, on the basis of observations of bouts of 2 day periodicity. It uses observations from Plasmodium knowlesi and chabaudi infections, showing in vivo evidence of the association between successive recrudescences of parasitaemia and expression of different variants of those parasites PfEMP1 analogues [231, 232] to incorporate assumptions of the appearance of variant strains of parasite, expressing variant PfEMP1. This was necessary to account for multiple episodes of apparent clearance of parasitaemia, followed by a recrudescence. This occurred as many as 14 times in an individual patient. The variables of interest in their model therefore, are parasite variant multiplication rates (which they assume to be normally distributed over a large range), and three functions describing the effect on parasite multiplication of the immune response, divided into three elements:

- innate and variant transcending,
- acquired and variant specific,
- acquired and variant transcending.

The effects of these immune responses are formulated as probabilities (the probability that the parasites escape their effects) and the product of these three functions gives the probability that the parasite successfully escapes the effects of all three. The model simulates infections as if they are monoclonal, with a single pool of 50 variants. They did find a large range of parasite variant growth rates, which appeared dependent on both host and parasite factors. Their main focus however, was on accurately modelling the expressions of variant strains of parasite, based on the assumptions of a biologically plausible mechanism. The model itself is not directly applicable to the data obtained in our malaria vaccine

trials, as the length of infection is rarely sufficient to allow emergence of new strains of parasite, and certainly not long enough for spontaneous resolution of the sort seen in these patients to occur. An investigational vaccine may also alter the pattern of emergence of variant strains of parasite, and this would also need to be included if this model were to be applied to data from vaccine trials. The model is included in this comparison as the main example from this category. It also forms the basis of the most comprehensive model yet published, described below.

Smith et al. have published a series of papers developing a dynamic model for prediction of the epidemiologic and economic impact of a malaria vaccine [233]. Their model aims to take into account the characteristics of individual P. falciparum infections, short and long term effects on the vaccinated individual, the interdependence of hosts and epidemiological impact of a vaccine on transmission, and a cost effectiveness analysis. This is a complex model, and in order to perform the computational analysis, they divided it into a number of sub-models, which are then combined. An important strength of this framework is that it ties together an ensemble of interconnected sub-models validated against actual field data from various settings across Africa. This is the most comprehensive population-based simulation of malaria yet developed, and so might be expected to be useful in this context. However, it is designed for use in studying the effect of a malaria vaccine on an African population, and not really for use in analysing data obtained from vaccine trials in the UK. The sub-model relating to the early stages of blood stage infection [234], that could potentially be relevant in this scenario, is largely based on that originally published by Molineaux et al. [223], described above. The variables of interest here are four parameters: the multiplication factor, the time of onset of adaptive immunity, and the parasite densities at which the innate and adaptive immune responses become active. Although useful in modelling the effects of hypothetical blood-stage vaccines, for example, and informing decisions about the endpoints and outcomes used in trials of such vaccines, it is not possible to compare this model usefully to the available data because of the number of variables involved and the complexity.

6.2.3 Probability Density Functions

More recently, Hermsen et al. [203] have published a statistical method for calculating parasite growth rates. Their data comes from volunteers participating in challenge studies to test malaria vaccines, such as those described in this thesis, and they have used it to develop a statistical model, allowing estimation of various parameters including length of parasite life cycle and multiplication rates. Their model is of parasite densities expressed as a non linear function of time (days) since infection. They assume that the timing of appearance of the first and subsequent generations of parasites will be normally distributed; for example, that the timing of hepatocyte rupture, and first release of parasites into the blood, will be normally distributed, and that it may therefore be modelled using a normal probability density function. The duration of the presence of a given ring form is also assumed to be normally distributed and so is modelled in the same way. The convolution of both probability density functions thus gives a model of the timing of the disappearance of ring forms. They then perform a summation over all the parasite stages to estimate the total number – this is illustrated in figure 6.2.

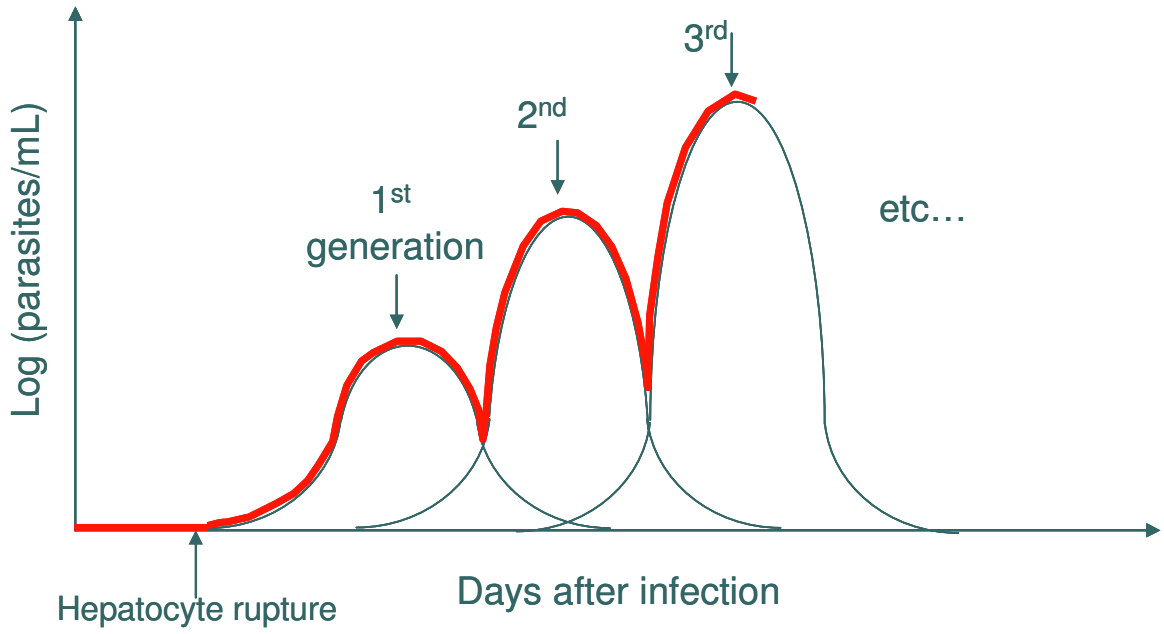

Figure 6.2: Hermsen model

Using the Hermsen model results in this graph of expected parasite density (log parasites/ml) in days following malaria infection. It shows the appearance and

disappearance (sequestration) of successive generations of blood stage parasites. The red line is the sum of all blood stage parasites from all cycles.

Similar biologically plausible assumptions made about the timings of the appearance and disappearance of subsequent generations of ring forms produce a statistical model which is then fitted to real data using a least squares optimization routine, and produces estimates of various parameters. The main drawback of this model is the number of parameters included in it – there are as many as nine unknown variables. This means that more data points are required in order to find a solution. In their paper, the authors fix some of these to constrain the optimizations. However, this model is still the one which most closely matches the problem of modelling parasite densities early in malaria infection, prior to volunteers becoming thick film positive. The model is fitted to data from volunteers participating in challenge studies, without any problems of poor fit, and by incorporating the variability of some parameters makes fewer assumptions about the behaviour of the parasites.

6.3 Modelling of PCR Data from Clinical Trials

The Bejon variant of the Simpson model, and that published by Hermsen were selected for further comparison. The Bejon variant was selected as it had been used to analyse data in Oxford previously, and is representative of the Simpson approach, and, after the Hermsen method, it is the best suited to analysing data from early in infection and calculating the variables that we are particularly interested in. The Bejon method is appealing because it is simple, although it has no clear foundation in biological terms. The main appeal of the Hermsen model, is that the terms have clear biological meaning and represent biological quantities. An additional advantage of the latter, because it relates clearly to the biology, is that it has potential for modelling the effects of a malaria vaccine, if terms predicting immune responses could be included, although it has not been used this way in this study.

For the comparison itself, data obtained in the vaccine trial VAC030, described in chapter 4 of this thesis, was used. Both models were incorporated in an Excel™

spreadsheet and the built in constrained optimisation algorithm was used to compare the values calculated by the relevant model to measured data for each individual. The optimisation routine calculated the sum of squared differences between the model and measured data, and changed the variables included in each model within specified, realistic limits, in order to minimise the sum. The constraints that were included within the optimisation routine are described in table 6.1. The variances were preset at the values shown, these were derived from the original paper published using this model [203]. No limits were required for the Bejon model.

Parameter	Notation	Units	Constraints
Number of infected hepatocytes	X		
Ring forms per hepatocyte per ml blood	B1		
Total no. of ring forms released from liver per ml blood	X*B1	number/ml	>1, <1500
Multiplication factor relating max. no. of ring forms of one cycle to previous cycle	B2		>1, <50
Mean duration until sequestering of ring forms	mu2	days	>0.8, <1.5
Variance in duration until sequestering of ring forms	Sig2^2	days^2	0.004
Mean duration until appearance of 1st generation ring forms in blood	mu1	days	>6.3, <7
Variance in duration until appearance of 1st generation ring forms in blood	Sig1^2	days^2	0.03
Mean duration until appearance of new generation ring forms in blood	mu3	days	>0.8, <1.5
Variance in duration until appearance of new generation ring forms in blood	Sig3^2	days^2	0.004

Table 6.1: Hermsen model parameters

This table shows the parameters, notation and limits used to perform the constrained optimisation routine using the Hermsen model.

In order to demonstrate this comparison, the following graphs are representative of data from 3 volunteers from VAC030. Each graph is of parasite density against

time, and the measured PCR data is plotted, along with the data calculated using each of the models.

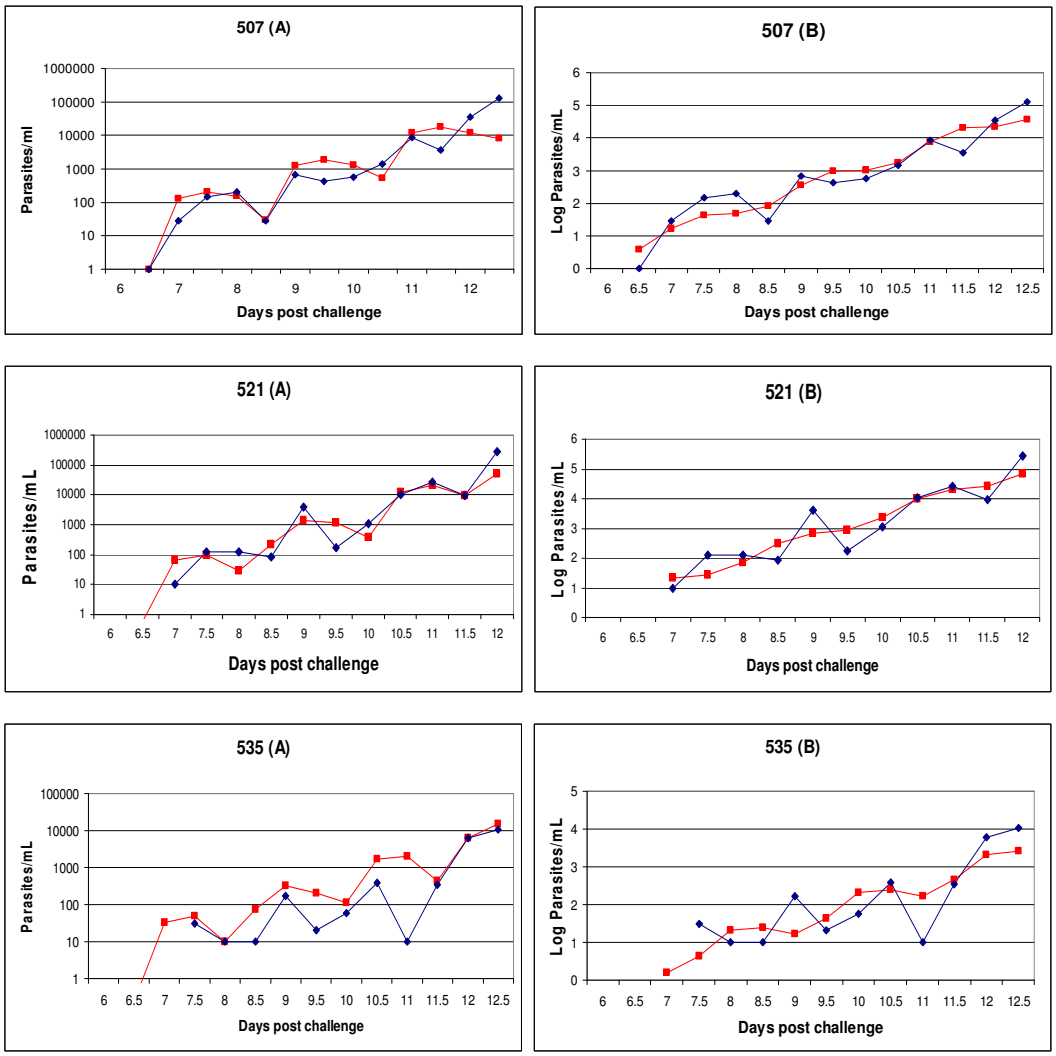

Figure 6.3: Graphs of data from VAC030.
The measured PCR data is plotted on each graph in blue, and the model in red. For each volunteer graph (A) shows the result of fitting the Hermsen model to the data, whilst graph (B) shows the Bejon model.

For these three subjects at least, the Hermsen model appears to visually fit the raw data from early malaria infection better than the Bejon model. The 'goodness of fit' can be compared statistically, using the sum of squared errors for each

model, and in this case the Hermsen model gives lower values (i.e. is a closer fit of the data) than the Bejon model. This is also demonstrated in table 6.2, which gives the values of the sum of the squared error for each model by volunteer for those illustrated, and the summed value for all volunteers.

Volunteer	A (Hermsen)	B (Bejon)
507	1.84	2.44
521	1.84	2.67
535	1.61	4.56
SUMMED ERROR (all volunteers)	47.7	70.9

Table 6.2: Error values for each model

Similar comparisons were made for all subjects from VAC030, and the squared error was lower with the Hermsen model than the Bejon model in the majority of the subjects, and gave a lower total summed error. Although growth rates calculated using these two models were different, they did have a positive correlation (see graph; Pearson correlation 0.63, $p < 0.01$).

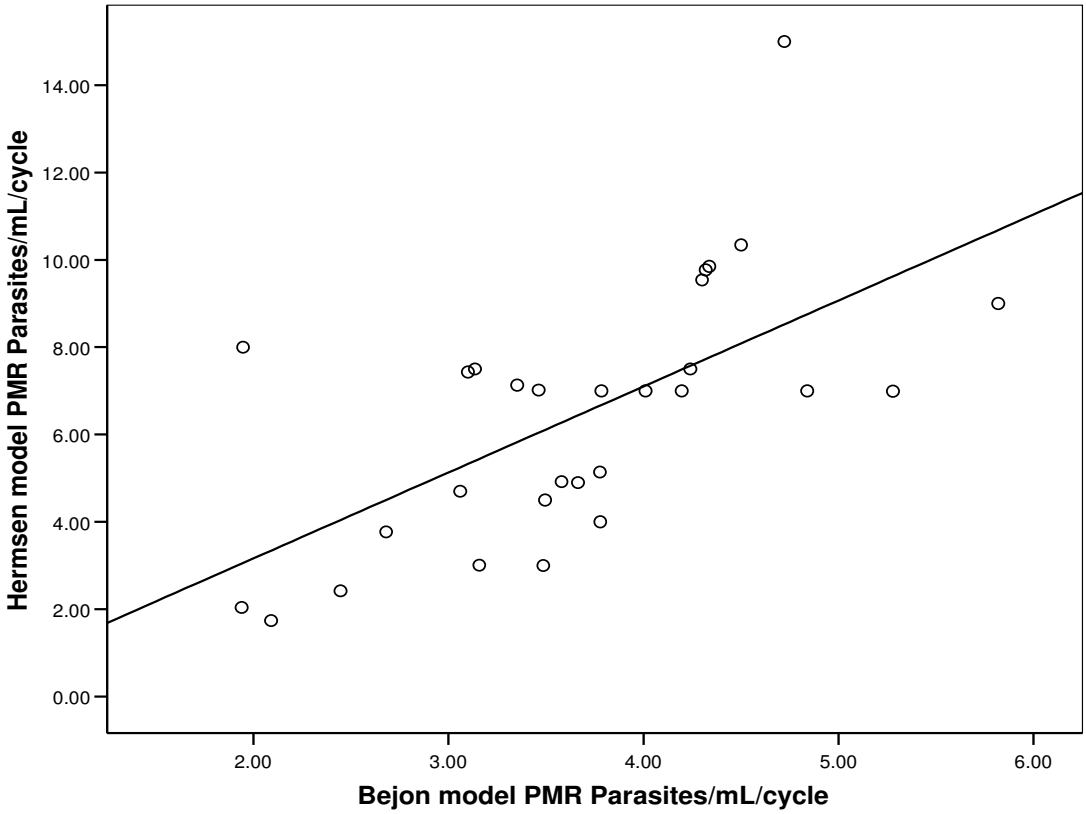

Figure 6.4: Scatter plot of parasite growth rates (PMR)
Scatter plot demonstrating the positive correlation (shown by best fit straight line) of values calculated by the Bejon and Hermsen methods. Data from VAC030.

In order to provide another independent method to assess these two models, an estimate of the initial number of parasites released into the blood of each volunteer was made using each one. This was then used to estimate the number of infected hepatocytes (making the standard assumptions that each infected hepatocyte releases 20,000 merozoites, and that the circulating volume is 5 litres). Volunteers receiving liver stage targeting vaccines might be expected to mount an immune c response against infected hepatocytes, so this number may be used as a surrogate marker of efficacy of these vaccines. However estimates of numbers of infected hepatocytes varied substantially from one model to the other, and there is no correlation between the two (see graph; Pearson correlation coefficient 0.069, p = 0.7).

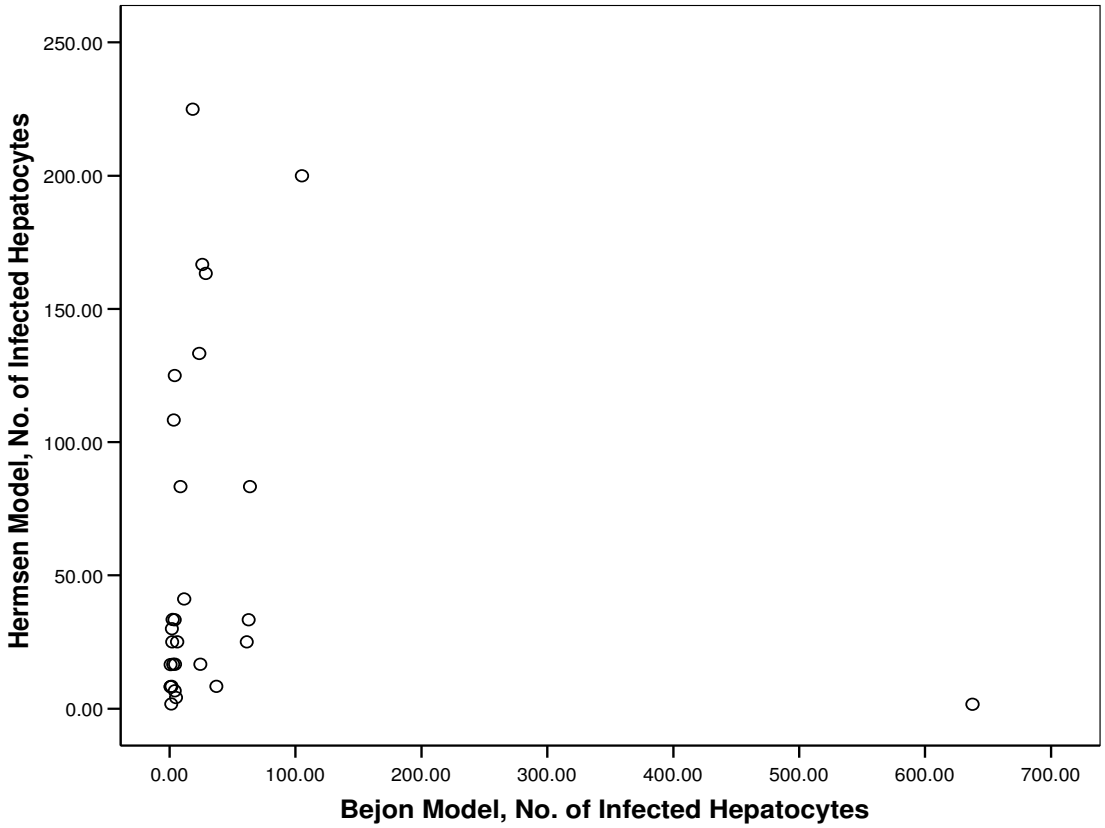

Figure 6.5: Scatter plot of numbers of infected hepatocytes
This scatter plot shows the estimated numbers of infected hepatocytes calculated using each model, and demonstrates the lack of correlation between the two models. Even if the one outlier is removed, the correlation between the two models is poor (Pearson correlation coefficient 0.4). Data from VAC030.

These estimates of infected hepatocyte numbers were then plotted against the day of diagnosis. Volunteers with high numbers of infected hepatocytes would be expected to have higher parasitaemias earlier in infection, and hence to be diagnosed before those with a lower infectious burden. A negative correlation between liver parasite burden and day of diagnosis would be expected. Using Spearman's correlation, for both models, the number of infected hepatocytes correlates with day of diagnosis (statistical model – Spearman's correlation coefficient = 0.77, $p<0.01$, sine model – Spearman's correlation coefficient = 0.386, $p = 0.038$). However with the Bejon model, this is a positive correlation, suggesting the model is wrong. Scatter plots of the data are shown for comparison – the following graphs show the data from the Bejon model, with a positive

correlation, and that from the Hermsen model. Best fit straight lines through the data points illustrate the positive and negative correlations.

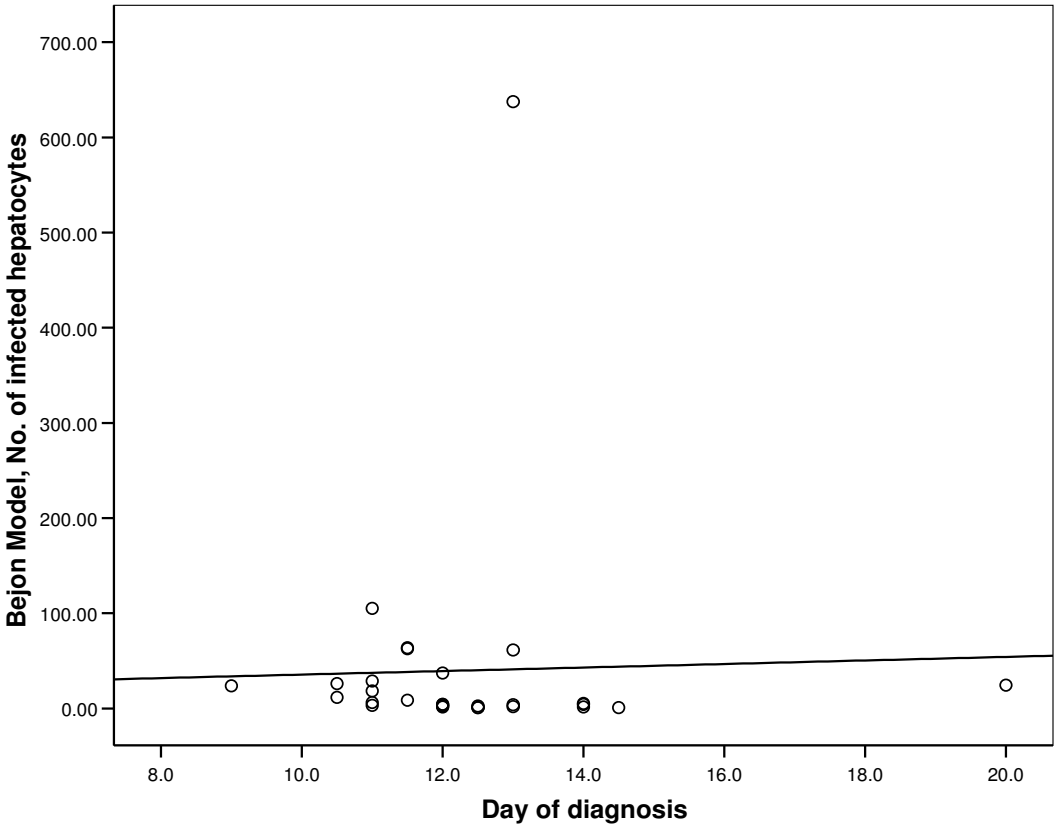

Figure 6.6: Scatter plot of infected hepatocytes (Bejon) and day of diagnosis.
The lack of correlation between the estimated number of hepatocytes calculated using the Bejon method and the day of diagnosis is shown. If the one outlier is removed, the gradient does become negative, however there is no correlation (Pearson correlation coefficient 0.165, p = 0.4) Data from VAC030.

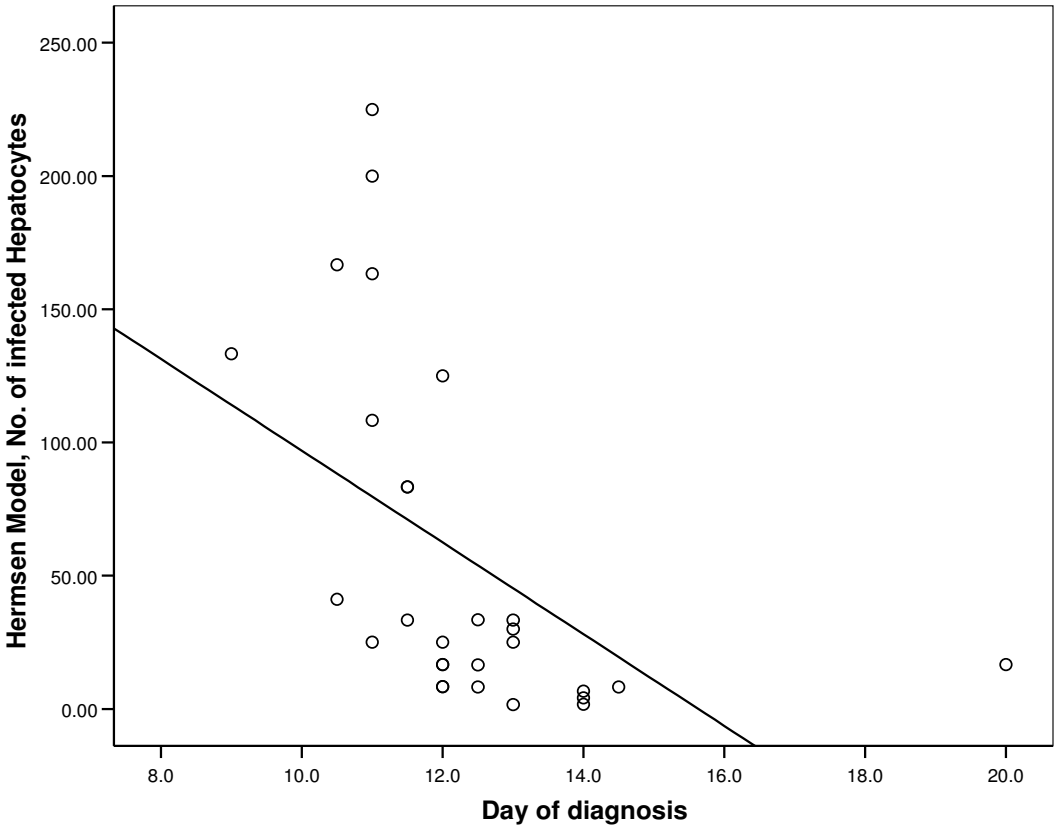

Figure 6.7: Scatter plot of infected hepatocytes (Hermsen) and day of diagnosis
The number of infected hepatocytes calculated using Hermsen's model, correlates with the day of diagnosis, as shown by the best fit line.

6.3.1 Delay to Parasitaemia and Liver Parasite Burden

As many of the vaccines used in trials in Oxford have been primarily targeting liver stage antigens, it is particularly useful to be able to obtain an estimate of the initial number of infected hepatocytes. While we assume that any significant reduction in the liver parasite burden might result in a delay in the time to patent parasitaemia, it is possible that in the stringent challenge used in these studies, there are so many liver stage parasites that they overwhelm any induced immune response. In order to examine this in more detail, the data collected from two previous studies was examined. The trials, in each of which a group of vaccinated volunteers received FFM ME-TRAP, demonstrated a delay in time to patent parasitaemia in vaccinated volunteers in these groups, suggesting a vaccine induced impact on the liver stage burden. The Hermsen method was used to model PCR data from

volunteers involved in this study, and the estimated number of infected hepatocytes was calculated for each volunteer. One would expect the group of vaccinated volunteers, in which a delay to diagnosis was seen, to have significantly fewer parasites released from the liver, resulting in a reduction in the calculated number of infected hepatocytes. Sporozoites which infect hepatocytes but which fail to transform into merozoites because of an induced immune response would not be included in the model because there would be no measurable effect on the numbers of blood stage parasites, so the estimated number of infected hepatocytes is a measure of successfully infected hepatocytes.

PCR data from 19 vaccinated volunteers and 8 controls was used for these calculations; the volunteers participated in trials VAC012 and VAC015. The malaria challenge was performed as described elsewhere in this thesis. Of the 19 vaccinees, data was available for only 16, (for 1 blood was collected by finger prick therefore there was no PCR data available, and for 2 there was insufficient data – only one or two positive PCR results). The mean number of infected hepatocytes for the 16 vaccinated volunteers was 66 (95% confidence intervals 30 – 101, standard deviation 67) and for the 8 controls volunteers it was 168 (95% confidence intervals 145 – 191, standard deviation 28). This does represent a significant reduction in infected hepatocytes in vaccinated volunteers (Mann Whitney two tailed $p = 0.005$) of 61%. The following box plot demonstrates the differences.

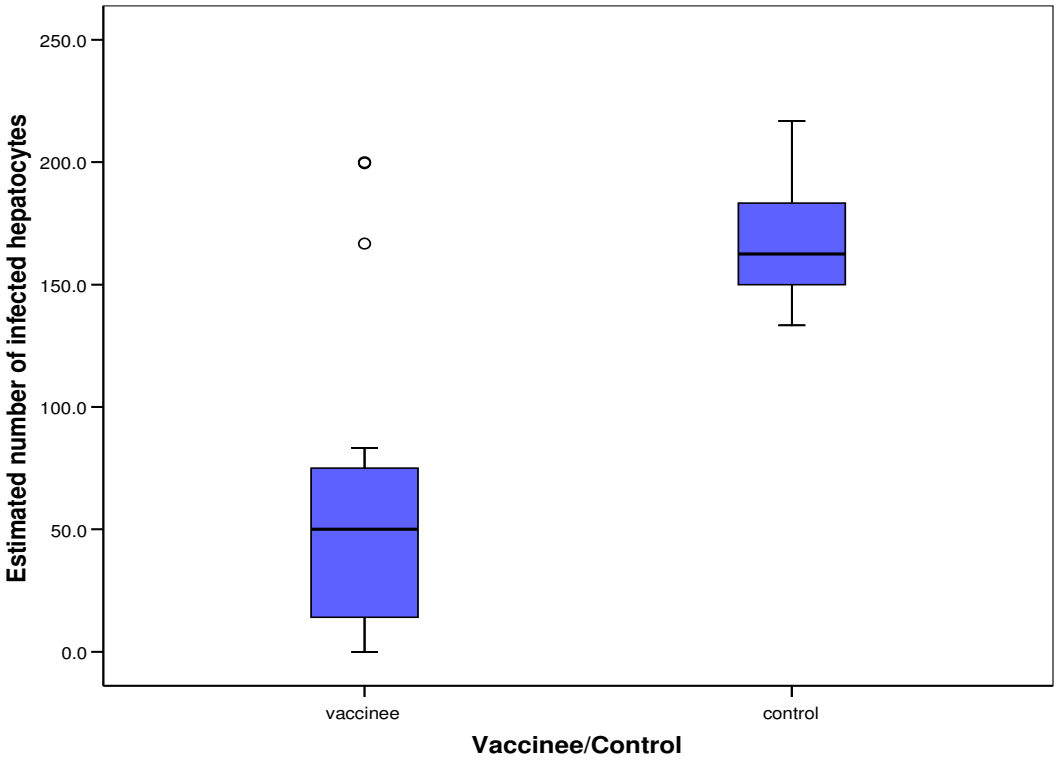

Figure 6.8: Box plot of infected hepatocytes in vaccinees and controls
The estimated number of infected hepatocytes for vaccinated volunteers and controls participating in VAC012 and VA015. For Vaccinees n=16 and for Controls n=8. The outlying points in the Vaccinee box represent 3 volunteers, there were only 5-7 positive PCR results available for these volunteers, so while it is possible to fit the model, it is hard to be sure of the accuracy.

6.3.2 Conclusion from Comparison of Methods and Modelling Data

The Hermsen model seems to be the most likely to give realistic results, as it is based more upon the biological information we do have about parasite growth and invasion. The Hermsen model therefore appears to be superior to that of Bejon et al. in several important respects:

i) The assumptions made fit the biology of the malaria parasite life cycle as it is currently understood.

ii) Visually, the model fits the data from trial volunteers more closely, and the summed squared errors calculated are consistent with this better fit.

iii) Using the model to estimate the number of infected hepatocytes (a procedure that requires the same assumptions to be made, whichever model is used) produces a number that correlates with the day of diagnosis of the volunteer with malaria. This shows that the model reflects the in vivo situation more accurately than the Bejon model.

When the Hermsen model is used to calculate numbers of infected hepatocytes in groups of volunteers in whom a delay in time to patent parasitaemia was recorded, a reduction in the number of infected hepatocytes is seen in these volunteers versus controls, as would be expected. This is also true when the Bejon model is used to perform this calculation.

On the basis of this, the Hermsen model was used for further analysis of parasite growth rates. Using this statistical model, the rates of parasite growth in groups of vaccinated and control volunteers for various studies are compared.

6.4 Results

6.4.1 VAC030 Results

Using the Hermsen model to fit data obtained from volunteers taking part in VAC030 allowed estimation of parasite growth rates for each one. These are shown in figure 6.9, along with mean rates for each group of volunteers.

As described in Chapter 4, Group 1 were vaccinated with PEV3A alone, Group 2 received both PEV3A and FFM ME-TRAP, and they were all challenged along with a group of unvaccinated control volunteers. The mean rate of blood stage parasite growth in volunteers in group 1 was 5.7 parasites per ml per cycle (95% confidence intervals 4.1 - 7.3; standard deviation 2.6), for group 2 this was 6.3 (95% CI 4.0 - 8.5; SD 3.5) and for controls it was 8.7 (95% CI 7.2 - 10.2; SD 1.2) (figure 6.9). Comparing the growth rates of vaccinated volunteers, those in group 1 are not significantly different to those of volunteers in group 2 (Mann Whitney U test; two-tailed $p=0.63$). Comparing group 1 to controls, growth rates are

significantly lower in group 1 volunteers than in controls (Mann Whitney U; two-tailed p = 0.02). For group 2 versus controls, there is again a significant difference (Mann Whitney U; two-tailed p = 0.02). Grouping all vaccinated volunteers together, and comparing them to controls, there is again a significant reduction in parasite growth rates; (Mann Whitney U; two-tailed p = 0.012).

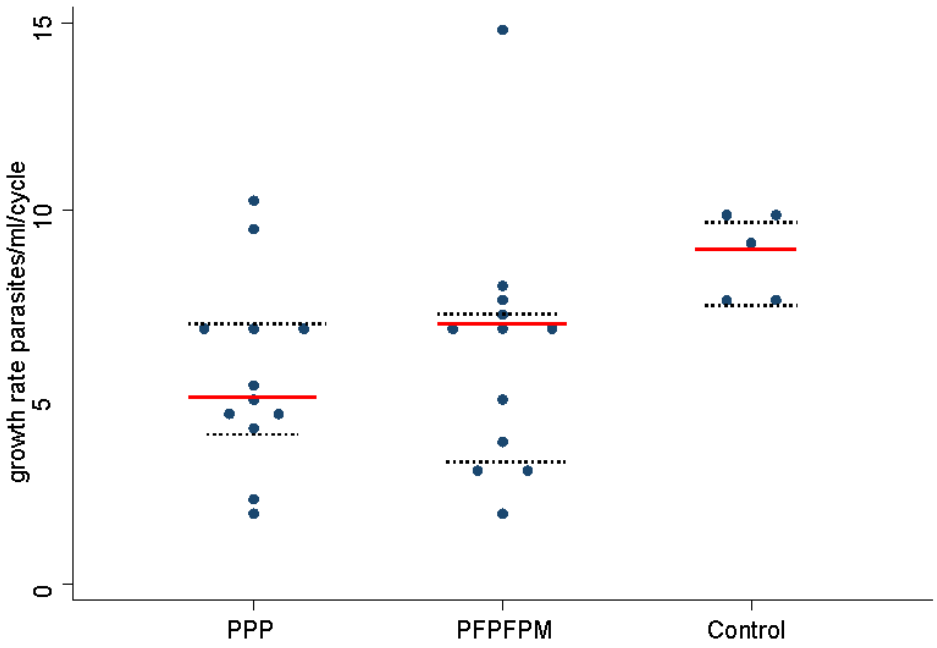

Figure 6.9: Scatter plot of growth rates by group
Red lines represent mean for each group, dotted lines are inter quartile ranges.

There is a significant difference in the parasite growth rates in vaccinated volunteers versus controls. This is observed in both groups of vaccinated volunteers, and is therefore likely to be related to vaccination with PEV3A rather than the FP9 or MVA ME-TRAP. This data brings us to the conclusion that PEV3A has induced an immune response that has an effect on parasite growth rates, although this was not sufficient to prevent or clear infection in this study.

Looking at the estimated numbers of infected hepatocytes in the different groups, the following diagram shows the individual data by group, again with red lines

representing the mean for each group. This suggests that there may be a difference between the groups, with volunteers in group 2 having lower numbers of infected hepatocytes – see figure 6.10. However, statistical analysis demonstrates no difference between groups 1 & 2 versus controls (Mann Whitney U test; two-tailed p=0.34) or between either group of vaccinated volunteers and controls (group 1 versus controls, Mann Whitney U test; two-tailed p=0.34, group 2 versus controls, Mann Whitney U test; two-tailed p=0.43).

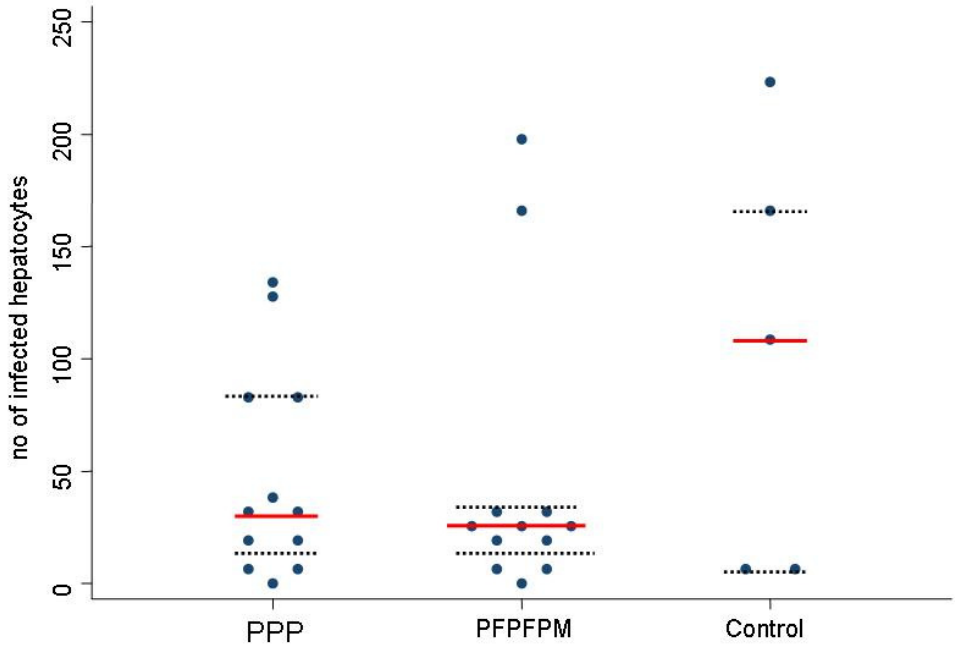

Figure 6.10: Scatter plot of numbers of infected hepatocytes by group.
Red lines represent mean for each group, dotted lines are inter quartile ranges.

6.4.2 VAC027 Results

Using the Hermsen model, the results from the challenge of VAC027 were also analysed. To recheck that the model remained valid for this data, the estimated number of infected hepatocytes was plotted against day of diagnosis, and as before there was the expected negative correlation between the two variables

(Spearman's correlation coefficient = 0.70, $p < 0.01$). This is demonstrated in figure 6.11.

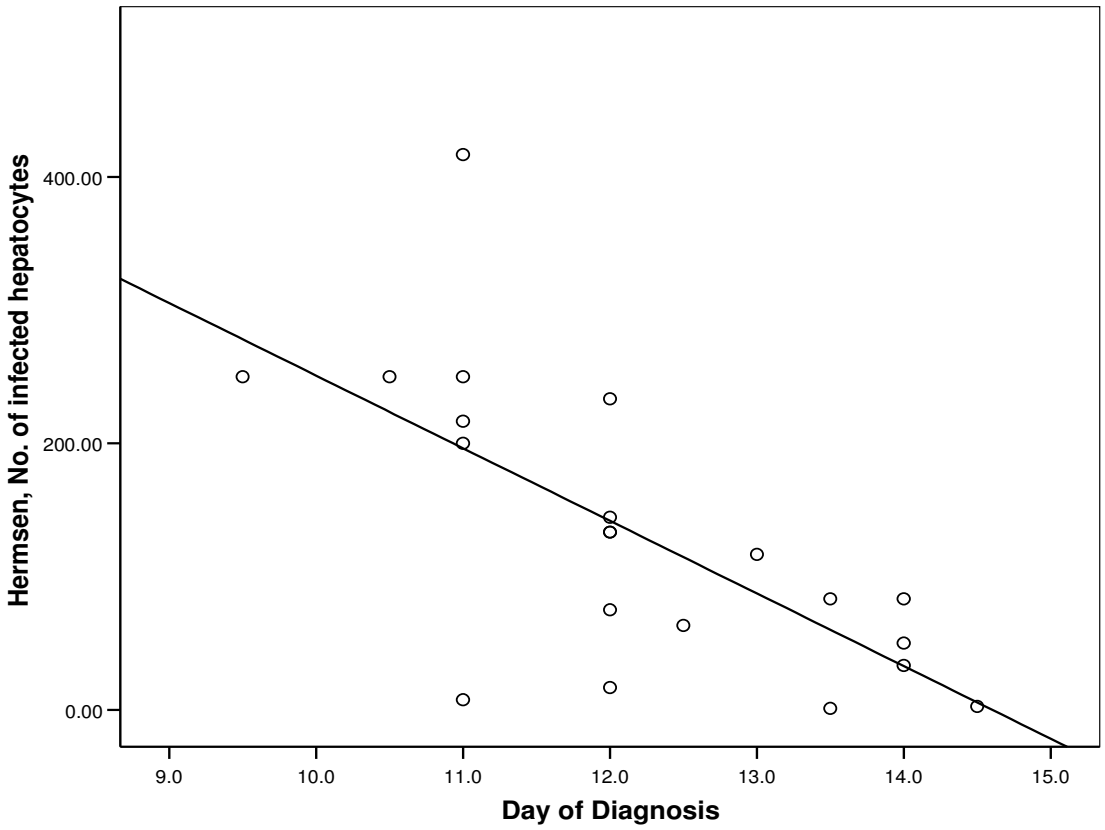

Figure 6.11: Scatter plot of number of infected hepatocytes vs. day of diagnosis Calculated using Hermsen model. Data from VAC027

The mean rate of blood stage parasite growth in volunteers in VAC027 were as follows: in group 6, 7.5 parasites per ml per cycle (95% confidence intervals 3.9 – 11; standard deviation 4.2), for group 7 this was 7.4 parasites per ml per cycle (95% CI 4.1 – 10.7; SD 3.5) and for controls it was 9.1 parasites per ml per cycle (95% CI 7.4 - 10.8; SD 1.6).

There was no significant difference in growth rates between different groups of vaccinees, (Group 6 vs. Group 7, Mann Whitney U test, two tailed $p = 0.95$) or between control volunteers and those who received either vaccine regimen (Group 6 plus Group 7 vs. Controls, Mann Whitney U, two tailed $p = 0.19$).

As the vaccines used in VAC027 have both a liver stage and a blood stage component, as well as looking at the blood stage parasite growth rates, it is also useful to examine the numbers of infected hepatocytes in each group of volunteers. In this case, the numbers of infected hepatocytes were as follows:
Group 6 mean 146 95% CI 60 - 232 SD 103
Group 7 mean 133 95% CI -3 - 270 SD 148
Controls mean 109 95% CI 33 - 183 SD 71

There was no significant difference in the numbers of infected hepatocytes, either between group 6 and group 7 (Mann Whitney U, two tailed $p = 0.56$), or between all vaccinated volunteers together and controls (Group 6 + Group 7 vs. Controls, Mann Whitney U, two tailed $p = 0.76$).

6.5 Discussion

A selection of the most relevant published models have been described, and the major assumptions used discussed. The Hermsen model was selected as the most appropriate for use with the data collected during the clinical trials performed in Oxford following direct comparison with the Bejon model, previously used for this purpose. Using this Hermsen model, it has been possible to show that some of the candidate malaria vaccines tested in these studies in Oxford do exert some effect on blood stage parasite growth.

VAC030 was the first to provide evidence of vaccine-induced blood stage protection in a healthy volunteer challenge study. An effective immune response against blood stage parasites has previously been achieved in a rodent malaria model by subpatent infection by blood stage malaria parasites [235], and recurrent subpatent infections of human volunteers has also been shown to induce protection from a subsequent challenge [109]. A study evaluating the efficacy of a candidate AMA1 vaccine in monkeys demonstrated some good protection from P. falciparum blood stage challenge [236]. This vaccine has been tested in a Phase I

clinical trial in humans, and has been shown to elicit functional antibody responses [206], however as yet there has been no evidence of vaccine induced protection from infection in humans.

Low levels of immunogenicity were measured throughout VAC027. It is perhaps therefore not surprising that no measurable effect on numbers of infected hepatocytes was seen. The blood stage components of the vaccine did not appear to have any effect on blood stage parasite growth either. The use of this model enables the conclusion that no part of the vaccine (neither the pre-erythrocytic components nor the blood stage antigens) has induced any useful immune response, even an only partially protective one.

While it is useful to be able to model blood stage infections in this way it is also useful to be able to estimate the effect vaccines may have on liver stage parasites. In natural infection, it is unlikely that an individual would be exposed to such a high parasite load simultaneously. The estimated numbers of infected hepatocytes therefore, are useful in this context, as many of the vaccines that have been investigated in Oxford target liver stage antigens, and would so be expected to produce an effect at this stage rather than have any impact on blood stage parasite growth. In the studies where a delay in parasitaemia has been observed, this is associated with a reduction in the estimated numbers of infected hepatocytes as calculated using the Hermsen model.

Chapter 7 Challenge Controls

7.1 Introduction and Literature Review

The challenge studies that are performed in Oxford play a vital role in establishing the potential of a particular vaccine regimen to provide any protection from malaria infection. As part of each challenge study, a number of healthy control volunteers are recruited, and infected with malaria, without prior administration of any vaccine. The purpose of these volunteers is to prove the integrity of the infectious system, and also to act as a comparison group when calculating parasite growth rates and time to patent parasitaemia in vaccinated volunteers. The data collected from these controls represents a valuable model in which to study malaria infection, at an earlier stage than they would usually present for medical attention.

Before examining the symptoms that volunteers report during the challenge phase of these studies, it is important to note that none of the volunteers studied here become as ill as those with malaria described in the Oxford textbook of medicine (below). As discussed in Chapter 2 of this thesis, when considering carrying out work of this sort, considerable care must be taken to ensure the safety of the participants involved.

In all of the challenge studies performed by the Oxford group, over 230 volunteers have been infected with falciparum malaria. All have subsequently been successfully treated, and have completely recovered from the infection. Only 2 have ever required admission to hospital, one who became dehydrated as a result of nausea and vomiting, and was admitted for intravenous fluid rehydration, and the second following an idiosyncratic reaction to chloroquine.

The symptoms of malaria infection are well described in the medical literature. Fever, rigours, arthralgia, nausea and vomiting, anaemia caused by haemolysis, haemoglobinuria, and convulsions are those commonly listed. This classical

description of the clinical syndrome of malaria infection is from the Oxford Textbook of Medicine:

“Several days of prodromal symptoms such as malaise, headache, myalgia, anorexia, and mild fever are interrupted by the first paroxysm. Suddenly the patient feels inexplicably cold (in a hot climate) and apprehensive. Mild shivering quickly turns into violent shaking with teeth-chattering. There is intense peripheral vasoconstriction and gooseflesh. Some patients vomit…. The rigor lasts up to 1 h and is followed by a hot flush with throbbing headache, palpitations, tachypnoea, prostration, postural syncope, and further vomiting while the temperature reaches its peak. Finally, a drenching sweat breaks out and the fever defervesces over the next few hours. The exhausted patient sleeps. The whole paroxysm is over in 8 to 12 h, after which the patient may feel remarkably well. Classical tertian or subtertian periodicity (48 and 36 h between fever spikes) is rarely seen with falciparum malaria. A high irregularly spiking, continuous or remittent fever, or daily (quotidian) paroxysm, is more usual. Other common symptoms are headache, backache, myalgias, dizziness, postural hypotension, nausea, dry cough, abdominal discomfort, diarrhoea, and vomiting. The non-immune patient with falciparum malaria usually looks severely ill, with ‘typhoid’ facies and, in dark-skinned races, a curious greenish complexion. Commonly, there is anaemia and a tinge of jaundice, with moderate tender enlargement of the spleen and liver. Useful negative findings are the lack of lymphadenopathy and rash (apart from herpes simplex ‘cold sores’) and focal signs [8]”

It is important to review our experience of this method for testing potential vaccine candidates, in order to ensure continued safety, efficiency and reproducibility of the technique. We have not previously reviewed the normal clinical course of malaria during these studies. It is useful to review this data, partly to explain to prospective volunteers what to expect from taking part in a study, but also to assess its safety and reliability.

There is very little published literature relating parasitaemia to symptoms in malaria infection. As mentioned elsewhere parasitaemia generally correlates with severity of infection [237], so patients with very high parasite burdens tend to have

more severe disease, and one might assume therefore, more symptoms. However, little is known about the relationship between parasitaemia and symptoms at the other end of the disease spectrum.

The objectives of this study were therefore to collate the data gathered from all control volunteers who have taken part in malaria challenge studies in Oxford. The aim was to describe the symptoms and signs reported by these volunteers, and any laboratory abnormalities reported during the follow up period. A secondary objective was to examine if there was any correlation between symptoms and levels of parasitaemia as determined by PCR, at these low levels of infection.

7.2 Methods

7.2.1 Challenge Method

The protocol for the challenge phase of each vaccine study has remained largely unchanged throughout the many different trials that have been carried out. The model itself is derived from one originally described by Chulay et al. [202]. The P. falciparum strain used is the 3D7 clone of strain NF54, isolated by Prof. D. Walliker. 3D7 parasite cultures are maintained in a continuous culture system in a medium containing 10% v/v human AB serum (from the Blood Transfusion Service, Colindale, UK) heat inactivated at 56℃ for 30 min. The blood products come from volunteers who have been screened as per UK Blood Bank (antibodies for HIV, HBV, HCV and syphilis). A large master seed lot of 3D7 has been prepared for the human studies under GMP conditions and only parasites from this master seed are used to infect mosquitoes.

Anopheles stephensi (originally obtained from Nijmegen-strain SF500) are infected with the cultured material 17 days from the beginning of gametocyte culture when sufficient mature gametocytes are present, as indicated by the ability of the microgametocytes to exflagellate. The mosquitoes are allowed one blood meal at this time, given via a membrane feeder. This contains the cultured parasite material (at 17 days), plus fresh red cells from a donor and AB human serum. Again, the blood products come from volunteers who have been screened as per

UK Blood Bank (antibodies for HIV, HBV, HCV and syphilis). The fed mosquitoes are then maintained on a fructose/ paba solution. 7-9 days after the infective feed, samples of the mosquitoes are checked for the presence of oocysts. 16 days after the blood meal, samples of the mosquitoes are again checked, this time for the presence of sporozoites in the salivary glands. The remaining infected mosquitoes are then transferred to small pots containing 1-5 mosquitoes ready for feeding on the volunteers.

Each volunteer is taken into the insectary at Imperial College in London, and given a cardboard container, covered with netting, initially containing five mosquitoes. The subject rests his or her forearm on the screened top of the carton. The mosquitoes are allowed to feed undisturbed for 5 minutes. After this period the cup is removed, and fed mosquitoes, as indicated by the presence of a blood meal in the abdomen, are individually dissected, and the paired salivary glands of each fed mosquito are transferred to a microscope slide. A cover slip is placed on the slide and the salivary glands are gently squashed to release the sporozoites. Under the microscope, glands are rated 0 to +4 according to the gland rating index for sporozoites: any mosquito with a gland rating of +2 (i.e. more than 11 sporozoites present) or more qualifies as being infective. The scale is illustrated in table 7.1.

Gland rating	No. of sporozoites
0	No sporozoites observed
+1	1 – 10 sporozoites observed
+2	11 – 100 sporozoites observed
+3	101 – 1000 sporozoites observed
+4	>1000 sporozoites observed

Table 7.1: Salivary gland sporozoite rating index

If by this method the volunteer is found to have been inoculated by fewer than five infected mosquitoes, further infected mosquitoes are allowed to feed on the volunteer until a total of 5 infected mosquitoes have fed. New mosquitoes are added to a carton depending on the number of infective bites still required. The

bite-challenge procedure continues until each subject has received bites from 5 infectious mosquitoes.

These challenge procedures are performed within the insectary of the Alexander Fleming Building, Imperial College London. The insectary is one of only 3 such facilities worldwide, and is isolated by vacuum traps to prevent escape of any uncontained mosquitoes.

7.2.2 Challenge Follow-up and Treatment

From the evening of day 6 after the day of challenge, each volunteer is followed up twice daily for a maximum of 7 days, and then once daily for up to another 7 days. At each visit, volunteers are asked about symptoms of malaria infection using a structured questionnaire. An example of the questionnaires used is shown. The replies are recorded in the case record forms for each volunteer, and blood is taken for a blood smear and PCR for parasite DNA.

Symptoms	Tick if YES
NO SYMPTOMS	
Feverish	
Chills	
Rigours	
Sweats	
Headache	
Anorexia	
Nausea / Vomiting	
Diarrhoea	
Myalgia / Arthralgia	
Low Back pain	
Other (specifiy)	

Table 7.2: Symptom questionnaire used during challenge study

When one viable parasite was detected by microscopy in the blood of a volunteer, they were commenced on treatment. In the early challenge studies, the treatment given was Chloroquine. Chloroquine 150mg (base) was administered as 4 tablets orally at time 0, then 2 tablets at 6 hours, 2 tablets at 24 hours and 2 tablets at 48 hours. In 2004, this was changed to artemisinin combination therapy (ACT) with oral Riamet®, partly to minimise reported toxicity, and also because of the shorter reported half life of Riamet® (4.5 days) [238] compared to that of chloroquine (60 days) [239]) This was to minimise the effect of previous antimalarial treatment on vaccinated volunteers that might be re-challenged (for example, if they were protected at first challenge, a subsequent challenge enables the duration of protection to be established). This change was also in line with the updated WHO guidance on the treatment of uncomplicated falciparum malaria [240]. Riamet® is a combination drug consisting of artemether (20 mg) and lumefantrine (120 mg)

per tablet. A treatment course of Riamet® consists of 6 times 4 tablets. The first 4 tablets were given when diagnosis was made, followed by additional doses after 8, 24, 36, 48 and 60 hours. At least 3 out of the 6 doses were observed by the investigators. Regardless of the treatment given, slide reading was continued daily until two consecutive slides were negative for parasites.

7.2.3 Slide Reading Methods

To assess the development of parasitaemia after challenge, microscopy was performed on thick smears using a standard operating procedure by two experienced microscopists. Blood collected by venepuncture into an EDTA tube was prepared by spreading approximately 10 µl over one thousand high powered fields on a glass microscope slide. Slides were allowed to dry, and then stained with Field's stain. Field's stain was applied by dipping the slide into Field's stain A for 3 seconds, then into tap water for 3 seconds (with gentle agitation), into Field's stain B for a further 3 seconds and then washing gently in tap water to remove excess stain. The slide was then air dried for at least 30 minutes. After drying, they are then examined under oil immersion (Microil, BDH) at high power (magnification X1000). A minimum of 200 high power fields must be examined before a film can be declared negative, this ensures at least 2 µl of blood are examined for parasites.

7.2.4 PCR Method

During the challenge follow up, in addition to the thick film, blood was taken for PCR analysis for parasite DNA. This was performed in real time, although the clinicians assessing the volunteers were blinded to the results. The method used is described in detail in [194].

7.2.5 Data Collation and Analysis

Case record forms completed during each study for each volunteer were reviewed. The detailed answers to the malaria symptom questionnaire on each visit from Day 6 post infection, initially twice daily, then once daily, either until Day 21, or

until the day of diagnosis with malaria. Adverse event records were collated into an Excel ™ spreadsheet.

The incubation period is defined as the time from challenge until the first onset of two or more symptoms of malaria, and the pre-patent period is the time until first positive blood smear. Clinical laboratory data from blood samples collected during the challenge period was also examined. PCR data calculating the numbers of parasites per mL of blood for each volunteer at each time point during the follow up period was collected for inclusion in the analysis.

7.3 Results

A total of 50 control volunteers were included in this analysis. All volunteers were healthy, malaria naïve individuals aged 18-50 years. They participated in nine different malaria challenge studies that took place in Oxford between August 2001 and December 2006. All volunteers provided fully informed consent to participate in each study by signing a written consent form. Exclusion criteria included a prior history of malaria, risk of exposure in the preceding 6 months, immunosuppression, epilepsy, infection with Hepatitis B, Hepatitis C or HIV, pregnancy, drug or alcohol abuse, significant psychiatric disorder or other significant illness. All studies received ethical approval from the Oxfordshire Research Ethics Committee. The studies that were included, their ethics reference number, and the volunteer numbers of the controls analysed here, are listed in table 7.3.

STUDY Ethics ref.	**VAC012** C01.111	**VAC015** C02.069	**VAC017** C02.069	**VAC018** C02.153	**VAC021** C02.268	**VAC022** C02.305	**VAC023** C03.088	**VAC027** 06/Q1604/55	**VAC030** 05/Q1604/69
Volunteer Numbers	126	160	185	348	399	376	458	588	537
	143	179	311	350	410	377	459	591	538
	145	180	326	352	411	378	462	592	539
	149	190	331	354	417	380	463	596	542
	151	191	335	355	435	381	464	600	544
					451	383	466	605	546

Table 7.3: Malaria challenge studies

Challenge studies used in this analysis, and volunteer numbers of control subjects included

The mean pre-patent period for all controls was 11.5 days (range 8 – 14.5), with a standard deviation of 1.49 days. The summary statistics for the pre-patent period for control volunteers from each study are shown in table 7.4.

STUDY	VAC012	VAC015	VAC017	VAC018	VAC021	VAC022	VAC023	VAC027	VAC030
Mean (days)	11.3	11.6	10.4	11.6	10.7	12.0	11.8	12.8	11.2
95% CI Lower	9.5	10.5	8.3	9.5	9.3	10.8	10.9	11.4	8.9
Upper	13.1	12.6	12.5	13.7	12.1	13.2	12.8	14.3	13.4
Median	11.0	12.0	10.0	11.0	11.3	11.8	11.8	12.8	11.0
S.D.	1.4	0.8	1.7	1.7	1.3	1.1	0.9	1.4	2.1
Min	9.5	10.5	9.0	9.5	9.0	11.0	11.0	11.0	8.0
Max	13.0	12.5	13.0	14.0	12.0	14.0	13.0	14.5	14.5

Table 7.4: Summary statistics for pre-patent period
Statistics from control volunteers from each study included in this analysis, demonstrating no heterogeneity.

There is no significant difference between the pre-patent periods in any of the trials included in this analysis (one way ANOVA p =0.2). The spread of the data is represented in the following bar chart, showing that the majority of volunteers are diagnosed on day 11.

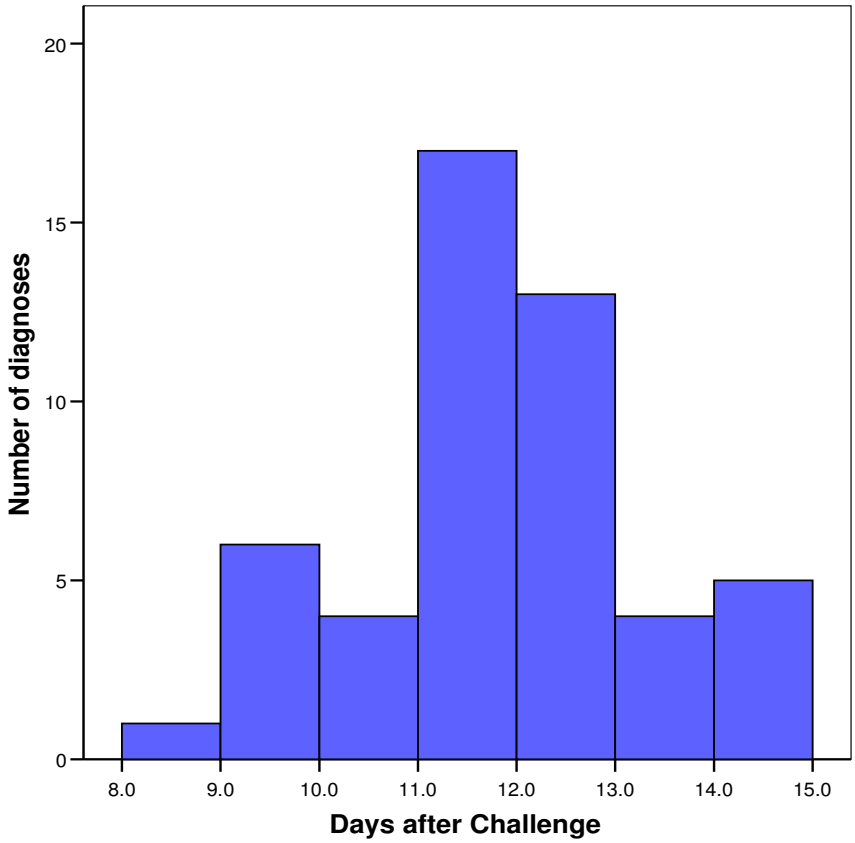

Figure 7.1: Bar chart of numbers diagnosed each day after challenge
The number of control volunteers diagnosed on each day after challenge is shown, n=50.

7.3.1 Symptoms

The mean incubation period was 9.6 days, (range 6.5 – 14 days) with a standard deviation of 1.9 days. Thus on average, volunteers reported symptoms for 2 days prior to a diagnosis of malaria being made.

Summarising all the solicited symptoms over the period of the challenge follow up, the most commonly reported symptom was that of headache, comprising nearly 20% of all symptoms. Each symptom is recorded at each clinic visit, so if one subject reports any symptom twice in one day, then it is included as two separate reports of that symptom. The frequency of each solicited symptom is presented in table 7.5. Low back pain was sometimes reported as a form of myalgia/arthralgia. The severity of each symptom was not recorded, however all volunteers were

managed successfully as outpatients indicating that all reported symptoms were mild or moderate.

Day	Feverish	Chills	Rigors	Sweats	Headache	Anorexia	Nausea/ Vomiting	Diarrhoea	Myalgia/ Arthralgia	Low back pain	Other
6.5	1	0	0	0	5	1	2	3	3	2	2
7	3	1	0	0	4	2	4	3	2	2	3
7.5	1	2	0	0	7	4	5	1	2	0	3
8	1	1	0	0	6	1	4	1	3	6	1
8.5	2	0	0	0	7	4	4	1	3	6	3
9	3	3	1	1	6	4	3	0	7	7	2
9.5	4	3	2	2	12	2	5	0	10	6	2
10	8	2	2	3	5	4	5	0	9	5	4
10.5	4	1	0	2	11	5	5	0	6	4	6
11	6	5	2	5	11	7	7	1	8	11	3
11.5	1	7	0	1	8	5	3	0	8	7	2
12	5	6	1	6	10	4	4	0	6	6	2
12.5	6	5	1	4	6	4	3	0	5	4	1
13	3	1	1	3	3	2	4	0	2	3	1
13.5	2	2	1	2	2	2	1	0	3	2	0
14	3	2	1	2	4	2	2	0	2	2	0
14.5	2	2	1	1	2	2	1	0	2	1	0

Table 7.5: Frequency of each solicited symptom by day after challenge

Figure 7.2 illustrates the relative proportions of each of the reported symptoms in this analysis.

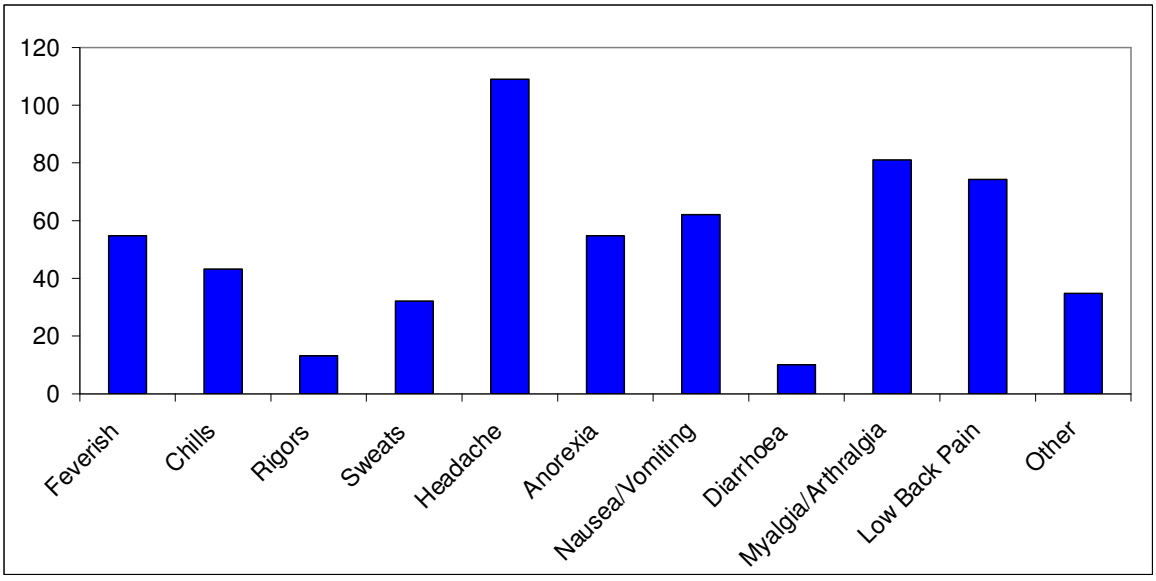

Figure 7.2: Bar chart of reported symptoms

'Other' includes: dizziness, tiredness / fatigue and malaise.

The number of symptoms reported each day gradually increased from the first day (6.5) up to a peak on day 11, which corresponds with the mean day of diagnosis for this group of volunteers.

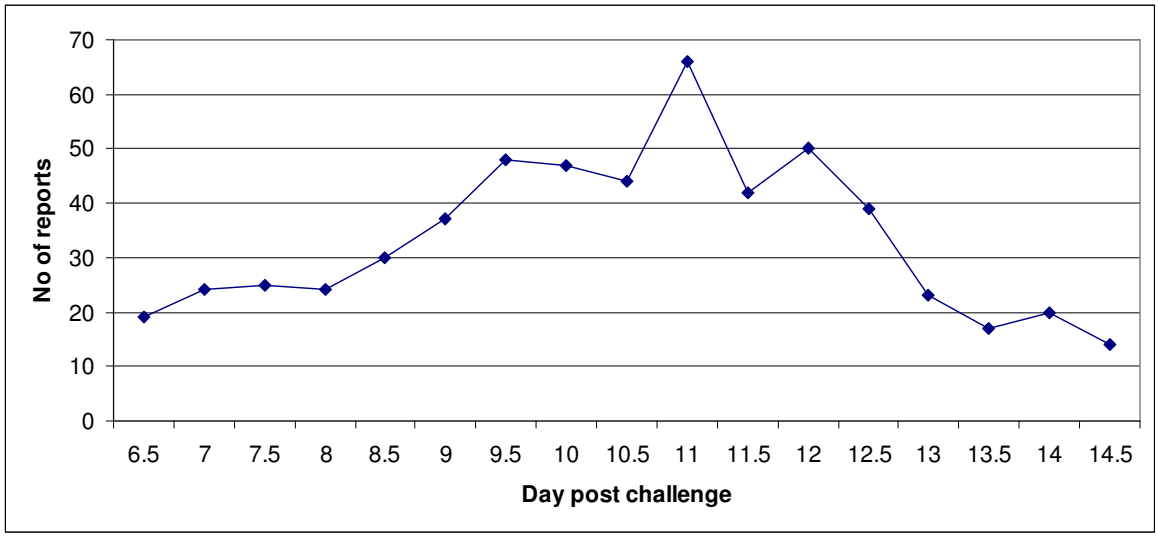

Figure 7.3: Total number of symptoms reported each day.
The peak number of symptoms is reported on day 11, corresponding with the peak day of diagnosis.

The frequency of reports of each symptom varies, although most rise with time after challenge. The following two graphs demonstrate this.

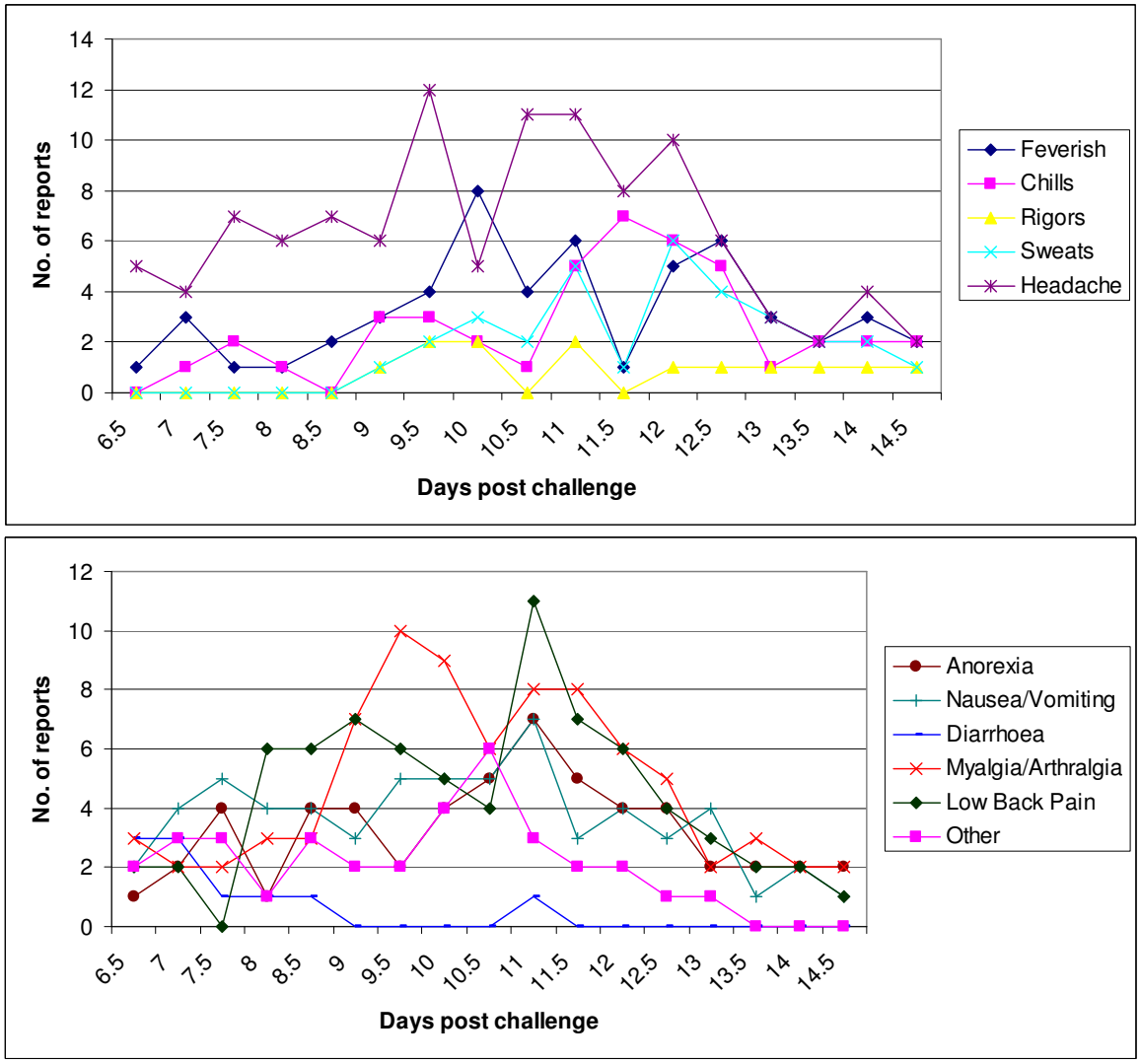

Figure 7.4: Number of reports of each symptom by day

From these graphs, it can be seen that the common early symptoms are those of headache, myalgia, arthralgia. As time progresses, reports of headache rise, but so too do those of low back pain, feverishness, chills and sweats.

Examining the data by volunteer rather than by total number of reports, a total of 43/50 (86%) of control volunteers reported headache at least once during the follow up period, meaning that headache is also reported by a majority of volunteers, rather than just a few, but on several occasions. Feverishness, chills, myalgia / arthralgia, and low back pain are also reported by 50-60% of volunteers representing the next most commonly reported symptoms. Rigours are experienced by relatively few people, only 10/50 in this series (20%). The majority

of these symptoms are relatively short lived, all having a mean and median duration of less than 1 day (<24 hours). Table 7.6 lists the frequency of each symptom and their duration.

Symptom	Frequency (%)	Duration, days	
		Median (range)	Mean (SD)
Feverish	28/50, (56)	1, (1 – 3)	0.76, (0.51)
Chills	26/50, (52)	1, (1 – 1.5)	0.74, (0.34)
Rigors	10/50, (20)	1, (1 – 1)	0.59, (0.20)
Sweats	17/50, (34)	1, (1 – 2.5)	0.84, (0.60)
Headache	43/50, (86)	1, (1 – 3)	0.84, (0.54)
Anorexia	26/50, (52)	1, (1 – 1.5)	0.78, (0.40)
Nausea/Vomiting	22/50, (44)	1, (1 – 2.5)	0.87, (0.61)
Diarrhoea	8/50, (16)	1, (1 – 1)	0.56, (0.18)
Myalgia/Arthralgia	30/50, (60)	1, (1 – 5.5)	0.91, (0.85)
Low Back Pain	29/50, (58)	1, (1 – 5.5)	0.90, (0.90)
Other	18/50, (36)	1, (1 – 1.5)	0.68, (0.33)

Table 7.6: Solicited symptoms

Frequency and duration of all solicited symptoms, n=50

Only one volunteer out of 50 (2%) reported no symptoms of malaria at all up to the time of diagnosis, but 11/50, (22%) reported 3 different symptoms or fewer at any time during the challenge. Most volunteers (52%) reported more than 5 symptoms, however, only 9/50 (18%) reported nine or 10 different symptoms.

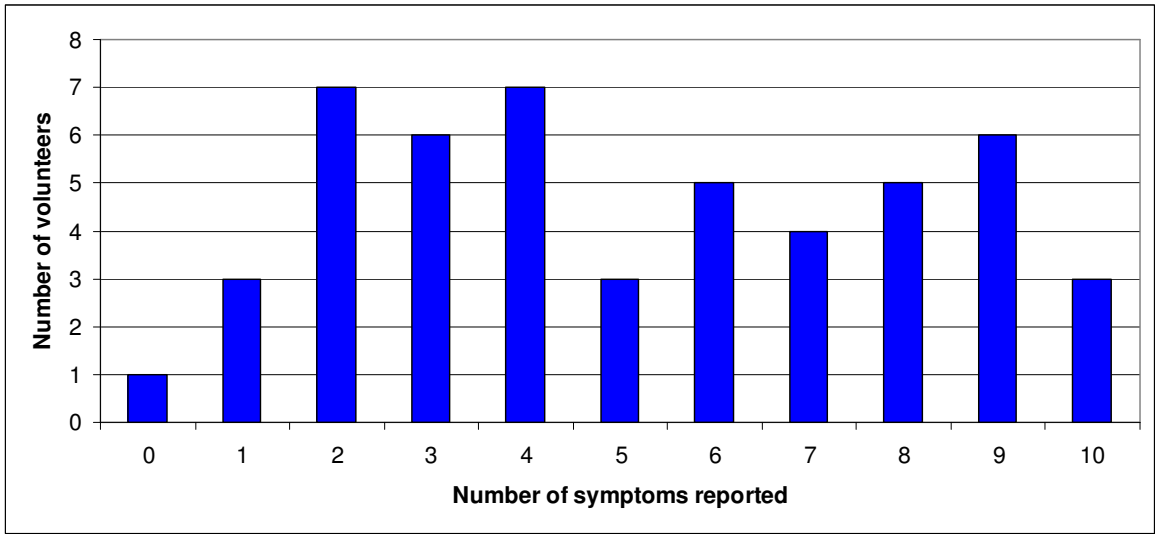

Figure 7.5: Number of symptoms reported by each volunteer

Total number of symptoms reported by each volunteer during the challenge follow up period

7.3.2 Parasite Density and Symptoms

To examine if there was any relationship between parasite density and number of reported symptoms, the parasite density measured by PCR at each time point was plotted against the number of symptoms reported at that visit by each volunteer. There is a poor positive correlation between the individual values for each volunteer (data not shown), and the Pearson correlation coefficient is 0.11 (not significant).

However, if this data is analysed by the day of peak PCR measurement for each volunteer, and this is compared with the day that volunteer reported the most symptoms, there is a correlation between the two. So, while the numbers of symptoms reported on a daily basis do not correlate with parasite density, the worst symptoms (i.e. maximum number) do correlate with the highest parasite density. This is the first time such a correlation has been shown at such low parasite densities. The data is shown in figure 7.6, and the Pearson correlation coefficient is 0.67

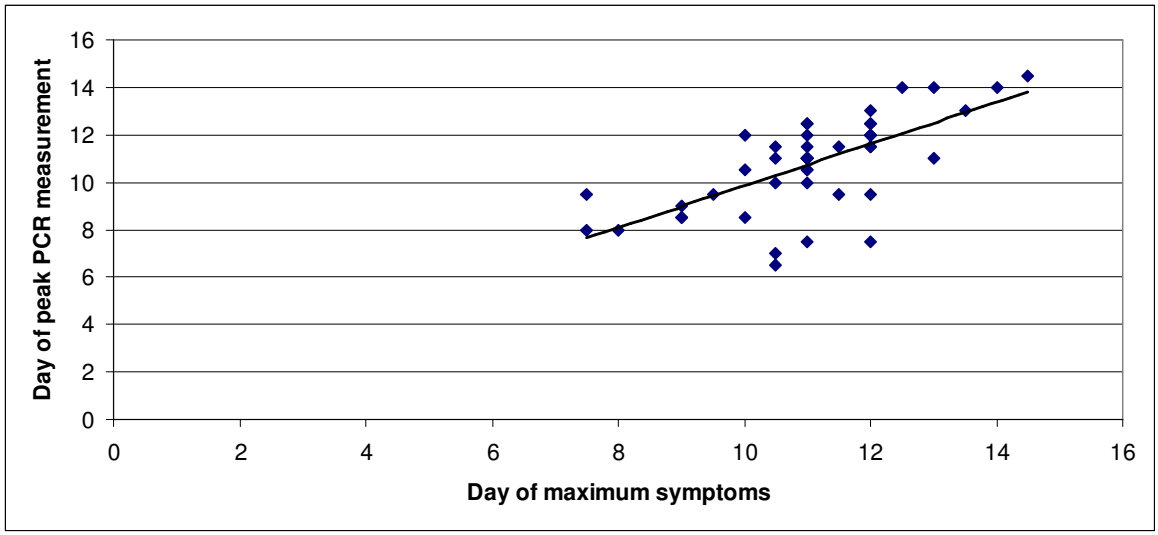

Figure 7.6: Scatter plot of peak PCR measurement vs. maximal symptoms.

7.3.3 Laboratory Results

The schedule for collection of blood for monitoring of routine haematological and biochemical parameters has varied between studies. Table 7.7 outlines the principal time points for sampling for control volunteers in the trials under consideration.

STUDY	VAC012	VAC015	VAC017	VAC018	VAC021	VAC022	VAC023	VAC027	VAC030
Sampling time points	S	S	S	S	S	S	S	S	S
								C	C
	C +7	C +7		C +7	C +7	C +7	C +7		
								C + 35	C + 35
				C + 90	C + 90	C + 90	C + 90	C + 90	C + 90

Table 7.7: Time points for safety blood collection
S = Screening, C = Challenge, C + 7 = 7 days post challenge etc.

There have been few clinically significant alterations in safety blood parameters during these challenge studies. Table 7.8 demonstrates all out of range blood tests for these control volunteers throughout each challenge study. Of the clinically significant abnormalities, only two were thought to be related to malaria infection. These included a mild neutropenia, reflected in a low white cell count, another

volunteer had a transient rise in AST, which resolved on repeat testing. A raised creatine kinase, of up to 1642, was temporally related to a period of vigorous exercise, and this was thought to be the likely cause. None of the other out of range values were thought to be clinically significant

Subject id	Study	Time point	Safety Test	Reference Range	Value	Clinical Details
126	VAC012	Screen	Potassium	3.5-5.0 mmol/l	3.1	Subject well, K 3.6 on retesting.
179	VAC015	DOC+7	White cell count	3.0-14.0 x 10^9/l	2.29	Subject suffering from malaria
191	VAC015	DOC +7	Potassium	3.5-5.0 mmol/l	3.1	Subject well, not clinically significant
352	VAC018	DOC + 90	Potassium	3.5-5.0 mmol/l	3.2	Subject well, not clinically significant
411	VAC021	Screening, DOC +7	Gamma GT	<50 IU/l	57, 60	Subject well, 33 and 44 on repeat testing
377	VAC022	Screening, DOC+90	Creatine Kinase	<100 U/l	413, 1642	Subject well, related to vigorous exercise on both occassions
378	VAC022	Screening	Potassium	3.5-5.0 mmol/l	3.1	Subject well, 3.6 on repeat testing
378	VAC022	DOC +7	AST	>80 IU/l	86	Subject suffering from malaria. 29 on repeat testing
380	VAC022	DOC +7	Creatine Kinase	<100 U/l	328	Subject well, exercise related
596	VAC027	Screening	ALT	≤60 IU/l	61	Clinically well. Transiently raised prior to enrolment in trial only.
539	VAC030	Screening	Potassium	3.5-5.0 mmol/l	3.1	3.6 on repeat testing

Table 7.8: Out of range lab values

All out of range lab values for control volunteers participating in challenge studies are listed, n = 50.

7.4 Discussion

No volunteer in these studies has developed severe malaria, and all subjects were successfully treated as outpatients. No symptoms were considered serious or life threatening. There have been no cases of recrudescence of malaria after successful treatment.

The mean pre-patent period for these volunteers is 11.5 days, which is slightly longer than that reported by the US Navy in their recent publication reviewing their experience in studies of this sort [172].

Analysis of the commonly reported symptoms following malaria infection in a challenge study has shown that the most prominent symptom is that of headache. All other expected symptoms of malaria are reported, although some rarely – there are only 10 reports of diarrhoea, making up less than 2% of the total number of symptoms. The majority of volunteers taking part in the challenge study will report at least 5 or more symptoms of malaria infection, with the majority of these being reported on the day of diagnosis (data not shown). On an individual volunteer level, the symptoms do often appear to occur in paroxysms, as described in the medical textbooks; however, when examining the symptoms of the whole group, this effect is lost.

Thick films are used in this study to examine a larger volume of blood, and therefore detect parasites at a lower density than would be possible using thin films. It has been shown that thick films consistently underestimate the parasite density when compared to PCR [241].

At the low parasite densities seen in volunteers taking part in these challenge studies, however, there is a correlation between the peak parasite density and peak number of symptoms reported. Previous work looking at the relationship between parasitaemia and clinical symptoms indicates that this is complex, even in well studied populations such as P. falciparum infected children in sub-Saharan Africa, and much of the published evidence appears contradictory [242]. A recent

study used data from malaria treated neurosyphilis patients to examine the relationship between fever, parasitaemia and gametocyte development and density [242]. This shows a link between the development of gametocytaemia and fever, rather than parasitaemia as whole. Epstein et al. [172] failed to demonstrate a correlation between parasitaemia and severity of symptoms in their report.

The analysis here confirms that this experimental challenge method employed in the evaluation of malaria vaccines is a safe and reliable method. The pre-patent and incubation period was remarkably constant between studies. In our experience, experimental infection of healthy volunteers with malaria is a safe, reproducible, efficient, and cost effective method for the testing of new malaria vaccines. It requires that volunteers are closely monitored throughout the challenge period. All volunteers should expect that they are likely to become mildly to moderately ill, and to remain ill for several days, but to continue to be able to perform the activities of daily living. They may also experience mild derangements in the values of haematological and biochemical indices, which are generally transient.

In contrast to the recently published experience of the Naval Medical Research Centre in the States, [172], control volunteers participating in our studies appear to have fewer symptoms, and they last for shorter periods of time. For direct comparison, their data is shown in table 7.9, alongside the data obtained from the work described in this chapter.

Site	Symptom	n	%	Duration (days)		Mean (days)	St. dev.
				Median	Range		
Oxford University n = 50	Chills	26	52	0.5	1,2	0.74	0.34
	Rigors	10	20	0.5	1,1	0.59	0.20
	Headache	43	86	0.5	1,3	0.84	0.54
	Anorexia	26	52	0.5	1,2	0.78	0.40
	Nausea/Vomiting	22	44	0.5	1,3	0.87	0.61
	Diarrhoea	8	16	0.5	1,1	0.56	0.18
	Myalgia/Arthralgia	30	60	0.5	1,6	0.91	0.85
Naval Medical Research Centre n = 47	Chills	40	85	2	1,6	2.28	1.26
	Rigors	18	38	1	1,3	1.50	0.71
	Headache	47	100	3	1,9	3.75	2.20
	Nausea	29	62	1	1,6	1.83	1.20
	Vomiting	6	13	1	1,2	1.68	0.41
	Diarrhea	12	26	1.5	1,4	1.67	0.89
	Myalgia	38	81	3	1,9	3.00	1.82
	Arthralgia	17	36	1	1,9	2.35	2.29

Table 7.9: Comparison of symptoms

Symptoms of malaria infection reported by subjects participating in studies at different sites. The data shown is only for those symptoms that are directly comparable between the two groups. Differences in recording methods mean that it is not possible to compare all of the reported symptoms.

This demonstrates that in the US Navy studies, generally more volunteers report each of the symptoms, and they last for a longer period. Overall, the numbers of volunteers reporting each symptom are not significantly different. However, the mean duration of symptoms reported by the American group is significantly longer than that reported in our group (mean duration for US Navy volunteer is 2.3 days, whilst that for Oxford University volunteers is 0.8 days, two tailed t test $p = 0.001$). There is a possibility that this difference in duration could be due to the different protocols for challenge visits. Volunteers participating in studies run by the US Navy are reviewed only once a day, and so the minimum duration for any reported symptom is one day, whereas those in Oxford were reviewed twice daily.

However, even if this is taken into account by rounding up the duration of symptoms for the Oxford data the difference in duration is still significant (two tailed t test p = 0.004).

This is possibly related to the strain of malaria used; the Navy use the NF54 strain of P. falciparum rather than 3D7, which is a laboratory clone of NF54. It would seem that our experience lends credence to the belief that 3D7 challenge, whilst remaining a stringent test for a potential vaccine candidate, appears to cause a similar number or fewer symptoms, and these last for a shorter period of time. Data concerning the severity of reported symptoms has not been collected during the Oxford challenges, so it is not possible to perform a direct comparison of severity. The Navy report an incidence of severe symptoms of 21%, although the definition of a severe symptom is not clear.

In our overall experience, less than 1% (0.85%) of all challenged volunteers have been hospitalised (none of those analysed here – both were vaccinated volunteers), so this is unlikely, and the likelihood of developing severe malaria is extremely unlikely (none of those in our experience). Volunteers should continue to be informed of these potential risks of participation, but reassured by the low frequency with which they have occurred up to now.

Chapter 8 Conclusions

8.1 Vaccine trials

8.1.1 VAC027

This trial examined the safety and immunogenicity of the novel candidate malaria vaccines FP9 and MVA PP, encoding a string of six pre-erythrocytic antigens [167]. It was hoped that a broad response against multiple parasite epitopes might enhance the immune response and provide immune protection. However, the immunogenicity following vaccination in all study groups was disappointing, perhaps relating to inadequate expression of this very large insert to allow good immunogenicity in humans. Both vaccines were tested for potency prior to use in the study, and both passed. However, in keeping with the low immunogenicity observed, no efficacy was demonstrated in the sporozoite challenge study. All volunteers succumbed to malaria infection by day 14.5 after the challenge.

The clinical safety profile of these vaccines was good. 34 volunteers were enrolled and 31 (91%) completed the trial successfully. The safety profile reported provides support for an ongoing program to develop effective vaccines against malaria, tuberculosis and HIV employing these vectors, and also for exploring the use of other recombinant viral vectors to enhance immunogenicity. There are no further plans for use of the polyprotein construct at present, although it is possible that using these antigens in a construct with a more potent vector may produce the desired broad response to multiple epitopes.

8.1.2 VAC030

This trial was the first to investigate the approach of combining two existing vaccine strategies, one T cell inducing and one antibody inducing, in the hope that they would work synergistically to improve protection. This hypothesis was based on studies in an animal model [169]. Some evidence of vaccine-induced blood stage parasite activity and partial protection was observed, for the first time in a

healthy volunteer challenge study such as this. As described, two major observations led to this conclusion. However, the dramatic increase in immunogenicity and protection seen in the murine model was not observed in this human study. In fact, as the positive results were distributed across all vaccinees, it would seem that any efficacy demonstrated was a result of the antibody inducing vaccine PEV3A, and the contribution of FP9 and MVA ME-TRAP appears to have been minimal.

The reason for this may be related to the batch of vaccines used, or to the approach. In the successful animal model, the best results (i.e. highest levels of protection from a subsequent malaria challenge) were obtained when the vaccines were mixed physically in the same syringe and administered at the same site. Both T cells and antibodies were shown to be important in mediating this protection. Administering the vaccines at separate sites should have compartmentalised the vaccine induced responses (opposite arms have different draining lymph nodes) and thence may have led to the lack of synergism. It is possible that the immune responses induced by one antigen interfered with the induced response to the other. However, as observed in VAC027, the immunogenicity produced by the T cell inducing component of the combination vaccine was disappointing. Again, both vaccines (FP9 and MVA ME-TRAP) were tested for potency prior to use in the study, and were found to be immunogenic , so it appears likely that co-administration of these different vaccines, even using different immunisation sites had an adverse effect of the immunogenicity of the poxvirus vaccines in humans.

An effective malaria vaccine may need to target multiple parasite antigens from different stages of the life cycle. This study provides evidence of a vaccine-induced blood stage protection for the first time, and it supports the further development of the virosomal system as an effective vector for malaria vaccines. A Phase IIB study, using the vaccine candidate PEV3A, is currently being planned in Africa, and Pevion also have plans to produce virosomal vaccines containing multiple blood stage antigens.

8.1.3 Vaccine Issues

Previous human trials of poxviruses encoding P. falciparum antigens have produced specific T cell responses of the order of 400 spot forming units per million PBMC. The most effective prime-boost group in VAC027 achieved a summed response to the whole insert of just under 100 spots per million PBMC at the peak time point, whilst in VAC030, the peak response was even lower, at under 50 spots per million PBMC. The reasons for the poor immune responses generated by these vaccines are not clear. All vaccines were stored as recommended by the manufacturer and remained infectious and potent when tested prior to use. The intradermal route has proved the most immunogenic in previous trials of poxvirus vaccines encoding malaria antigens in humans (a previous study [216] compared intradermal and intramuscular routes, although again poor immunogenicity complicated these results). It is possible that the low immunogenicity is a result of changes in the manufacturing process. These vaccines were all manufactured under contract by IDT, Germany, and significant changes have been introduced in recent years in order to scale up their manufacturing processes. Further work to determine if any vaccine related stability issues could have contributed to the reduction in immunogenicity has been undertaken without identifying any evidence of loss of potency with time.

8.2 Lab Work

In this study, T cell, and antibody responses to polyprotein antigens are identified in samples from and African population. T cell, but not antibody, responses are shown to play a role in protection from malaria infection. However, caution is required in interpreting results obtained from samples such as these, as malaria infection itself may have modulated the observed response. This work provides a clue that responses to these antigens may be important, but in order to extend this, a longitudinal study of responses would be required. Unfortunately, it was not possible to find the samples necessary to perform a study such as this. This data adds to understanding of the development of clinical immunity. It also provides further support for the approach of developing T cell inducing vaccines in order to protect against disease.

8.3 Parasite Life Cycle Modelling

Multiple models of parasite growth have been published. The Hermsen model was selected from these as the most appropriate for use with the data collected during the clinical trials performed in Oxford, and using this Hermsen model, it was possible to show that some of the candidate malaria vaccines tested in these studies in Oxford do exert an effect on blood stage parasite growth. Examining previous studies where pre-erythrocytic efficacy has been observed further validated the use of this model. In the studies where a delay in parasitaemia has been observed, as expected this is associated with a reduction in the estimated numbers of infected hepatocytes calculated.

8.4 Challenge Safety Data

Analysis of the data obtained from challenging 50 malaria naïve, unvaccinated individuals provides an insight into the course of early malaria infection. The data presented confirm that this is a safe method for testing the efficacy of candidate malaria vaccines, and that the pre-patent and incubation periods remained remarkably constant throughout multiple trials. A correlation of the peak in parasitaemia with the peak of reported symptoms was observed for the first time at the relatively low parasite densities reached in these studies. A comparison with published data from the US Navy demonstrates that the Oxford challenge appears to be better tolerated. This may be related to the parasite strain used.

8.5 Overall conclusions

The two clinical studies presented in this thesis represent two successful trials, which were satisfactorily conducted, with no serious safety or other issues. The results of these trials were disappointing, in that immunogenicity of the vectored vaccines was in both cases lower than anticipated, and no complete protection from malaria was observed. However, they do provide us with important insights into the relative roles of T cell and antibody mediated immunity. The holy grail of

an immune correlate of protection remains to be elucidated, and in the study where some efficacy was demonstrated (VAC030), none of the measured immune responses correlated clearly with time to parasitaemia. The other work presented demonstrates the complexity of unravelling the immune response to malaria, and the mechanisms of clinical immunity. Modelling parasite growth is an important part of these clinical trials, and examining the relevant models in detail in order to select one has highlighted the many assumptions that need to be made, which may not be correct.

Malaria remains a serious problem throughout the world, and the work presented has contributed to our understanding of the immune response to malaria infection, and allowed selection of one vaccine candidate for further studies. It has also highlighted a problem of poor immunogenicity associated with the pox viral vectored vaccines studied, which has led to the development of alternative adenoviral vectors, that are currently undergoing clinical assessment.

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Appendix 1

Standard operating procedure for protocol development

Appendix 2

Consent forms, VAC027 and VAC030

Appendix 3

Polyprotein peptides

Appendix 4

Study personnel lists

Appendix 5

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