Immune responses to polymorphic antigens and protection against severe malaria in Kenyan children

Thesis

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IMMUNE RESPONSES TO POLYMORPHIC ANTIGENS
AND PROTECTION AGAINST SEVERE MALARIA IN
KENYAN CHILDREN

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MBChB, MRCPCH, MSc

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ABSTRACT

The mechanisms by which individuals acquire immunity to malaria are poorly understood and although antibodies are thought to be central, evidence that specific anti-malarial antibodies are associated with protection from clinical episodes of malaria has been conflicting.

I hypothesized that the breadth (number of important targets to which antibodies were made) and magnitude (antibody level measured in a random serum sample) of the antibody response were important predictors of protection from clinical malaria. I also investigated whether allele-specific antibodies protected children from developing clinical episodes of malaria associated with parasites bearing homologous alleles.

I analyzed naturally-acquired antibodies to five leading *P. falciparum* merozoite stage vaccine candidate antigens, and schizont extract, in Kenyan children monitored longitudinally for mild and severe malaria. I also genotyped parasites from clinical episodes to investigate allele-specific antibody-mediated immunity.

Serum antibody levels to apical membrane antigen 1 (AMA1), and merozoite surface protein antigens (MSP-1 block 2, MSP-2, MSP-3) were inversely related to the probability of developing malaria, but levels to MSP-119 and erythrocyte binding antigen (EBA-175) were not. The risk of mild malaria was also inversely associated with increasing breadth of antibody specificities, with none of the children who simultaneously had high antibody
levels to five or more antigens experiencing a clinical episode, (17/119, 15%) P=0.0006. Particular combinations of antibodies (AMA1, MSP-2, MSP-3) were more strongly predictive of protection than others. The results were validated in a larger, separate case-control study whose end-point was malaria severe enough to warrant hospital admission (n=387). I found little evidence that allele-specific antibodies conferred protection against clinical episodes associated with parasites bearing homologous alleles.

These findings suggest that under natural exposure, immunity to malaria may result from high titre antibodies to multiple antigenic targets and support the idea of testing combination blood stage vaccines optimized to induce similar antibody profiles.
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DEDICATION

To all those who inspired me....

"Where there is no vision, people perish"

Proverbs 29:18
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<table>
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<th>Description</th>
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<tr>
<td>ABRA</td>
<td>Acidic-basic repeat antigen</td>
</tr>
<tr>
<td>ACTs</td>
<td>Artemisin-based combination therapies</td>
</tr>
<tr>
<td>ADCI</td>
<td>Antibody dependent cellular inhibition</td>
</tr>
<tr>
<td>AER</td>
<td>Antigenic escape residues</td>
</tr>
<tr>
<td>AMA1</td>
<td>Apical membrane antigen 1</td>
</tr>
<tr>
<td>AMANET</td>
<td>African Malaria Network</td>
</tr>
<tr>
<td>BIC</td>
<td>Bayesian Information Criteria</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CSP</td>
<td>Circumsporozoite protein</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro-diphenyl-trichloroethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EBA</td>
<td>Erythrocyte-binding antigen</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal-like growth factor</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological Inoculation Rate</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme linked immune-sorbent spot</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno-adsorbant assay</td>
</tr>
<tr>
<td>EPI</td>
<td>Expanded Programme of Immunization</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antibody genes</td>
</tr>
<tr>
<td>FGT</td>
<td>Four gamete test</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross Domestic Product</td>
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<tr>
<td>GPIs</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HIV/AIDS</td>
<td>Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intra-cellular adhesion membrane protein 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IMCI</td>
<td>Integrated Management of Childhood Diseases</td>
</tr>
<tr>
<td>IPT</td>
<td>Intermittent preventative treatment</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide Treated Bed Nets</td>
</tr>
<tr>
<td>LSA</td>
<td>Liver stage antigen</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MBCA</td>
<td>Melanoma cell binding assay</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIM</td>
<td>Multilateral Initiative on Malaria</td>
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<tr>
<td>MMV</td>
<td>Medicines for Malaria Venture</td>
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<tr>
<td>MSPs</td>
<td>Merozoite surface proteins</td>
</tr>
<tr>
<td>MudPIT</td>
<td>Multidimensional protein identification technology</td>
</tr>
<tr>
<td>MVI</td>
<td>Malaria Vaccine Initiative</td>
</tr>
<tr>
<td>NKs</td>
<td>Natural Killer Cells</td>
</tr>
<tr>
<td>NKTs</td>
<td>Natural Killer T-Cells</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PAR</td>
<td>Population at Risk</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PfEMP-1</td>
<td><em>P. falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>RBM</td>
<td>Roll Back Malaria</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>Ro</td>
<td>Basic Reproduction Rate</td>
</tr>
<tr>
<td>RTS, S</td>
<td>CSP repeat region (R), T-cell epitopes (T), hepatitis B surface antigen (S), ratio of RTS to additional S antigen 1:4 (RTS,S)</td>
</tr>
<tr>
<td>SALSA</td>
<td>Sporozoite and liver stage antigen</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immune deficiency</td>
</tr>
<tr>
<td>SFU</td>
<td>Spot forming unit</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SP</td>
<td>Sulphadoxine-pyrimethamine</td>
</tr>
<tr>
<td>SPAM</td>
<td>Secreted polymorphic antigen associated with merozoites</td>
</tr>
<tr>
<td>SSA</td>
<td>Sub Saharan Africa</td>
</tr>
<tr>
<td>STARP</td>
<td>Sporozoite threonine- and asparagine-rich protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor – α</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombospondin-related anonymous protein</td>
</tr>
<tr>
<td>UIS</td>
<td>Up-regulated in infected sporozoites</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant Surface Antigen</td>
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PUBLICATIONS


Osier FH, Polley SD, Mwangi T, Lowe B, Conway DJ, Marsh K.

I begin in Chapter one with a broad overview of the burden of malaria in the context of the global disease burden, before focusing on current malaria statistics and control strategies. I then review malaria vaccines in more detail as vaccines have historically provided the most cost-effective interventions in the long-term against infectious diseases. This is followed by a review of naturally-acquired immunity to malaria, the understanding of which is essential to the rational development of vaccines.

In Chapter two I provide a comprehensive review of five leading malaria vaccine candidate antigens, MSP-1\textsubscript{19}, MSP-1 block 2, MSP-2, MSP-3 and AMA1. These antigens were the subject of my thesis. For each antigen I start from the beginning with antigen discovery, review the current understanding of antigen structure and processing, before critically reviewing the evidence that antibodies against the antigen play a role in providing protection from malaria. In particular I highlight reasons why data on these antigens from immuno-epidemiological studies has been conflicting using MSP-1\textsubscript{19} as an example. This sets the scene for the approaches used for my studies.

In Chapter three I outline my objectives and detail the methodology including, the study cohorts, recombinant antigens, antibody and cellular assays, parasite genotyping and sequencing. I also explain the key parameters used in molecular population genetic analyses to allow the non-specialist reader to follow the logic of the analyses.
The experimental work begins in **Chapter four** where I test the hypothesis that the breadth of antibody specificity and the magnitude of the antibody response are key determinants of protection from clinical episodes of malaria. I develop the analytical tools using data from one cohort and then validate these tools in a separate group of children. I found that serum antibody levels to some but not all antigens were inversely related to the probability of developing malaria. The risk of disease was also inversely associated with increasing breadth of antibody specificities and particular combinations of antibodies (AMA1, MSP-2, MSP-3) were more strongly predictive of protection than others. Interestingly in this analysis, protection did not seem to depend on the allelic version of the antigen suggesting that there may be significant cross-allele protection to clinical episodes. This was investigated in more detail in chapter six.

**Chapter five** is a sub-analysis exploring whether the quality and/or quantity of antibodies can distinguish children responding to a malaria challenge by developing mild disease (a more efficient response) or severe malaria (a less efficient response). Antibodies collected at the time of the clinical episode and at convalescence did not distinguish between these two groups of children.

**In Chapter six** I investigate allele-specificity by determining whether pre-existing allele-specific antibodies protect against clinical episodes associated with parasites bearing the homologous parasite alleles. I used competition ELISA to dissect out antibodies to allele-specific epitopes and PCR to genotype parasites, and found little evidence of allele-specific protection.
Alleles of AMA1 do not cluster into major allelic families like the other antigens analyzed here and a different set of analyses were applied for this antigen. In Chapter seven I used several definitions of 'haplotypes' of AMA1 alleles to investigate whether particular alleles were over-represented among children presenting with mild or severe malaria, but found no evidence for this.

In Chapter eight I used molecular population genetics analyses to identify regions of ama1 that were under balancing selection from alleles sequenced from Kenyan isolates. In agreement with previous studies, I identified a strong signature of balancing selection within domains I and III of the surface-exposed ectodomain. The signal of selection was strongest in domain III and I investigated whether this was immune-mediated by conducting humoral and cellular assays. As had been previously observed, I found little evidence of antibodies targeted to domain III. However, my preliminary study provides some evidence that T-cell responses may drive the selection observed in domain III and this will be pursued in comprehensive studies.

Chapter nine briefly summarizes the key findings.
1. INTRODUCTION

1.1 THE GLOBAL BURDEN OF DISEASE

In high-income countries non-communicable diseases such as depressive disorders and cardiovascular diseases are the leading causes of morbidity and mortality, and mainly affect adults. In contrast, in low-income countries communicable diseases, perinatal conditions and malnutrition are largely responsible for the burden of morbidity and mortality, and apart from HIV/AIDS, these affect children predominantly. This translates to a high infant and childhood mortality with low-income countries accounting for over eighty percent of all global deaths in children under the age of five years. These deaths are largely attributable to preventable infectious diseases such as malaria, measles, pertussis, HIV/AIDS, pneumonia, diarrhoeal diseases and tetanus. In Sub-Saharan Africa (SSA), the three leading causes of disease are HIV/AIDS, lower respiratory tract infections and malaria, with each of these accounting for 17%, 10% and 8.5% of total disability-adjusted life years (DALY), respectively (Lopez and Mathers 2006) (Figure 1.1.1). Malaria deaths alone (excluding morbidity and disability) are responsible for almost 3% of the world’s and greater than 10% of Africa’s DALYs (Breman, Alilio et al. 2004).

DALY: Disability Adjusted Life Years - the number of healthy years of life lost due to premature death and disability
Figure 1.1.1 The burden of disease

The 10 leading causes of the burden of disease, measured in disability-adjusted life years (DALY) in sub-Saharan Africa in 2002 (DALY figures taken from Lopez 2006)
1.2 THE GLOBAL BURDEN OF MALARIA

1.2.1 FACTS AND FIGURES

Four species of Plasmodia are responsible for causing disease in humans and include *P. malariae, P. ovale, P. vivax* and *P. falciparum*. The most severe forms of malaria are caused by *P. falciparum*, and nearly one-third (2.5 billion people) of the world's population is exposed to this parasite. Estimates of the population at risk (PAR), which take into account the effects of human population density on malaria risk, indicate that South East Asia has the highest PAR with 1.25 billion people exposed to *P. falciparum* in 2005. This is followed by the African region where 500 million persons were exposed to *P. falciparum* during the same period. Other exposed populations include the Western Pacific (400 million), the Eastern Mediterranean (245 million), and South America (50 million) (Guerra, Snow et al. 2006). Although South East Asia has the highest population at risk of falciparum malaria, 70% of disease episodes attributable to this parasite occur in Africa (Snow, Guerra et al. 2005). The reasons are unclear but are likely to include environmental, as well as parasite and host factors. Notably, malaria deaths in Africa account for 86% of global malaria mortality with an estimated three million people dying each year (Breman, Alilio et al. 2004). These figures are nearly three times higher than the World Health Organisation (WHO 2002) estimates because they take into account both the direct and indirect effects of malaria, long-term effects, as well as it's effect on enhancement of the severity of other childhood diseases. Malaria has extensive effects on health and has been shown to significantly increase all-cause mortality in children under the age of five years (Snow, Korenromp et al. 2004).
1.2.2 TRENDS IN MALARIA MORTALITY IN SUB-SAHARAN AFRICA

At the turn of the 21st century, it was clear that malaria mortality in Africa was on the increase. The proportion of deaths attributable to malaria had risen from 18 – 37% in the preceding twenty years. Malaria mortality in East and Southern Africa was up from 6.5 per 1000 child-years between 1982 and 1989 to 11.9 per 1000 child-years between 1990 and 1998 (Korenromp, Williams et al. 2003). Concurrent declines in all-cause child mortality and non-malaria mortality, indicated that factors specific to malaria were responsible for this rise. Failing drug efficacy, (at that time chloroquine resistance) was widely believed to be the main explanation for the observed trends and continues to pose a significant threat to people living in malaria-endemic areas (White, Nosten et al. 1999). At present, anecdotal reports that the prevalence of malaria is declining in many parts of Africa are widespread, conceivably due to the intensified global efforts to control the disease. Published data on trends in malaria mortality since the year 2000 are scarce because of the time it takes to introduce interventions, scale-up and evaluate coverage, before finally assessing efficacy. Additionally, for effects on mortality to be detected, populations need to be exposed to interventions for a reasonable period of time (Rowe, Steketee et al. 2007). However, there is some indication that the anecdotal reports are true. Paediatric malaria admissions in three district hospitals along the Kenyan coast declined by as much as 63% between January 1999 and March 2007. Non-malaria paediatric admissions at the same time increased, or remained constant, showing that this decline was unique to malaria. The change was attributed to expansion in the coverage of interventions, such as the use of insecticide-treated bed nets and availability of anti-malarial medicines (Okiro, Hay et al. 2007).
1.2.3 MALARIA MORBIDITY

The clinical effects of malaria are far-reaching and include acute clinical manifestations, with accompanying short- and long-term sequelae, as well the effects of chronic parasitization on health and productivity. More recently, co-existence of severe malaria and other serious co-morbidities in African children have become apparent (Berkley, Mwarumba et al. 1999) with the growing realization that malaria is an important risk factor for other childhood morbidities (Snow, Korenromp et al. 2004). Figure 1.2.1 summarizes the major effects of acute and chronic malaria in children and pregnant women, many of which overlap. Malaria-induced anaemia causes more deaths than any of the other manifestations of the disease (Murphy and Breman 2001). Perinatal mortality rates in malaria-endemic areas are nearly three times higher than they are in non-malarious countries, even after controlling for socio-economic status (van Geertruyden, Thomas et al. 2004). The long term neuro-cognitive effects of severe malaria, including severe motor deficits, behavioral difficulties and epilepsy have been documented, with estimates indicating that between 1300 and 7800 children will develop neurologic sequelae following cerebral malaria each year in areas with stable endemic malaria (Mung'Ala-Odera, Snow et al. 2004). Pregnant women co-infected with HIV and malaria have consistently more peripheral and placental malaria, higher parasite densities, more febrile illnesses, severe anaemia, and adverse birth outcomes (low birth weight, prematurity, intra uterine growth retardation) than HIV uninfected women (ter Kuile, Parise et al. 2004).
Acute febrile illness

- Cerebral malaria
- Respiratory distress
- Severe anaemia
- Hypoglycaemia
- Co-morbidity

Chronic malaria

- Fetal
  - Low birth weight
  - Abortion
  - Still-birth

Malaria in Pregnancy

- Maternal
  - Acute illness
  - Anaemia

Long term sequelae

- Death

Long term sequelae

- Neurological deficits
- Developmental deficits
- Schooling, Employment & Productivity

- Anaemia
- Neuro-cognitive effects
- Developmental
- Malnutrition

- Infant and fetal mortality
- Long-term sequelae

- Long-term sequelae
1.2.4 ECONOMIC COSTS OF MALARIA

Nations affected by malaria are the poorest in the world. The average gross domestic product (GDP) per capita in 1995 for countries affected by malaria was $1,526, compared to $8,268 in malaria-free countries. Economic growth in malarial countries is five times lower than that in malaria-free countries. At a national level, malaria reduces annual economic growth of African countries by 1.3% and is estimated to cost African governments $12 billion annually (Gallup and Sachs 2001). At the household level, direct and indirect costs of malarial illness ranged from 2 – 6% of household income across Africa and Asia. The poorest families are the hardest hit, and in the lowest socio-economic groups the average total cost burden is often catastrophic (defined as greater than 10% of annual income) (Russell 2004). It is a vicious cycle, with poverty causing disease, and disease causing poverty.
1.3 MALARIA CONTROL STRATEGIES

1.3.1 TRANSMISSION DYNAMICS

Malaria is transmitted through specific mosquitoes of the *Anopheles* species. The *Anopheles gambiae* complex and *Anopheles funestus* are the most efficient vectors for *P. falciparum* and are widespread throughout tropical Africa, where warm temperatures, heavy rainfall and high humidity make ideal conditions for mosquito breeding. In order to interrupt transmission and thus eradicate malaria, the basic reproduction rate ($R_0$) – the number of infections transmitted in a non-immune population from each infected person - needs to be reduced to less than 1. However, the sigmoid relationship between $R_0$ and malaria prevalence (Figure 1.3.1) means that in areas of high malaria transmission where $R_0$ is high, large reductions in $R_0$ lead to only modest declines in malaria prevalence. In contrast, in areas with intermediate to low transmission intensity, reductions in $R_0$ lead to more substantial drops in malaria prevalence. This partly explains why eradication programs in SSA have been less successful than those in the areas with low to intermediate transmission (Walther and Walther 2007). Moreover, successful malaria eradication requires a well designed and managed health infrastructure at a regional level within countries, detailed logistical organization and financial resources to ensure sustainability (Beales and Gilles 2002), all of which are lacking in SSA. Consequently, more emphasis is currently placed on malaria control, as opposed to eradication.
1.3.1 Malaria prevalence and the basic reproduction rate

The sigmoid relationship between malaria prevalence and basic reproduction rate ($R_0$). Compared to areas with moderate to low malaria transmission, in areas with a high transmission intensity (and high $R_0$) show only modest reductions in malaria prevalence following large reductions in $R_0$. (Figure taken from Walther 2007)

1.3.2 CURRENT MALARIA CONTROLS STRATEGIES IN AFRICA

1.3.2.1 Insecticide treated bed nets (ITNs)

The efficacy of ITNs in reducing severe and uncomplicated malaria, as well as all-cause childhood mortality in children under the age of five years is not in doubt (Lengeler 2004). Similar gains have been realized in pregnant women, with ITNs significantly reducing morbidity and adverse birth outcomes (Miller, Korenromp et al. 2007). In spite of this, ITN coverage in malaria endemic countries has been extremely low with the median proportion of children sleeping under an ITN being just 3% across 34 countries (WHO 2005). Cost has
been one of the main barriers to widespread ITN use with many arguing strongly for the free provision of bed nets to rural communities (Curtis, Maxwell et al. 2003; Roberts 2007), and others for 'social marketing' where advertising campaigns promote bed-net use and local manufacture is encouraged (Lines, Lengeler et al. 2003). In a recent study in Kenya, the benefit of providing free ITNs was clearly demonstrated. When ITNs were only available via the commercial sector, 7% of children under the age of five-years, across four districts in the country slept under ITNs. This improved to 24% when subsidized nets became increasingly available through government health clinics. Importantly, the largest increment in coverage of 66% was observed following a free ITN distribution campaign (Noor, Amin et al. 2007). Increased coverage translates to a reduction in mortality, with an estimated seven deaths averted for every 1000 ITNs distributed (Fegan, Noor et al. 2007).

1.3.2.2 Indoor Residual Spraying (IRS)

IRS has historically been successful mainly in areas of low to moderate transmission, discrete, accessible communities such as islands and refugee camps, as well as in epidemics. In Africa pilot projects undertaken to eradicate malaria between the 1950s and 1970s demonstrated that it was possible to reduce but not to interrupt transmission in high transmission areas. To-date, although the WHO recommends its widespread use, including in high transmission areas, few African countries have taken up IRS (WHO 2006). Challenges include funding, sustainability and infrastructure necessary for this tool to be effective in high malaria transmission areas (Kolaczinski, Kolaczinski et al. 2007), not to mention environmental concerns regarding the use of chemicals such as dichloro-diphenyl-trichloroethane (DDT) (Rogan and Chen 2005).
1.3.2.3 Malaria Treatment

1.3.2.3.1 Home treatment of fevers

For most sick children in Africa, treatment begins at home with shop-bought brand name drugs, often with incorrect or sub-optimal dosing regimens (Marsh, Mutemi et al. 1999). This realization has prompted efforts to train local mother co-ordinators (Kidane and Morrow 2000), or shopkeepers (Marsh, Mutemi et al. 1999) to teach mothers how to correctly administer anti-malaria medications. In Kenya, the shopkeeper training program that was initiated in Kilifi district was found to be highly effective in terms of cost per DALY averted (Goodman, Mutemi et al. 2006), and has since been successfully implemented in several other districts within the country (Abuya, unpublished).

1.3.2.3.2 Hospital treatment of fevers

For sick children arriving at health facilities in SSA, the WHO has introduced the Integrated Management of Childhood Diseases (IMCI). This is a strategy for integrated case management of the five most important causes of childhood mortality; acute respiratory infections, malaria, measles, diarrhoeal disease and malnutrition (Nicoll 2000). It also includes prompt referral of children with danger signs, which is particularly important for malaria where most deaths occur within the first 24 hours of arriving to hospital (Idro, Aketch et al. 2006). Recognition and treatment of other serious childhood infections that co-exist with severe malaria will also lead to improved case management (Berkley, Mwarumba et al. 1999).
1.3.2.3.3 Intermittent preventative treatment

Intermittent preventative treatment (IPT), where a therapeutic course of an anti-malarial drug is administered at pre-determined intervals regardless of infection, is a promising option for malaria control. It is effective in reducing both the maternal and foetal adverse outcomes that arise due to malaria in pregnancy (Shulman, Dorman et al. 1999; ter Kuile, van Eijk et al. 2007). Similarly in infants, IPT appears to be effective although its protective efficacy estimates against clinical malaria (22% to 63%) vary considerably in the studies reported to date (Munday 2007). Few studies have reported the effects of IPT in older children but even in this age-group, it appears that IPT will be of benefit with a protective efficacy against clinical malaria of up to 83% (Cisse, Sokhna et al. 2006; Greenwood 2006). The main challenges facing the widespread use of IPT are the choice of drug, the development of resistance (discussed below) and the uncertain effects that IPT in infants and children may have on the development of natural immunity, particularly given that the mechanism(s) by which it prevents malaria are not fully understood (Munday 2007).

1.3.2.3.4 Anti-malarial drug resistance

Wherever anti-malarial drugs are administered, either as prophylaxis or for treatment, or for the treatment of bed-nets, drug resistance continues to be a major challenge. It arises and spreads by the evolutionary selection of spontaneously arising mutants that are drug-insensitive. At the molecular level, the principal mechanism employed by *P. falciparum* appears to be point mutations in genes that directly affect drug binding or transport with consequent changes in drug accumulation or efflux within the erythrocyte, or reduced drug affinity for the target molecule (Hyde 2007). The understanding of the molecular basis of
drug resistance is facilitating the rational development of new drugs, and enabling the engineering of older ones to regain their efficacy (Nzila 2006). Resistance to chloroquine is widely blamed for the rise in malaria mortality that was seen in SSA in the 1980's and 1990's. Resistance to sulphadoxine-pyrimethamine, which replaced chloroquine in many countries in SSA spread even faster (Sibley, Hyde et al. 2001). Recommended first-line treatments in most countries are currently based on combination therapies, to help delay or prevent resistance. These usually include a rapidly acting drug (quine or artemisinin) and a slower acting drug that often has a different mechanism of action (lumefantrine, tetracycline) (WHO 2005). However, many argue that resistance will inevitably develop even to artemisinin-based combination therapies (ACTs) whose use is currently widely promoted (Duffy and Sibley 2005; Hastings and Ward 2005). One of the major concerns is the over-diagnosis of malaria in endemic countries which is not surprising, as they commonly lack basic laboratory facilities and equipment (Makani, Matuja et al. 2003; Reyburn, Mbatia et al. 2004). Presumptive treatment of all acute childhood fevers with an anti-malarial in areas of high malaria endemicity as recommended by the WHO unfortunately, further compounds this problem (Hastings, Korenromp et al. 2007).

1.3.2.4 Environmental management

Larval control aims to reduce malaria transmission indirectly by reducing the vector population density near human habitations. It includes environmental modification (drainage, land leveling and filling), environmental manipulation (changes to the aquatic environments in which larvae develop, including chemical and biological larvicides) and modification of human habitations or behaviors. Although it is a well proven preventive
method, it is not currently being implemented in most of SSA. Major issues include a paucity of information about the distribution and behavior of vector larvae which varies between species and even within different populations of the same species, the wide range of larval habitats and an upsurge of human activities leading to an increase in human-made breeding habitats particularly in growing urban centres, around dams and irrigations sites. A recent review highlighted the substantial gaps in the scientific literature, both in control of malaria vector larvae and on the larval ecology of African vectors, information critically needed for designing effective control programs based on these approaches. Nevertheless, the limited research indicates that interventions against larval anophelines are beneficial in SSA, particularly when implemented hand in hand with other control tools targeting adult mosquito vectors (Walker and Lynch 2007).

1.3.2.5 Initiatives to combat malaria

Over the past twenty years there has been a proliferation of international, multi-lateral initiatives to co-ordinate and consolidate the efforts to control malaria. Roll Back Malaria (RBM) was launched by the WHO, World Bank, the United Nations Children’s Fund and other partners and aims to reduce the malaria burden in half by 2010 mainly through treatment and prevention strategies. The Global Fund aims to fight AIDS, Tuberculosis and Malaria and to reduce poverty. The Multilateral Initiative on Malaria (MIM) was created to promote greater research and leadership in Africa through capacity building and the facilitation of global collaboration. Medicines for Malaria Venture (MMV) is a private-public partnership whose goal is to develop at least one new affordable anti-malarial drug or drug combination. The Malaria Vaccine Initiative (MVI) speeds up the development of
promising malaria vaccine candidates. The African Malaria Network (AMANET) focuses on regional training and organization of clinical and vaccine trials within the continent. These, and other partnerships against malaria not mentioned here reflect the growing global commitment to significantly reduce the burden of malaria (Alilio, Bygbjerg et al. 2004).

1.4 MALARIA VACCINES

1.4.1 INTRODUCTION

Vaccines are the most effective, cost-effective and logistically feasible public health interventions against infectious diseases (Andre 2003). This is exemplified by the eradication of small pox, the near-eradication of polio, and the achievements of the Expanded Program of Immunization (EPI) in resource-poor countries (Ada 2005). Immunization is currently estimated to save the lives of three million children every year (Andre 2003). Although an increased number of tools for the control of malaria are now available, the majority of these are at best, partially effective and require substantial training and resources to implement at national levels (Greenwood and Mutabingwa 2002). Many malaria control experts argue that effective vaccination against malaria is in fact, the only realistic long-term solution for resource-poor countries (Tongren, Zavala et al. 2004). To date, in spite of many years of dedicated and high quality research, there is still no effective malaria vaccine but optimism that it is "just round the corner" remains high. Most efforts to develop a malaria vaccine are focused on *P. falciparum* (Figure 1.4.1) as it is responsible the most severe forms of the disease. The belief that it will be possible to
develop an effective vaccine for malaria stems from three principal observations. First, complete protection against experimental sporozoite challenge has been achieved in rodents (Nussenzweig, Vanderberg et al. 1967), non-human primates (Collins and Contacos 1972) and humans (Clyde, Most et al. 1973; Hoffman, Goh et al. 2002). Second, individuals living in endemic areas naturally acquire non-sterile immunity to malaria in which they are protected from severe illness and death, while remaining susceptible to infection (Marsh 1992). Third, in classic experiments, passively transferred antibodies from malaria ‘immune’ individuals were effectively used to treat both children and adults with severe malaria (Cohen, McGregor et al. 1961; McGregor and Carrington 1963; Sabchareon, Burnouf et al. 1991). In spite of this compelling evidence that an effective malaria vaccine should be possible, numerous challenges need to be overcome. The parasite expresses over 5500 proteins, many of which are stage specific, vary between parasite ‘strains’, and even within a single ‘strain’ through antigenic variation. Protective immunity is poorly understood and may be mediated by different immune mechanisms at different points in the life cycle. To further compound matters, the host response to the parasite is extremely variable, with manifestations ranging from asymptomatic infection and mild clinical episodes to severe, life threatening disease and death. The factors governing this range of outcomes are not well understood but undoubtedly include both parasite and host determinants. These challenges notwithstanding, two main strategies for malaria vaccine development are currently being pursued, largely in parallel; the sub-unit approach and the whole organism approach.
1.4.2 **SUB-UNIT VACCINES**

The rationale behind these vaccines includes the ability to block the molecular interactions (e.g., receptor-ligand binding) that are known to occur between the host and parasite, for example, during invasion of hepatocytes and erythrocytes by sporozoites and merozoites, respectively. It also includes the ability of single antigens to induce protective cell- or antibody-mediated immune responses. For example, antibodies that block the binding of merozoite surface proteins (MSPs) which mediate invasion of red blood cells could halt the proliferation of blood stage parasites, thereby preventing disease. Sub-unit vaccines can be made up of single or multiple antigens, and can target a single stage of parasite development or target multiple stages. Alternatively, they can be designed as multi-epitope vaccines, containing a string of immuno-dominant epitopes from a combination of antigens. Stage-specific vaccines can be classified as pre-erythrocytic, erythrocytic and sexual-stage based on the antigens they contain. This classification provides a logical approach to vaccine design though in reality it is increasingly clear from studies of the parasite genome, transcriptome and proteome, that many antigens are expressed in multiple stages (Florens, Washburn et al. 2002; Hall, Karras et al. 2005).
Figure 1.4.1 Life cycle of *Plasmodium falciparum*

On the right, the vaccine candidates from different stages that are currently under development (Figure taken from Okie 2005; list obtained from MVI).
1.4.2.1 Pre-erythrocytic vaccines

A pre-erythrocytic vaccine aims to completely prevent blood stage infection by blocking the invasion of hepatocytes by sporozoites, and/or preventing liver stage parasites from developing to maturity. This would be an ideal vaccine, preventing clinical symptoms that are generated by blood stage parasites, as well as disease transmission, in effect creating “sterile immunity” which is rarely, if ever, observed in nature. Research in this area came to the fore by serendipity. *P. gallinaceum* sporozoites from a carton of infected mosquitoes that had been left under ultra-violet (UV) light lost their infectivity to chickens but induced strong protection against subsequent challenge with virulent sporozoites (reviewed in (Druilhe and Barnwell 2007)). Although challenges remain, it is now well established both in mice and humans that immunization with radiation-attenuated sporozoites confers sterile protection against subsequent challenge with infectious sporozoites (Nussenzweig, Vanderberg et al. 1967; Hoffman, Goh et al. 2002). The mechanisms by which his protection is achieved are not completely understood but have been shown to involve cells (primarily non-cytolytic CD8+ T cells, but also CD4+ and γδ T cells), cytokines (principally IFN-γ, but also other others), antibodies and free oxygen radicals (Doolan and Martinez-Alier 2006). Similarly, the target(s) of immune responses at this stage are not well elucidated and in theory could be sporozoites, intra-hepatic parasites, or both.

Transcriptome and proteome analyses of *P. falciparum* indicate that there are numerous proteins expressed in the pre-erythrocytic stages (Florens, Washburn et al. 2002; Hall, Karras et al. 2005). The majority of these proteins have yet to be studied in detail but nonetheless, they could all be potential targets of protective immune responses. This fact was well illustrated when 16 of 27 putative *P. falciparum* proteins identified by
multidimensional protein identification technology (MudPIT) were recognized variably by antibodies in sera from volunteers immunized with irradiated sporozoites (Doolan, Southwood et al. 2003). Nevertheless, the best characterized antigens to-date include the circumsporozoite protein (CSP) which coats the sporozoite (also the first malaria antigen to be identified and cloned) (Ozaki, Svec et al. 1983) and the liver stage antigen 1 (LSA-1) which is expressed in the hepatic stages (Guerin-Marchand, Druihlé et al. 1987).

Of all the sub-unit pre-erythrocytic vaccine candidates currently under development (Figure 1.4.1), the one based on the CSP shows the most promise. In the RTS,S/AS02 vaccine, the central tandem (aparagine-alanine-asparagine-proline, NANP) repeat and carboxy-terminal regions of CSP are fused to the S antigen of hepatitis B virus (HBsAg) and co-expressed in yeast with un-fused HBsAg. The resulting complex is formulated with the adjuvant AS02 (GlaxoSmithKline Biologicals) which contains an oil-in-water emulsion and immuno-stimulants. In phase IIa trials, RTS,S had a protective efficacy of 41% (95% confidence interval (CI) 22-56%, p = 0.0006) against experimental sporozoite challenge of malaria-naïve volunteers (Kester, McKinney et al. 2001). In a phase IIb trial in Gambian adults, it had a modest protective efficacy against time to first infection of 34% (95% CI 8-53%), though this protection appeared to be short-lived (Bojang, Milligan et al. 2001).

In African children however, the results of both phase I and IIb trials have been incrementally encouraging. In this population, RTS,S has been shown to be safe, well-tolerated and immunogenic both in older children aged 1-4 years (Alonso, Sacarlal et al. 2004; Bojang, Olodude et al. 2005; Macete, Aponte et al. 2007; Macete, Sacarlal et al. 2007)
and in infants (Aponte, Aide et al. 2007). Although its reported vaccine efficacy against clinical disease has been modest (30-35%), its protective efficacy against severe disease has been substantial and sustained, 58% (95% CI 16-81%) at 6 months, 49% (95% CI 12-71%) at 18 months (Alonso, Sacarlal et al. 2004; Alonso, Sacarlal et al. 2005). Large-scale, multi-centre phase III trials are now planned (Bojang 2006).

Some are skeptical about RTS,S, in part because its precise mechanism of action remains unclear (Snounou, Gruner et al. 2005; Druilhe and Barnwell 2007). While there is evidence that it induces high levels of IFN-γ producing CD8+ (non-cytolytic) and CD4+ T cells, as well as antibodies, particularly in the presence of key adjuvants, these immune responses have not consistently correlated with protection (Lalvani, Moris et al. 1999; Sun, Schwenk et al. 2003). Others have shown using transgenic parasites that sterile protection against malaria can be obtained independently of immune responses to CSP. Mice immunized with irradiated wild type *P. berghei* were completely protected against challenge with parasites in which *P. berghei* CSP had been replaced with that of *P. falciparum*, a result which could not be accounted for by cross-reactivity of responses to CSP (Gruner, Mauduit et al. 2007). From a separate viewpoint, unlike other malaria vaccine candidates, there is little evidence that immune pressure from the human host has driven the polymorphisms observed in CSP in natural infections (Kumkhaek, Phra-Ek et al. 2005; Weedall, Preston et al. 2007), neither is there evidence that RTS,S-induced immune responses select for parasites bearing divergent CSP alleles (as opposed to those contained in the vaccine) as might have been anticipated (Enosse, Dobano et al. 2006). These reports notwithstanding, there is little
doubt that RTS,S has provided the most promising results of any malaria vaccine trial conducted in the field to date.

1.4.2.2 Erythrocytic stage vaccines

Clinical symptoms of malaria manifest during the blood-stage of the infection when the asexual-stage parasites multiply exponentially within red blood cells. Erythrocytic or blood-stage vaccines therefore aim to prevent disease or to reduce the severity or complications of disease, including death. They are predominantly being developed for children and pregnant women living in malaria-endemic areas. The cornerstone of research on blood-stage vaccines is the demonstration that the passive transfer of antibodies from semi-immune adults in malaria-endemic areas to malaria-infected patients resulted in both clinical and parasitological resolution of the disease, both in children and adults (Cohen, McGregor et al. 1961; McGregor and Carrington 1963; Sabchareon, Burnouf et al. 1991). Neither the mechanism(s), nor the target(s) of these 'protective' antibodies have been conclusively demonstrated, although many have been proposed (reviewed in (Marsh and Kinyanjui 2006; Schofield and Mueller 2006)). Two leading mechanisms include the inhibition or blocking of obligate receptor-ligand interactions, such as are required for merozoite invasion (Cowman and Crabb 2006), and antibody-dependent cellular inhibition (ADCI), in which antibodies mediate their inhibitory effects in conjunction with other immune effector cells (Bouharoun-Tayoun, Attanath et al. 1990). Potential antibody targets are numerous, and include merozoites (or merozoite proteins involved in red cell invasion) (Cowman and Crabb 2006), as well as parasite-derived proteins located on the erythrocyte surface (Bull, Lowe et al. 2002).
Many erythrocytic-stage vaccines are currently under development (Figure 1.4.1). The furthest along the line of vaccine development are based on MSP-1, -2, -3, apical membrane antigen 1 (AMA-1) and glutamate rich protein (GLURP) (Genton and Reed 2007). The first field trial of an asexual blood-stage vaccine was that of the Combination B vaccine, which contains MSP-1 (a 175 amino acid fragment from the relatively conserved blocks 3 and 4 of the K1 parasite line), MSP-2 (the 3D7 allelic type, nearly full length protein), CSP (a T cell epitope), and the ring-infected erythrocyte surface antigen (RESA, containing 70% of the native protein from the C terminal end of the molecule). In a phase 1-2b trial, 120 children in Papua New Guinea were randomized to receive either vaccine or placebo. The 60 children in each arm were further randomized into two equal groups, one with pre-treatment with sulphadoxine-pyrimethamine (SP) to clear parasites at the start of the study (n=30), and the other to no-SP treatment (n=30). Although this vaccine had no effect on clinical disease, parasite densities were lower in the vaccine group compared to the placebo group (vaccine efficacy 62% (95% CI 13 – 84)), but only in children who were not pre-treated with SP (n=30). Interestingly, during the follow up period (weeks 8 to 76 post vaccination), compared to the placebo-group, symptomatic episodes in this sub-group of vaccinees were more likely to be caused by parasites bearing the FC-27 allele of MSP-2 (as opposed to the 3D7 allele contained in the vaccine). Fifteen (15) children in the vaccine group had parasites bearing the FC-27 MSP-2 alleles, compared to 8 children in the placebo group. Eleven (11) children in the vaccine group had parasites bearing 3D7 MSP-2 alleles compared to 7 in the placebo group. Clearly, the sample sizes in this study preclude the drawing of any firm conclusions from the data. No such differences were observed in the vaccinated but SP-pre-treated group (n=30). Based on these results, the authors
concluded that the MSP-2 component of the vaccine had a specific effect on parasite growth and multiplication, and moreover had induced selection pressure on the parasites (Genton, Betuela et al. 2002). In the same study, high 3D7-specific antibody titres were obtained post vaccination in both the SP and No-SP pre-treatment groups, casting doubt on the mechanism by which vaccine-induced antibodies reduced parasite densities only in the latter group (Fluck, Smith et al. 2004). Although widely quoted, this combination B vaccine study provides remarkably weak evidence in support of arguments to include the major allelic types of antigens in blood-stage malaria vaccines.

At present, in endemic countries, the most advanced malaria vaccines have entered phase I and II clinical trials in multi-centre studies. These are based primarily on pre-erythrocytic or erythrocytic stage antigens, singly, or in combination. The map of Africa below (Figure 1.4.2) shows the countries in which malaria vaccine trials are currently being conducted, or recently completed, as listed in the worldwide registry of clinical trials (http://www.clinicaltrials.gov). Although at least thirty one different trials can be counted, the actual vaccines are comprised of two pre-erythrocytic antigens (CSP and ME-TRAP), and three erythrocytic antigens (AMA1, MSP-3 and GLURP), administered on different platforms, with different adjuvants, in semi-immune adults, or in infants and children.
Figure 1.4.2 Phase I and II malaria vaccine trials in Africa

Malaria-endemic countries in which Phase I and II malaria vaccine trials are being conducted. Colors indicate the countries, while numbers indicate the number of trials being conducted in those countries. Adapted from http://www.clinicaltrials.gov.
1.4.2.3 Sexual stage vaccines

In contrast to the pre-erythrocytic and erythrocytic stages, sexual-stage vaccines aim to block malaria transmission from infected hosts, thereby providing a herd benefit for future exposed populations. They would be targeted to people of all ages, particularly in areas with relatively low malaria transmission, as part of long-term, integrated malaria control programs. The concept of transmission blocking vaccines began with the observation that chickens immunized with *P. gallinaceum* gametocytes generated antibodies that failed to kill circulating gametocytes within the host but efficiently eliminated parasite gametes in the mosquito following a blood meal (reviewed in (Saul 2007)). Targets of such antibodies include Pfs 48/45 and Pfs 230 which are expressed by the parasite while within the vertebrate host and also exposed on the free-living gamete. As with other stages of the parasite life cycle, genomic information will lead to the identification of many new potential vaccine candidates (Florens, Washburn et al. 2002). The only published study on transmission blocking vaccines in humans has been conducted for *P. vivax* malaria, where vaccine induced antibodies to Pvs25 (a protein found on the surface of ookinetes) induced significant transmission blocking activity as detected by the membrane feeding assay (Malkin, Durbin et al. 2005).

1.4.2.4 Multi-stage, multi-component vaccines

Vaccines combining several key antigens from multiple stages would have the potential to interrupt the life cycle of the parasite at multiple points. In a multi-antigen, multi-stage vaccine for instance, parasites that survived the range of immune responses induced by pre-erythrocytic antigens, would then have to contend with those induced by blood stage
antigens. The different antigens could be selected to target discrete pathways essential to parasite survival within each stage, and ideally induce both antibody- and cell-mediated immunity. In addition, the antigens could be sufficiently diverse to overcome the genetic restriction of the host immune response and polymorphism of critical target epitopes. While attractive conceptually, few human trials with multi-stage, multi-component vaccines have actually been performed.

The first synthetic malaria vaccine (SPf66) to be tested in malaria-endemic areas contained multiple components from both the pre-erythrocytic and erythrocytic stages of *P. falciparum* (Patarroyo, Romero et al. 1987). Despite initial promise, a recent meta-analysis of ten trials conducted in malaria-endemic areas found no evidence of protection conferred by vaccination with SPf66 (Graves and Gelband 2006). Of the many lessons that could be learned from the ‘failure’ of Spf66 (Snounou and Renia 2007), perhaps the most important is the need to understand the mechanism(s) by which future vaccines exert their anti-malarial effects. Improvements to Spf66 were precluded in part, by a lack of understanding of its’ mode of action (Gilbert and Hill 1998). Indeed, vaccine induced total IgG antibodies to Spf66 did not correlate with the ability of sera to inhibit growth, or with partial clinical protection (Ferreira 1996).

In a separate attempt, vaccination with NYVAC-Pf7, a pox-vectored malaria vaccine that contained seven antigens from all three stages of the parasite life cycle within the human host (CSP, PfSSP2, LSA1, SERA, AMA1, Pfs25), also yielded disappointing results when volunteers were challenged (Ockenhouse, Sun et al. 1998). Potential explanations include;
the emphasis of the investigators on the induction of pre-erythrocytic cell-mediated immunity, the ethical constraints of the study design, precluding the investigation of blood-stage immunity, and probably a poor combination of antigens, delivered sub-optimally. This is an area that needs further study and development.

1.4.3 Whole Organism Vaccines

The whole organism vaccine approach shares the same foundations as the pre-erythrocytic vaccines ie, the demonstration of sterile immunity following immunization with radiation-attenuated sporozoites. In early studies in humans, this was achieved by a lengthy and potentially dangerous process. Volunteers were infected with *P. falciparum* and were treated with doses of chloroquine sufficient to suppress but not eradicate the parasites. Gametocytes were allowed to develop and then mosquitoes were fed on the volunteers (Clyde, Most et al. 1973). This approach was simplified with the advent of methods to culture *P. falciparum in vitro* (Trager and Jensen 1976), produce gametocytes in culture (Campbell, Collins et al. 1982) and infect mosquitoes from *in vitro* gametocyte cultures (Chulay, Schneider et al. 1986). More recent studies have confirmed that the method works in principle, but requires 1000 or more infective mosquito bites, making it logistically impractical to implement on a large-scale (Hoffman, Goh et al. 2002). Live sporozoites have to be used for immunization as the induction and maintenance of protective immune responses depends upon the presence of viable, but developmentally arrested liver stage parasites (Scheller and Azad 1995). As such, irradiation of sporozoites has to be carefully and reproducibly titrated, to allow effective immunity to develop, without permitting break-through parasites that could lead to potentially lethal infections (Mellouk, Lunel et al.
1990). A further limitation is the inability to define the effects of irradiation at a molecular level, to ensure that the vaccine contained well defined parasites. Remarkably many of these logistic and technical challenges are being overcome and efforts to develop radiation-attenuated sporozoites that can be practically administered, produced in sufficient quantities and meet regulatory, potency and safety requirements (Luke and Hoffman 2003) are coming to fruition, with human trials with attenuated sporozoites planned in the 2009.

An alternative strategy for the whole organism approach is the use of genetically-attenuated parasites. In place of radiation, genetic engineering is used to attenuate sporozoites such that they remain viable, are able to infect hepatocytes, but unable to develop into mature pre-erythrocytic forms. Importantly, this can be reproduced consistently and in a standardized fashion. In a mouse model infected with *P. berghei*, inactivation of liver-stage specific genes, *UIS3* and *UIS4* (up-regulated in infective sporozoites) or the sporozoite-specific gene P36p, render parasites incapable of completing their intra-hepatic development, but induce immune responses that confer sterile protection when challenged with wild type infectious sporozoites (Mueller, Camargo et al. 2005; Mueller, Labaied et al. 2005; van Dijk, Douradinha et al. 2005). The immune mechanisms underlying this protection continue to be elucidated, with early studies conducted in immune-deficient mice indicating that adaptive T and B cells, as well as interferon-γ may be important (Mueller, Deckert et al. 2007).
1.4.4 THE CHALLENGES

It is clear that numerous hurdles lie ahead for malaria vaccine development, both for sub-unit vaccine, as well as whole organism vaccines. The situation is not made easier by the poor understanding of the mechanisms underlying naturally acquired immunity, which could strategically inform vaccine design and guide vaccine improvement (Marsh and Kinyanjui 2006). The lack of relevant animal models in which to dissect out protective immunity is an additional setback. Immune mechanisms in mice and primate models of malaria do not reliably generalize to the human immune system (Druilhe 1997). The lack of immune correlates of protection means that candidates for vaccine development are selected on the basis of supportive data rather than formal evidence of a protective role in humans, and protective efficacy will only truly be established in vaccine trials in malaria-endemic countries.

1.5 NATURALLY ACQUIRED IMMUNITY TO MALARIA

1.5.1 DEFINING IMMUNITY TO MALARIA

In populations continuously exposed to malaria, several types or levels of immunity against *P. falciparum* co-exist. Immunity to severe and life threatening clinical episodes (including cerebral malaria, severe anaemia, metabolic acidosis, and other severe manifestations) is observed in older children and adults, is acquired relatively early (usually complete by the age of five years), and lasts for life (Marsh 1992; Baird 1995). Immunity to mild clinical episodes takes longer to establish. Young adults remain susceptible often until their middle to late twenties. Sterile immunity to infection with parasites is rarely, if ever,
observed. Indeed, the prevalence of *P. falciparum* parasitaemia increases steeply in early childhood, remaining high for a period of time after, immunity to severe and mild disease is well established (Figure 1.5.1). Thus, the expression of the acquisition of immunity appears to be sequential, with the ability to limit parasite growth and multiplication, followed by essentially complete protection against severe and then mild clinical disease, and culminating with partial protection against infection. Of particular interest are children under the age of five years, who paradoxically are at risk of severe disease and death caused by malaria parasites, while at the same time, able to harbor large numbers of parasites without showing any obvious symptoms (Baird 1998; Marsh and Kinyanjui 2006). It is not immediately apparent from these epidemiological descriptions whether the children who proceed from asymptomatic infection, through mild to severe and life-threatening malaria are a distinct sub-set of a larger pool of children who are able to control their parasites or whether in-fact, under similar environmental conditions, all children under five years of age are equally at risk of clinical episodes.
Population indices of immunity to malaria - Kilifi

Figure 1.5.1 Population indices of immunity to malaria

Population indices of immunity to malaria, taken from representative studies conducted in Kilifi, Kenya. The age pattern of asymptomatic parasite prevalence and the period prevalence of both severe and mild clinical malaria are shown in relation to maximum immunity. Immunity to severe malaria is established at a time when asymptomatic parasite prevalence is rising and susceptibility to mild malaria is constant (Taken from Marsh 2006).
1.5.2 MEASURING IMMUNITY TO MALARIA

The first point in any discussion of immunity must be the definition of the outcome against which this immunity is directed. Infection with malaria ranges from being asymptomatic, to mild febrile episodes, through to severe and life-threatening clinical disease. However, for any of the possible 'outcomes' or 'end-points' of infection with malaria parasites, there are no robust immune correlates of protection. Two fundamental issues contribute to this. The first relates to the identification of individuals that are 'protected' from any given outcome. The standard approach used to identify protective immune responses involves longitudinal monitoring of cohorts in malaria endemic areas for defined periods of time, to identify those that are protected (Marsh, Otoo et al. 1989). Logistical and financial constraints often dictate that such studies are conducted for limited periods of time.

Immune responses detected against the malaria antigen of interest are then compared using a wide range of statistical analytical strategies, among protected and susceptible individuals, "during the period of observation". Such studies have often produced conflicting results when repeated in different malaria endemic populations, as illustrated by studies of the effect of antibodies to the best characterized merozoite surface protein, MSP-1\textsubscript{19}. Antibodies to this antigen have been associated with protection in some studies, but not in others (Riley, Allen et al. 1992; Hogh, Marbiah et al. 1995; al-Yaman, Genton et al. 1996; Egan, Morris et al. 1996; Branch, Udhayakumar et al. 1998; Dodoo, Theander et al. 1999; Conway, Cavanagh et al. 2000; Cavanagh, Dodoo et al. 2004; Perraut, Marrama et al. 2005). The key limitation of this approach is that it introduces a misclassification bias at several levels, leading to an underestimation of the potential protective effects of antibodies. First, individuals protected during follow-up could actually have been
identified as susceptible, had the period of observation been longer. Second, it is never
absolutely certain that each person identified as protected, was actually challenged during
the observation period. Third is the fact that most assays are conducted at a single time
point, and do not take into account the ability of individuals to respond had they been
challenged, termed ‘immune-responsiveness’ (Marsh and Kinyanjui 2006). This effect is
well demonstrated, especially for antibody responses to malaria antigens, where the
proportion of responders and antibody amounts are consistently higher in individuals who
were parasitaemic at the time the serum sample was collected, even though individuals
probably move frequently between being parasitized and non parasitized (Muller, Fruh et
al. 1989; Fruh, Doumbo et al. 1991; Tolle, Fruh et al. 1993; Polley, Mwangi et al. 2004;
Polley, Conway et al. 2006; Osier, Polley et al. 2007).

The other fundamental problem is the poor understanding of how immunity to any given
outcome of malaria is acquired in nature. Assuming that protected and susceptible
individuals for a particular outcome had been indisputably identified, what measure of
immunity should then be made? What are the mechanisms that lead to protective
immunity? Are there different mechanisms for the range of clinical outcomes, and are they
detectable at any given time? Do the same mechanisms that operate in adults, similarly
function in children? Why are children who are susceptible to death from severe malaria
nevertheless able to asymptotically tolerate much higher parasite burdens compared to
adults in the same area? What role does continuous exposure to diverse malaria parasites
and other infections have on these mechanisms? Do the mechanisms vary under different
intensities of malaria transmission? The rest of the discussion will review what we know,
or think we know, about the key mechanisms underlying natural acquired immunity to malaria.

1.5.3 Humoral immunity against malaria

1.5.3.1 Key evidence

The central evidence for the role of antibodies in mediating protection to malaria is that obtained from studies on the passive transfer of immune antibodies (discussed above). These studies are often interpreted as demonstrating that immunity (prevention of the unfavorable outcomes that could arise from infection with malaria parasites) can be passively transferred. This principle of the passive transfer of antibodies is supported by epidemiological observations that infants born to mothers in malaria-endemic areas are protected in the first few months of life (Brabin 1990), even though the evidence that this protection is antibody-mediated is weak. Placentally transferred malaria specific antibodies were associated with a lower risk of clinical disease in only one of four studies (Hogh, Marbiah et al. 1995; Achidi, Salimonu et al. 1996; Kitua, Urassa et al. 1999; Riley, Wagner et al. 2000), and with a lower risk of infection in one small study where twenty infants were monitored longitudinally (Branch, Udhayakumar et al. 1998). It is often overlooked that what the passive transfer studies actually did show is the therapeutic ability of immune antibodies. This is an important distinction, as the recipients of the immune sera were never challenged with malaria parasites at that time. It is conceivable that the mechanisms responsible for preventing disease in the first place, are distinct from those involved in controlling already established disease. This does not however exclude the possibility that antibodies mediate protection in both directions, ie preventing disease
and controlling established pathological disease processes. It is unknown whether passively transferred immune antibodies would prevent disease in proven susceptible individuals upon challenge. Another fact often overlooked is that the doses of human immunoglobulin that were required to achieve control of the disease and infection were massive (approximately half the total circulating immunoglobulin pool of the recipient), which might imply that immune adults actually have low titres of protective antibodies (Cohen, McGregor et al. 1961). Additionally, there is some evidence that this protective effect was not limited to anti-malarial antibodies per se. In experiments that attempted to dissect out the properties of 'protective antibodies', in comparison to serum that was pre-absorbed with a mixture of malaria antigens, non-absorbed sera was more effective at inhibiting parasites, as measured by reduced leucine incorporation, a finding the authors reported but did not discuss (Cohen and Butcher 1970).

1.5.3.2 Potential mechanisms and supporting data from studies in malaria-endemic areas

1.5.3.2.1 Invasion-inhibition

The concept that antibodies could inhibit invasion has its origins in the work that followed on from the passive transfer experiments. In one of these follow-on studies (Cohen and Butcher 1970), it was observed that immune serum had no effect on the growth of intra-erythrocytic parasites but inhibited growth at the time of merozoite release. *P. knowlesi* cultures in the presence of immune or normal serum (controls) were started at the trophozoite or early schizont stage and growth was monitored by the inhibition of uptake of labelled leucine. Neither immune, nor normal serum had any effect on parasite growth
before schizogony. In both groups, leucine uptake remained constant during schizogony.

After schizogony, leucine uptake remained constant or decreased gradually in adequate concentrations of immune serum, while it increased in a linear fashion in cultures maintained in normal serum. The authors proposed that in vitro immune serum somehow neutralized parasites but could not directly demonstrate how this was achieved (Cohen and Butcher 1970). This neutralization effect of immune serum was lost when univalent Fab + Fc antibody fragments were used, but retained with bivalent F(ab)2, leading them to postulate, as was later demonstrated (Miller, Aikawa et al. 1975), that antibody mediated agglutination of merozoites was a probable immune mechanism in vivo (Cohen and Butcher 1970).

With the advent of monoclonal antibodies (mAbs), it became possible to directly demonstrate that certain mAbs significantly reduced invasion (Freeman, Trejdosiewicz et al. 1980; Epstein, Miller et al. 1981), raising this as another potential mechanism for the 'neutralization effect' of immune antibodies. Interestingly, to-date, complete blockage of invasion has not been demonstrated using antibodies to a range of malaria antigens thought to be targets of invasion-inhibiting antibodies and therefore malaria vaccine candidates (Deans, Alderson et al. 1982; Epping, Goldstone et al. 1988; Clark, Donachie et al. 1989; Blackman, Heidrich et al. 1990; Sim, Orlandi et al. 1990; Locher, Tam et al. 1996). The same phenomenon (reduction, as opposed to abrogation of invasion/parasite growth) was also observed in the studies by Cohen et al. on the properties of protective antibodies, and was found to be dose dependent (Cohen and Butcher 1970; Cohen and Butcher 1970). However, even in the highest serum concentrations tested, the inhibition of leucine uptake
was never absolute, and declined gradually, suggesting that some parasites were still viable (Cohen and Butcher 1970). A parallel observation can be made in the passive immune-antibody transfer studies where total eradication of parasites was never achieved, in spite of repeated treatments (Cohen, McGregor et al. 1961; Sabchareon, Burnouf et al. 1991).

At present, numerous proteins located on, or associated with, the merozoite surface, or found within its apical organelles are thought to be involved in invasion, and are therefore potential malaria vaccine candidates. Key evidence often quoted for each of these antigens is the demonstration that monoclonal or affinity purified antibodies against the specific targets inhibit (reduce) invasion (Deans, Alderson et al. 1982; Epping, Goldstone et al. 1988; Clark, Donachie et al. 1989; Blackman, Heidrich et al. 1990; Sim, Orlandi et al. 1990; Locher, Tam et al. 1996). Interestingly, attempts to disrupt many of these genes, individually, have revealed a remarkable redundancy in merozoite-invasion pathways (Cowman, Baldi et al. 2002; Cowman and Crabb 2006). Furthermore, invasion-inhibition assays performed using sera from malaria immune adults, have consistently demonstrated wide variability (0-100%) in the ability of individual sera to inhibit invasion of a specific isolate, and variability even within a given sera against a range of laboratory or field isolates (Brown, Anders et al. 1983; Singh, Ho et al. 1988). If a particular threshold of protective antibodies is required to provide immunity, it may be expected from the epidemiology of malaria that all immune adults would have achieved this threshold, and thus, one would expect the majority, if not all, sera to inhibit invasion of a wide range of parasite strains. It is possible that methodological limitations in assays of invasion-inhibition may account for some of these discrepancies, and efforts are underway to
address these (Persson, Lee et al. 2006). Nevertheless, this brings into the question the importance of invasion-inhibition as a mechanism for natural immunity. While it appears to be logical, given our understanding of the parasite life-cycle, and we can demonstrate it to varying degrees in vitro, to date, there is little evidence that it correlates with immunity against any outcome of malaria infection in vivo.

1.5.3.2.2 Antibody-dependent cellular inhibition (ADCI)

Work in this area is partly fuelled by the inability to reproduce the in vivo findings from the passive immune antibody transfer experiments in vitro. On the one hand, passively transferred immune antibodies significantly reduced parasitaemia and alleviated clinical symptoms in patients with malaria (Cohen, McGregor et al. 1961; Sabchareon, Burnouf et al. 1991), on the other, in vitro invasion-inhibition by sera from malaria immune adults is highly variable, some sera have no effect in the assay, others differ considerably in their invasion-inhibition ability, while others somewhat counter-intuitively, appear to promote parasite growth (Phillips, Trigg et al. 1972; Mitchell, Butcher et al. 1976; Wilson and Phillips 1976; Brown and Smalley 1981; Shi, Udhayakumar et al. 1999).

In ADCI, antibodies exert their inhibitory effects, not in isolation, but in conjunction with monocytes (Khusmith and Druilhe 1982; Khusmith, Druilhe et al. 1982; Khusmith and Druilhe 1983). In a typical assay, parasite growth is compared in cultures containing immune sera with or without monocytes, and similarly, in non-immune sera, with or without monocytes. In the first description of this assay, effective inhibition of parasite growth was observed consistently only with the combination of immune serum and
monocytes, and was thought to be mediated via the Fc portion of immune antibodies. Monocytes phagocytosed mainly merozoites, as opposed to schizont-infected erythrocytes, and this increased with crude measures of increasing levels of immunity (Khusmith, Druilhe et al. 1982). The targets of antibodies that are able to inhibit parasite growth by ADCI have since been identified, and at present include MSP-3 (Oeuvey, Bouharoun-Tayoun et al. 1994), GLURP (Theisen, Soe et al. 1998), and SERP (serine repeat protein) (Soe, Singh et al. 2002), and in all these studies, the in vitro assays have correlated with either being susceptible or immune to clinical attacks of malaria. In spite of this, several unrelated groups have tried to reproduce the ADCI assay with unsatisfactory results (Rzepczyk, Lopez et al. 1988; Shi, Udhayakumar et al. 1999; Tebo, Kremsner et al. 2001).

One reason for this is the fact that it is a tedious assay, requiring meticulous calibration of culture conditions, and therefore unusually prone to operator error. A particular issue highlighted in these studies (Shi, Udhayakumar et al. 1999; Tebo, Kremsner et al. 2001) and often discussed by malaria researchers, is the heterogeneity of anti-parasitic activity in monocytes from individual donors.

1.5.3.2.3 Adhesion-inhibitory antibodies

Unlike other human malarias, P. falciparum has the unique ability to modify the surface of infected red cells, enabling the parasite to cytoadhere (stick) to a range of cells, including endothelial cells, cells from the syncytiotrophoblast, P. falciparum infected, and non-infected erythrocytes. Cytoadherence is thought to contribute directly and indirectly, to many of the severe clinical manifestations of P. falciparum infection, that are not observed in the other human malarias (reviewed in (Miller, Baruch et al. 2002; Mackintosh, Beeson
et al. 2004)). Cytoadherence is mediated by parasite proteins that are inserted onto the surface of infected erythrocytes by maturing parasites and collectively referred to as variant surface antigens (VSAs), because they are encoded by multigene families and undergo clonal antigenic variation. The best characterized VSA is PfEMP-1 (*P. falciparum* erythrocyte membrane protein 1) which binds to a wide range of host receptors, such as ICAM-1 (intra-cellular adhesion membrane protein 1), thereby contributing to disease pathogenesis (Deitsch and Hviid 2004). VSAs are key players in host immune evasion, maintaining chronic infections through clonal antigenic variation (Brown and Brown 1965; Butcher and Cohen 1972). At the same time, they are thought to contribute significantly to the pathogenesis of severe malaria, largely but not only, due to cytoadherence. Not surprisingly, they are also thought to be the key targets of protective antibody responses, which would reasonably be presumed to ‘block’ cytoadherence, amongst other functions (Hviid 2005). However, although antibodies to VSAs can be readily demonstrated in residents of malaria-endemic areas, and even correlated with immune status (Marsh and Howard 1986; Bull, Lowe et al. 1998), experiments to demonstrate their mechanisms of action have not yielded consistent results.

In a model of cytoadhesion, *P. falciparum* trophozoite and schizont infected red blood cells can unquestionably be shown to ‘stick’ to endothelial cells of various origins, including those taken from humans (Udeinya, Schmidt et al. 1981; Schmidt, Udeinya et al. 1982). This cytoadherence can be inhibited, and even reversed by homologous immune serum (Udeinya, Schmidt et al. 1981; David, Hommel et al. 1983). In an experiment that yielded dramatic and exciting results, immune serum was transferred into two *Saimiri sciureus*
monkeys infected with a strain of *P. falciparum* that had been adapted to the squirrel monkey. Prior to the administration of immune serum, few trophozoite- or schizont-infected erythrocytes were seen in the peripheral blood. However, in concordance with the *in vitro* melanoma cell binding assay (MBCA), this number increased sharply within minutes of receiving intra-venous immune serum and only declined gradually after the first half hour (David, Hommel et al. 1983), raising expectations that the same effects could be observed in humans with cerebral malaria (thought to be caused partly by sequestration, as a result of cytoadherence in small capillary beds in the brain). However, when human immune sera were tested in the MBCA, widely varying results were obtained. Briefly, a minority (21%) of sera showed significant inhibition of cytoadherence to at least one of five parasite strains tested, the majority of which had high titre antibodies (11/12), but overall, the majority of sera (36/47) containing high titre antibodies showed no significant adhesion inhibitory/reversal activity (Singh, Ho et al. 1988). This situation is reminiscent of the studies on invasion-inhibitory antibodies in immune sera from endemic populations.

1.5.3.2.4 Summary

Only the key or leading mechanisms thought to underlie naturally acquired immunity have been reviewed here, and this is by no means comprehensive. Nevertheless, a common theme emerges, which is, while antibodies to a range of parasite targets can often (not always) be demonstrated to be present, and in high titres in immune compared to non-immune individuals, the mechanisms by which they are proposed to mediate protection have not yielded consistent results, particularly when tested in residents of malaria endemic regions. Hence the lack of immune correlates of protection stems in-part, from the
inability to reproducibly marry immune mechanisms demonstrable in vitro, with levels of immunity observed in vivo.

1.5.4 Cellular responses against malaria

1.5.4.1 Key evidence

Apart from the fact that T cells are absolutely essential for the switch of antibodies from the IgM to IgG isotype, and therefore contribute directly to the effects of antibodies, the best direct evidence that T cells are important for protection in malaria comes from experiments in murine models of malaria. These experiments established that under certain conditions, it was possible for protective immunity against malaria to develop in the absence of antibodies. In the first of these studies (Grun and Weidanz 1983), the development of B-cells was inhibited using repeated doses of specific antiserum (anti-\( \mu \)) which leads to a severe B-cell deficiency. The "\( \mu \)-suppressed" mice were then actively immunized using intravenous or intraperitoneal injections of parasitized erythrocytes from a range of Plasmodial species, (\( P. yoelii \), \( P. vinckei \), \( P. berghei \), and \( P. chabaudi \)), and the course of infection compared with that in control 'intact' mice (not "\( \mu \)-suppressed"). In "\( \mu \)-suppressed" mice antibody-independent immunity was demonstrated against re-infection, but only with homologous parasites and under certain conditions. First, following initial challenge, the mice had to be treated with sub-curative doses of clindamycin, as the protective immunity depended on the presence of chronic low grade parasitaemia. Second, protection depended not only on the genotype of the mouse, but also on the infecting species of plasmodia (i.e. protection was observed in mice of a particular genetic background and not others, and with some, but not all the species of Plasmodia tested).
Furthermore, in chronically infected mice, challenge with parasites from heterologous species produced variable results, with protection being observed for some combinations, and not others. The tables below adapted from Grun 1983, summarize the results of these experiments.

**Table 1.5.1 Outcome of infection in B-cell deficient mice of the same genetic background**

<table>
<thead>
<tr>
<th>Infecting plasmodial species</th>
<th>Host Immune status (mice)</th>
<th>Outcome of Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. yoelii</em></td>
<td>Intact</td>
<td>Non-lethal</td>
</tr>
<tr>
<td></td>
<td>B-cell deficient</td>
<td>Uniformly lethal</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>Intact</td>
<td>Delayed death</td>
</tr>
<tr>
<td></td>
<td>B-cell deficient</td>
<td>Early death</td>
</tr>
<tr>
<td><em>P. chabaudi</em></td>
<td>Intact</td>
<td>Non-lethal</td>
</tr>
<tr>
<td></td>
<td>B-cell deficient</td>
<td>Non-lethal</td>
</tr>
<tr>
<td><em>P. vinckei</em></td>
<td>Intact</td>
<td>Lethal</td>
</tr>
<tr>
<td></td>
<td>B-cell deficient</td>
<td>Lethal</td>
</tr>
</tbody>
</table>

In mice of the same genetic background, varying levels of B-cell independent immunity were observed with some (*P. chabaudi, P. vinckei*), but not all (*P. yoelii, P. berghei*) plasmodial species.
Table 1.5.2 Proportion of B-cell deficient mice of different genetic backgrounds surviving following homologous challenge

<table>
<thead>
<tr>
<th>Infecting plasmodial strain</th>
<th>Mice (%)</th>
<th>Mice (%)</th>
<th>Mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/10</td>
<td>(C57BL/10 x BALB/c) F1</td>
<td>BALB/c</td>
</tr>
<tr>
<td><em>P. yoelii</em></td>
<td>100%</td>
<td>35%</td>
<td>80%</td>
</tr>
<tr>
<td><em>P. chabaudi</em></td>
<td>10%</td>
<td>0%</td>
<td>3%</td>
</tr>
</tbody>
</table>

The proportions of B-cell deficient mice surviving homologous challenge varied, depending on the genetic background of the mice.

Table 1.5.3 Specificity of antibody-independent immunity to malaria in B-cell deficient mice of the same genetic background

<table>
<thead>
<tr>
<th>Infecting species</th>
<th>Challenge with:</th>
<th><em>P. yoelii</em></th>
<th><em>P. chabaudi</em></th>
<th><em>P. berghei</em></th>
<th><em>P. vinckei</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. yoelii</em></td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Acute infection*</td>
<td></td>
</tr>
<tr>
<td><em>P. chabaudi</em></td>
<td>Death</td>
<td>Resistant</td>
<td>Death</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td><em>P. vinckei</em></td>
<td>Death</td>
<td>Resistant</td>
<td>Death</td>
<td>Resistant</td>
<td></td>
</tr>
</tbody>
</table>

Chronically infected mice were challenged with parasites from heterologous species and the course of infection monitored. * Mice developed significant parasitaemia but survived acute infection.

Although protective immunity could be achieved under these conditions, the mice were unable to completely eliminate parasites and instead developed chronic relapsing parasitaemias (Grun and Weidanz 1983). These observations were subsequently confirmed in mice genetically incapable of making B cells (van der Heyde, Huszar et al.)
Further clear evidence comes from separate experiments in which protective immunity that had been induced by sporozoite immunization could be passed on to naïve mice, by the adoptive transfer of splenic cells and not serum. This protection was lost if splenic cells were depleted of T cells in vitro, prior to adoptive transfer, demonstrating that immunity was T cell dependent (Spitalny, Verhave et al. 1977). More recently, the adoptive transfer of immune T cells into naïve mice protected against malaria in the absence of antibodies (Egan, Weber et al. 1987).

Although it is accepted that no single mouse model replicates all the features of human malarias either in terms of pathology or immune responses (Stevenson and Riley 2004), (Druilhe 1997), this approach nevertheless allows for a dissection of immune responses and mechanisms in studies that are impossible to conduct in humans, and may shed light on processes common to both hosts. While this section aims to briefly review key aspects of naturally-acquired cell mediated immune mechanisms against malaria in humans, it is impossible not to discuss murine and rodent malarias, as well as *P. falciparum* sporozoite challenge experiments in humans, because what is currently understood in humans has its foundations in these experiments.

1.5.4.2 Potential mechanisms and supporting data from studies in malaria endemic areas

1.5.4.2.1 CD4+ T-cell mediated mechanisms

Clear evidence for the protective role of parasite-specific CD4+ T cells in murine malaria is the demonstration that they can provide protective immunity when adoptively transferred
to reconstituted nude mice or those with severe combined immune deficiency (SCID) (Brake, Long et al. 1988; Taylor-Robinson, Phillips et al. 1993; Amante and Good 1997). These studies were conducted in mice of different genetic backgrounds, used different species of Plasmodia, and injected infected erythrocytes intravenously or intraperitoneally. As such, as discussed previously, the studies may have limited relevance to humans naturally infected with *P. falciparum* malaria. Furthermore, although protection was clearly achieved, the mechanisms underlying it are unclear since the animals were challenged with live blood stage parasites, yet red cells do not express the major histocompatibility (MHC) antigens and the targets of the protective T-cells remain unknown. However, at present, based on these and other studies mainly on murine malaria, a generally accepted model for antibody-independent CD4+ T-cell mediated parasite killing has been developed, and is illustrated in Figure 1.5.2. Dendritic cells (DCs) present parasite antigens to CD4+ T cells in the spleen, activating them to promote phagocytosis by macrophages, and/or to produce effector molecules (tumor necrosis factor α (TNF-α), gamma interferon (IFN-γ), oxygen and nitric oxide radicals) that in addition to promoting phagocytosis, also facilitate the killing of parasites within erythrocytes (reviewed in (Good 2001)).

In humans however, the role of CD4+ T-cells in immunity to malaria is less well understood. T-cells from both exposed and non-exposed donors proliferate and secrete IFN-γ in response to a range of malaria antigens *in vitro*, including those from the pre-erythrocytic, as well as those from erythrocytic stages (Zevering, Amante et al. 1992; Good 1994), (Ndungu, Sanni et al. 2006). Potential explanations for this include, mitogenic or
superantigenic activation by malaria parasites (Ballet, Druilhe et al. 1981), cross-reactive epitopes shared with other organisms (Currier, Sattabongkot et al. 1992) and, the engagement of T-cell receptor independent mechanisms (Ndungu, Sanni et al. 2006). The responses to the pre-erythrocytic antigens are the best studied, in this regard. CD4+ T cells that recognize multiple pre-erythrocytic antigens are present in sporozoite immune human volunteers and semi-immune residents from Kenya and the Irian Jaya (Doolan, Southwood et al. 2000). The frequency and magnitude of these T helper responses depended on the intensity of exposure to *P. falciparum* sporozoites (Doolan, Southwood et al. 2000). However, until recently, there has been little evidence to suggest that these play an important role in providing protection in humans. Although studies have reported higher frequencies of lympho-proliferative CD4+ T cell responses to specific malaria antigens (CSP) in individuals protected from malaria compared to those who are not (Hoffman, Oster et al. 1989), such cells are not cytolytic, and no specific correlation exists between proliferation and induction of cytokines that may mediate protection. Indeed, in a study of T-cell effector functions against PfCSP in malaria-exposed individuals, it was found that although responses assayed by the ex-vivo IFN-γ ELIspot, cultured IFN-γ ELIspot and lymphoproliferation assay were predominantly CD4+ T cell mediated, they were not correlated as might have been expected for any given peptide, resulting in the conclusion that each assay identified unique effector mechanisms (Flanagan, Lee et al. 2001). Perhaps the most credible evidence that CD4+ T cells are important in mediating naturally acquired immunity comes from the study of Reece *et al.* (Reece, Pinder et al. 2004), where the cultured ELIspot assay was used to detect IFN-γ secreting, central memory type CD4+ T
cells in response to a conserved sequence from PfCSP. The presence of such cells was strongly predictive of protection from infection and disease.

Finally, in a comprehensive review of data from children, adults, pregnant women and neonates, infection with the human immunodeficiency virus (HIV/AIDS) was found to have had less impact on human malaria than might have been anticipated given the epidemiological overlap of the two diseases and the fact that HIV causes low CD4+ T-cell counts (Butcher 2005). This is in contrast to mice where, early studies demonstrated clearly that T-cell depletion resulted in high and prolonged parasitaemias, and often led to fatal severe anaemia (Brown, Allison et al. 1968).
Figure 1.5.2 Antibody-independent cell-mediated immunity

Proposed mechanism of action of antibody-independent cell-mediated immunity, occurring mainly in the spleen. CD4+ T cells are activated by mature dendritic cells leading to macrophage activation, phagocytosis of parasitized erythrocytes, secretion of cytokines and other inflammatory mediators. IFN-γ, interferon-γ, IL-12, interleukin-12, MHC II, major histocompatibility complex class II, TCR, T cell receptor, TNF-α, tumor necrosis factor-α.

(Good 2001)
1.5.4.2.2 CD8+ T-cell mediated mechanisms

The best evidence for CD8+ T-cell mediated immunity against malaria comes from the sporozoite challenge experiments in rodent malaria models. The protective immunity that was obtained following radiation-attenuated sporozoites was lost when CD8+ T-cells were depleted *in vivo*. In these studies, protection was neither abrogated by the depletion of CD4+ T cells, nor affected by anti-sporozoite antibody titres, thus implicating CD8+ T cells as the critical effector cells (Schofield, Villaquiran et al. 1987; Weiss, Sedegah et al. 1988).

It was subsequently demonstrated that CD8+ T-cell clones raised against specific epitopes within the CSP protein conferred high levels of species- and stage-specific protection against sporozoite challenge when adoptively transferred into naïve mice (reviewed in (Hafalla, Cockburn et al. 2006)).

In humans, there is no direct evidence that CD8+ T-cells provide or contribute to protective immunity. Specific CD8+ T cells, particularly those to pre-erythrocytic stage antigens including, CSP, TRAP/SSP2, LSA1, and Exp-1 have been detected in humans either following immunization with radiation attenuated sporozoites, or following natural exposure (reviewed in (Doolan and Martinez-Alier 2006). However, the mere presence of malaria specific CD8+ T cells is not an indication that they have a role to play in providing protective immunity against malaria (Doolan and Martinez-Alier 2006), (Hafalla, Cockburn et al. 2006). Nevertheless, association studies conducted in malaria endemic areas, as well as human challenge experiments with radiation attenuated sporozoites suggest that they may be important. For example, the presence of parasite-specific CD8+ T-cells has been indirectly (via HLA-B53) associated with protection from severe malaria in Gambian
children (Hill, Allsopp et al. 1991). Interestingly, this association was not observed when a similar study was performed in a different malaria-endemic region (Yate 1994, unpublished). CD8+ T cells responses to certain peptides of Pf LSA-1 have been associated with protection from clinical episodes of malaria in a longitudinal study (John, Moormann et al. 2004). In the attenuated P. falciparum sporozoite challenge experiments, where humans were immunized with thousands of infected mosquitoes, the frequency and magnitude of CD8+ T cells was higher than that found in naturally exposed subjects who are usually immunized with ten to a hundred fold fewer sporozoites (Doolan and Hoffman 1997). The precise mechanisms by which CD8+ T-cells inhibit the growth of liver stage parasites are incompletely understood, but are thought to be mediated by the production of IFN-Y (Doolan and Hoffman 2000).

1.5.4.2.3 Cell-mediated immunoregulatory mechanisms

Immunoregulatory mechanisms ensure that invading pathogens are controlled or cleared with minimal damage to the host, a process often described as immune tolerance. Regulatory T cells (Tr) which may be naturally occurring or produced in response to specific stimuli are the key immunoregulators. The major subsets of Tr cells include; type 1 Tr cells (Tr1) which produce high amounts of interleukin-10 (IL-10) and low to moderate levels of transforming growth factor β (TGF-β); type 3 (Th3) which mainly secrete TGF-β, and the CD4+CD25+ T cells which inhibit immune responses through cell to cell contact (McGuirk and Mills 2002). In malaria, the roles of IL-10 and TGF-β in immunoregulation have been described as essential in mice, through experiments in which these cytokines are neutralized in vivo, or using mice that have the genes for the respective cytokines knocked
out. Lack of these cytokines results in increased pathology in vivo, which has been shown to be mediated by an excess of pro-inflammatory cytokines (Riley, Wahl et al. 2006). In both mice and humans, it is thought the variable outcomes of malaria infection are partly as a result of a delicate balance between pro- and anti-inflammatory cytokines (Artavanis-Tsakonas, Tongren et al. 2003). Severe manifestations of malaria have been associated with imbalances between pro- and anti-inflammatory cytokines (Day, Hien et al. 1999; Perkins, Weinberg et al. 2000), while clinical immunity is associated with down-regulated pro-inflammatory cytokine responses (Rhee, Akanmori et al. 2001).

1.5.4.2.4 Summary

Unlike antibodies which are relatively easy to study for a variety of reasons, not least that they are often abundant and readily accessible in peripheral blood, the study of cell-mediated immunity poses significant practical challenges. The most important cellular interactions occur in secondary lymphoid organs which are not accessible and cannot really be studied in humans infected with malaria. However, data from murine models suggest that although immune-effector cell populations are found at frustratingly low frequencies in the peripheral circulation, they are largely representative of processes in secondary lymphoid organs (Eunice Nduati, Phd thesis). Perhaps even more important is the complex interplay between cells involved in mediating protective immunity, the balance between the host of pro- and anti-inflammatory cytokines through which they act, the remarkable redundancy of various cells and cytokines in mediating their functions and finally the regulatory mechanisms that limit host damage. These interactions appear to be intricately linked and tightly regulated, and it is therefore challenging to dissect out the
1.5.5 INNATE DEFENCES AGAINST MALARIA

1.5.5.1 Key evidence

The role of the innate immune system in controlling infections by pathogens has gained prominence in recent years, with the growing acceptance of the fact that this arm of immunity was not completely non-specific as previously thought, but in-fact, able to discriminate between self and a variety of pathogens (Akira 2007). In malaria, more attention has understandably been paid to acquired immunity, and research into innate mechanisms that might contribute significantly to the rapid control of a malaria infection, early in the pathogenesis of disease, is only beginning in earnest (Stevenson 2004). In children, there is growing evidence that malaria impairs the innate mechanisms that would not only prevent or control the acute episode but also those required for effective ‘instruction’ of future adaptive immunity.

1.5.5.2 Potential mechanisms

1.5.5.2.1 Dendritic cells (DCs)

Dendritic cells have been described as the ‘sensors’ of the innate immune system, because of their ability to recognize microbes directly though a range of pattern recognition receptors (PRRs). They can also sense pathogens indirectly by detecting the inflammatory mediators produced by a range of other cells, including, macrophages, natural killer (NK)
cells, natural killer T cells (NKTs), mast cells and endothelial cells (reviewed in (Akira, Uematsu et al. 2006; Pulendran and Ahmed 2006)), the so-called 'danger' signals (Matzinger 1994). Dendritic cells are also thought to play an important role in the 'programming' of all arms of the adaptive immune system, to ensure the generation, regulation and maintenance of adequate amounts of high quality antibody and cell-mediated responses (Pulendran and Ahmed 2006).

Specific sub-sets of dendritic cells have been shown to induce immune responses to malaria parasites either via the scavenger receptor CD36 (Urban, Ferguson et al. 1999), or through toll-like receptor (TLR) mediated recognition of pathogen associated molecular patterns (PAMPs). In malaria, some of the PAMPs that induce immune responses are only just beginning to be identified and characterized, such as the unidentified component of schizont extract that binds to TLR9 of plasmacytoid DCs (Pichyangkul, Yongvanitchit et al. 2004), while more is known for a few others, such as, glycosylphosphatidylinositols (Gowda 2007) and haemozoin (Coban, Ishii et al. 2005). With regards to haemozoin, a recent study showed that it was the DNA attached to it, and not haemozoin per se, that bound to TLR9 in DCs (Parroche, Lauw et al. 2007).

In theory, abnormal DC function in malaria could arise as a consequence of impaired recognition of PAMPs (for example via TLRs), impaired antigen uptake following recognition or impaired maturation and antigen presentation. Some indirect evidence points to the fact that toll-like receptor mediated recognition per se, may be compromised in DCs of individuals susceptible to malaria compared to those who are not. In separate
studies, common polymorphisms in TLR4 and both TLR4 and TLR9, were associated with severe malaria in African children (Mockenhaupt, Cramer et al. 2006) and clinical episodes of malaria in pregnancy (Mockenhaupt, Hamann et al. 2006), respectively. By contrast, other evidence drawn from studies on the transcriptional profiles of peripheral blood mononuclear cells (PBMCs) from non-malaria immune individuals who were challenged with attenuated sporozoites suggests that blood stage parasites, at least, are recognized immediately. In these studies numerous genes related to innate immunity (including DCs) had been up-regulated even before parasitaemia became patent (Ockenhouse, Hu et al. 2006). Alternatively or additionally, the parasite may exert its effects predominantly on events subsequent to the initial recognition. It has been shown in vitro, that intact malaria-infected erythrocytes adhere to DCs and subsequently reduce their capacity to stimulate T cells (Urban, Ferguson et al. 1999). This would imply that impaired DC function may contribute not only to the immediate development of acute clinical episodes, but also have long term consequences on the secondary responses of adaptive immunity. Consistent with this idea, a study in Kenyan children found that compared to healthy children, those with acute episodes of malaria had reduced expression of HLA-DR on the surface of their peripheral DCs, which may indicate functional impairment of these instrumental cell populations (Urban, Mwangi et al. 2001).

1.5.5.2.2 Macrophages, Natural Killer (NK) cells and Natural Killer T-cells (NKT)

Macrophages or mononuclear phagocytes have important roles both in innate and adaptive immune responses in malaria. Macrophage mediated innate defenses include phagocytic uptake of infected red blood cells in the absence of cytophilic and opsonizing antibodies,
and antigen presentation (Serghides, Smith et al. 2003). The interaction between macrophages and infected red blood cells is thought to be via the scavenger receptor CD36 on the macrophage and Pfemp-1 on the surface of the infected erythrocyte. The role of natural killer (NK) and natural killer T (NKT) cells in mediating protection from malaria has been studied extensively in murine and rodent models of malaria, with limited data from small studies in humans from malaria-endemic areas. One study of NKT cells indicated that they are often the first cells to respond when infected erythrocytes are incubated with human peripheral blood mononuclear cells in vitro, but not all donors tested responded by the production of IFN-γ (Artavanis-Tsakonas and Riley 2002). The relevance of these studies with regards to NAI in humans remains to be established (Stevenson and Riley 2004).

1.5.5.3 Summary

Although by definition innate defenses are present from birth and have no ‘learned’ or memory component, there is considerable overlap between innate and adaptive immune mechanisms. As such, while NAI to malaria is usually concerned with adaptive immune responses, these cannot function without support from the innate system, and both these arms of immunity are intricately linked. More recently, the mechanisms by which the innate immune system ‘senses’ microbes, and vaccines, for that matter, have taken centre stage, particularly in the discipline of vaccinology, with the realization that innate responses have a profound role on subsequent adaptive immunity. It is envisaged that an improved understanding of the interplay between these two arms of the immune system will lead to improved vaccine design, particularly with the use of adjuvants that can be
engineered to steer the host immune responses in desired directions to improve efficacy and achieve optimal and long-lived protection (Pulendran and Ahmed 2006).
2 MALARIA VACCINE CANDIDATE ANTIGENS

Antigens on the merozoite surface, or those found within its apical organelles which are brought to it's surface at the time of erythrocyte invasion, are thought to be key targets of protective immune responses, particularly those mediated by antibodies. The antigens selected for study in this thesis are all considered to be leading malaria vaccine candidate molecules or antigens, based on the evidence presented below.

2.1 MSP-1

This was the first MSP to be described (Holder and Freeman 1981) and has been variously referred to as gp195, p190, PSA (polymorphic schizont antigen), PMMSA, MSA-1 (merozoite surface antigen 1), and MSP-1. It is also the most abundant protein on the merozoite surface and the most extensively characterized of the merozoite surface proteins. It is a large protein with a molecular weight of approximately 190 kDa which is synthesized during schizogony, during which time it can be found on the surface of the mature extracellular merozoite as a complex of non-covalently linked fragments derived from post-translational proteolytic processing (Holder, Sandhu et al. 1987; McBride and Heidrich 1987). At the molecular level it can be considered as seventeen distinct blocks, depending on whether the sequences within a particular block are variable, conserved or semi-conserved (Tanabe, Mackay et al. 1987; Miller, Roberts et al. 1993), and is illustrated in the schematic below (Figure 2.1.1). On the whole, MSP-1 is a dimorphic molecule, with variants falling into two distinct allelic families, as represented by the K1 and MAD20
malaria parasite 'strains'. Two regions of the gene will be discussed for the purposes of this thesis. The first is MSP-1\textsubscript{19} which is located within block 17 of MSP-1 and is largely conserved, containing only a few polymorphic residues. The second involves the entire block 2 of MSP-1 which is highly polymorphic, and variants at this locus are grouped into three main allelic types or families (K1-like, MAD20-like and RO33-like), based on the prototype of parasites in which they were described. More recently, a fourth allelic type has been reported called MR (thought to have a risen through recombination of the MAD20 and RO33 sequence types), and MR-like variants have been found in diverse geographical areas (Takala, Branch et al. 2002).

![Figure 2.1.1 Schematic of the msp1 gene of P. falciparum](image)

Block numbers are indicated. Colors: white, conserved blocks; blue, polymorphic block; black, dimorphic blocks; grey, semi-conserved blocks. Figure adapted from Cavanagh 1997

2.1.1 MSP-1\textsubscript{19}

2.1.1.1 Antigen discovery, location and structure

MSP-1 is synthesized as a precursor protein that subsequently undergoes two proteolytic processing events. The first occurs at the time of erythrocyte rupture, and yields a complex of several fragments (83, 42, 38 and 28-30 kDa) that are held together by non-covalent
bonds, and secured onto the merozoite surface by a GPI anchor. At the time of erythrocyte invasion, a second proteolytic event cleaves MSP-1_{42} into two fragments, MSP-1_{33} (shed off along with the other products of MSP-1 processing) and MSP-1_{19}. MSP-1_{19} is the 19kDa fragment that remains bound to the merozoite surface and is carried into the newly invaded erythrocyte (Blackman, Heidrich et al. 1990). Studies on the crystal structure of MSP-1_{19} reveal that it is composed of a compact, flat, disc-like structure, with two epidermal-like growth factor (EGF) domains that are folded back on each other in a side by side arrangement (Chitarra, Holm et al. 1999). At a molecular level, MSP-1_{19} contains approximately 100 amino acids, the majority of which are conserved in *P. falciparum* isolates from diverse geographical areas (Kang and Long 1995). However, it also contains six non-synonymous single nucleotide polymorphisms (SNPs), one is located at the first EGF-like domain and the remaining five are located at the second EGF-like domain, and at least 10 different alleles of MSP-1_{19} have been reported (Takala, Branch et al. 2002).

**2.1.1.2 Antigen function and effect of gene disruption**

Although the precise function of MSP-1 remains unknown, the fact that MSP-1_{19} contains EGF-like domains which are known to have essential binding functions in other molecules (Kansas, Saunders et al. 1994), suggests that it has a role to play in merozoite invasion of red blood cells. This is supported by its location on the merozoite surface, its abundance and the limited diversity in the sequence of MSP-1_{19}, which is thought to indicate functional constraints. Attempts to knock out MSP-1 have been unsuccessful (Cowman and Crabb 2006). However, allelic replacements in which MSP-1_{19} from *P. falciparum* is replaced the corresponding sequences from *P. chabaudi* or *P. berghei* are possible, and indicate that its
function is maintained across distantly related *Plasmodium* species (O'Donnell, Saul et al. 2000), (de Koning-Ward, O'Donnell et al. 2003). Allelic replacement has also been possible using the corresponding double EGF-like module of MSP-8 of *P. berghei* even though this sequence shares only low homology with MSP-1 of *P. falciparum* (Drew, O'Donnell et al. 2004). The fact that these parasites in which MSP-119 is radically altered retain the ability to efficiently invade erythrocytes makes it unlikely that it has a role in receptor-mediated binding.

2.1.1.3 Supportive evidence that antibodies play a role in protection against malaria

2.1.1.3.1 *In vitro* studies

Early studies employed monoclonal antibodies to characterize merozoite antigens and examine their roles in invasion. As such, mAbs were used to characterize what we now refer to as MSP-1, and demonstrated that certain mAbs against this antigen partially inhibited merozoite invasion (Pirson and Perkins 1985). Subsequent studies mapped the locations of the epitopes recognized by these monoclonal antibodies, and Blackman et al. (Blackman, Heidrich et al. 1990) showed that mAbs to epitopes located within MSP-119 specifically, inhibited merozoite invasion of erythrocytes. Two important points are worth noting with regards to the invasion inhibition using mAbs to MSP-119 reported in the study by Blackman et al.. The first is that to demonstrate invasion-inhibition, high titres (up to 500ug/ml) of mAbs were required, and although the amount of inhibition increased with increasing titre of antibodies, it never reached 100%, and it is not clear whether it saturated (no further increase in inhibition even with increasing concentrations of mAb). The highest degree of invasion-inhibition reported was 72% relative to control
immunoglobin (Ig), and using mAb12.10 at 500ug/ml against the T9-94 parasite strain (Blackman, Heidrich et al. 1990). The second point is that variable amounts of parasite inhibition were observed when the same mAb was tested against different parasite strains. For example, mAb 12.10 at 500ug/ml inhibited T9-96 parasites by 72% relative to control Ig, but only inhibited parasites of the T9-94 strain by 50%. It was not known at the time whether these two parasite strains differed as a result of sequence polymorphisms at the MSP-119 locus or not, and whether mAb 12.10 was directed against a conserved or polymorphic epitope. As such, while it is reasonable to conclude that antibodies to MSP-119 may interrupt the asexual blood stage cycle of the parasite, given the diversity that we now know exists even at the relatively conserved msp-1 locus, and the fact that invasion-inhibition is variable in different parasite isolates using well defined monoclonal antibodies, it would seem unlikely that this is the principal mechanism by which natural immunity is acquired.

However, other data demonstrate that anti-MSP-119 antibodies do contribute significantly to the invasion-inhibitory antibodies found in the sera of immune adults from malaria endemic areas. Using the parasites generated in the MSP-119 allelic replacement study mentioned above (O'Donnell, Saul et al. 2000), overall, there was a significant reduction in invasion-inhibition of immune sera tested against the transgenic parasites (containing P. chabaudi MSP-119), compared to control parasites (containing P. falciparum MSP-119) (O'Donnell, de Koning-Ward et al. 2001). Notably, although the majority of immune sera had demonstrable invasion-inhibitory ability, this varied widely between individuals. Additional variation between individual sera was observed in the amount of MSP-119.
specific invasion-inhibitory activity, with some sera having high amounts of invasion-inhibition that could not be attributed to MSP-119 antibodies (O'Donnell, de Koning-Ward et al. 2001). Another important finding in this study was the fact that MSP-119 specific antibodies as measured by ELISA did not correlate at all with invasion-inhibitory ability (R² values of 0.0003 and 0.013 for the two sets of immune sera tested), a fact that may contribute to the inconsistent results obtained in immuno-epidemiological studies designed to identify protective antibodies, as these commonly measure antibodies by ELISA (discussed below). A final point to raise from this study concerns the limited polymorphisms found within MSP-119. If indeed as their results suggest, it is an important target, it would follow that the locus was under strong immune pressure, which would be expected to result in greater within-locus diversity, as has been observed for other MSPs (Conway and Polley 2002).

Other studies have employed different strategies to investigate the function of MSP-119 specific antibodies. Chappel et al. (Chappel, Egan et al. 1994) affinity purified anti-MSP-119 antibodies from immune sera containing demonstrable parasite clearing activity. These sera had been used in the passive antibody transfer experiments in humans, in which hyper-immune sera from adult West Africans was administered to successfully treat patients with P. falciparum malaria (Sabchareon, Burnouf et al. 1991). Total IgG affinity-Selected on MSP-1-EGF-1 (AP-EGF1_IgG) recognized the native antigen, competed with protective antibodies but did not prevent invasion of red blood cells by merozoites in vitro, suggesting that this molecule did not include the target epitopes of invasion-inhibitory antibodies (Chappel, Egan et al. 1994). Given that previous studies using mAbs had
indicated that invasion-inhibitory epitopes were located within this EGF-like domain (Chappel and Holder 1993), the authors speculated that anti-MSP-119 antibodies induced by natural infection may have stimulated antibodies with different anti-MSP-1-EGF-1 idiotypes, with only a few of these possessing invasion-inhibitory activity (Chappel, Egan et al. 1994). This was supported by subsequent studies which showed that antibodies affinity purified on AP-EGF1-IgG contained a mixed population of antibodies, with inhibitory and blocking properties (Egan, Burghaus et al. 1999). In this study (Egan, Burghaus et al. 1999), human antibodies affinity purified on AP-EGF1-IgG were able to compete with both inhibitory and blocking antibodies, while those to the second EGF-like domains (AP-EGF2-IgG) were able to inhibit parasite growth in vitro suggesting that both EGF-like domains are important.

Antibodies to MSP-119 are further complicated by the demonstration of variation in their fine specificity. Apart from invasion-inhibitory antibodies described above, other MSP-119 specific antibodies have been shown to be inhibitory, blocking, or neutral (Uthaipibull, Aufiero et al. 2001). Inhibitory antibodies inhibit the processing of MSP-142 and thereby reduce erythrocyte invasion. Blocking antibodies compete with invasion-inhibitory antibodies, thereby blocking their binding and function. Neutral antibodies are neither inhibitory nor blocking. These variable specificities may be explained by the actual epitopes within MSP-119, to which the various antibodies bind, with neutral antibodies binding epitopes that are distinct from the overlapping ones targeted by blocking and processing-inhibitory antibodies (Uthaipibull, Aufiero et al. 2001). Again, the importance
the fine specificity of MSP-19 antibody response has been demonstrated in immunoepidemiological studies, discussed below.

2.1.1.3.2 Vaccination and passive antibody transfer experiments in animal models

In animal models of malaria, vaccination with MSP-19 confers partial or complete protection against challenge with fatal *P. yoelii* in mice (Daly and Long 1993; Ling, Ogun et al. 1994; Tian, Miller et al. 1996; Hirunpetcharat, Vukovic et al. 1999; Kumar, Jones et al. 2004) and *P. falciparum* in Aotus monkeys (Kumar, Yadava et al. 1995; Kumar, Jones et al. 2004). Protection has been associated with high anti-MSP-19 antibody titres (Kumar, Jones et al. 2004), although the specificity of the antibodies appears to be important. In one study the titres of invasion-inhibitory antibodies and not those of total MSP-19-specific immunoglobulin correlated with protection against homologous blood stage challenge in mice (de Koning-Ward, O'Donnell et al. 2003). However, in other studies vaccine-induced anti-MSP-19 invasion-inhibitory antibodies did not consistently correlate with protection (Egan, Blackman et al. 2000), and were present in comparable quantities in both protected and susceptible Aotus monkeys (Kumar, Collins et al. 2000). These animal immunization data are difficult to interpret due to the small numbers of animals routinely included in such studies, not to mention the fact that they do not routinely assess the fine specificity of vaccine-induced anti-MSP-19 antibodies.

Protective antibodies have also been shown to be conformation dependent, as the reduction of disulphide bonds abolishes the production of growth inhibitory antibodies (Locher and Tam 1993).
With regards to cellular responses, while it appeared that complete MSP-119 vaccine-induced immunity could be mediated independently of specific CD4+ T cells (Wipasa, Xu et al. 2002), passively transferred vaccine-induced antibodies to MSP-119 only conferred partial protection against challenge with P. yoelii (Tian, Miller et al. 1996), and only in naïve immuno-competent mice (Hirunpetcharat, Vukovic et al. 1999). High titres of anti-MSP-119 antibodies failed to protect naïve SCID, nude, CD4+ T-cell depleted and B-cell knock out mice, indicating a role for cellular, as well as humoral immune responses (Hirunpetcharat, Vukovic et al. 1999).

Antibodies to MSP-119 may act by inhibiting the proteolytic cleavage of MSP-142 (Blackman, Scott-Finnigan et al. 1994). It has therefore been of interest to determine whether vaccine-induced antibodies to MSP-142 also inhibit invasion as well as, or better than those to MSP-119, or whether such protection is accounted for by antibodies to epitopes within MSP-119, MSP-133 or both. These studies have provided mixed results. Both murine and simian models of malaria immunized with MSP-142 have been protected from lethal challenge with malaria (Kumar, Yadava et al. 1995; Chang, Case et al. 1996; Stowers, Cioce et al. 2001), with some constructs inducing high antibody titres to MSP-119 (Chang, 1996). However, in some studies, better protection was observed following immunization with MSP-119 than with MSP-142 (Kumar, Yadava et al. 1995). The opposite effect has been reported in other studies, with immunization with MSP-142 yielding better protection than immunization with MSP-119 (Stowers, Cioce et al. 2001). Such differences may be partly explained by the different vaccination regimens, adjuvants and expression systems used to generate the vaccine constructs. In a direct comparison of immunization with MSP-119 versus MSP-133, it was found that despite generating high titres of antibodies, immunization with MSP-133...
was not associated with protection, while immunization with MSP-119 was (Ahlborg, Ling et al. 2002).

2.1.1.3.3 Population genetic analyses

In contrast to other antigens selected for study as past of this thesis, the MSP-119 locus is relatively conserved. Genotype frequencies determined at this locus from molecular studies from multiple locations in Africa and the Brazilian Amazon suggest that the observed variation arises primarily as a consequence of genetic drift and that it is not under strong immune selection for diversity, based on low $F_{\text{ST}}$ values (Conway, Cavanagh et al. 2000; Silva, Silveira et al. 2000). Analysis of MSP-119 orthologs from seven species of *Plasmodia* revealed that sequences from primate (*P. vivax, P. knowlesi* and *P. cynomolgi*), rodent (*P. chabaudi, P. berghei, P. yoelii*) and human (*P. falciparum*) malarias were relatively conserved, and could essentially be considered as chimeras of two archetype sequences, suggesting that the diversity in this locus had ancient origins (Saul and Miller 2001). The authors argued that this was good news for an MSP-119 based vaccine, as functional constraints in the two EGF domains would restrict the generation of new sequence variants due to vaccine-induced immune pressure.

2.1.1.3.4 Immuno-epidemiological studies

The most extensively characterized and well studied merozoite surface antigen is MSP-1, and in particular MSP-119. I will discuss antibodies to MSP-119 in detail, to highlight some of the methodological issues that make interpretation difficult, and that will apply to some degree to many other malaria vaccine candidates. I identified over 40 studies where naturally acquired antibodies to MSP-119 were investigated. I begin by highlighting the
differences in multiple variables of interest in these types of studies, which undoubtedly account for (at least in part), the discrepant results often reported from immunoepidemiological studies of MSP-119. I raise these differences deliberately at the beginning of the review, rather than at the end, to allow for a careful consideration of the data and consequent cautious interpretation of the results (as opposed to suggesting them as explanations for the discrepant findings). It will become clear why for the majority of studies, it is difficult to make any meaningful comparison of the results, and even more difficult to summarize these across studies in order to draw general conclusions.

2.1.1.3.4.1 Variation in MSP-119 antigens

It is common practice in immuno-epidemiological studies to report the prevalence of the specific immune response under investigation in the population of interest. This is usually the first indication that the response may have a role in the pattern of disease observed in the population. In studies on antibody responses to malaria antigens, this is further supported by the demonstration that the specific immune response is not detectable in individuals that have not been exposed to malaria (for antibody responses at least). When I attempted to summarize the prevalence of antibodies to MSP-119 reported from malaria endemic areas I found that different studies measured antibodies to MSP-119 antigens that varied in several ways. This was surprising as MSP-119 is not only a relatively small protein, but it is also largely conserved among parasite isolates in the field (Kang and Long 1995; Qari, Shi et al. 1998). In reviewing these immuno-epidemiological studies, five important sources of variation for the MSP-119 antigen itself, were noted.
The first is due to naturally occurring polymorphisms in the molecule, which may occur in either one or both of the EGF-like domains (Tanabe, Mackay et al. 1987; Miller, Roberts et al. 1993; Kang and Long 1995; Qari, Shi et al. 1998). The second source of variation arises due to the different expression systems used to synthesize the recombinant MSP-119. The third comes from the design of the actual MSP-119 construct, with regards to the EGF-like molecules. The fourth source of variation comes mainly from older studies where antibodies were analyzed to varying fragments of the C-terminal of MSP-1. The final source of variation comes from studies where MSP-119 specific invasion-inhibitory antibodies are analyzed.

**Naturally occurring polymorphisms within MSP-119**

Different studies have used MSP-119 antigens based on a range of parasite 'strains' that contain different alleles of MSP-119. The reason for choosing one MSP-119 allele over another is not commonly reported, and may range from convenience (i.e. readily available), to using the one that is most prevalent is the geographic location where the study is conducted (Egan, Morris et al. 1996; Branch, Udhayakumar et al. 1998). ELISA OD reactivities against antigens based on different MSP-119 alleles were highly correlated in some studies (Egan, Chappel et al. 1995; O'Donnell, de Koning-Ward et al. 2001; John, O'Donnell et al. 2004), but not in others (Udhayakumar, Anyona et al. 1995; Shi, Sayed et al. 1996). Even when good correlation is observed between different alleles, a proportion (minority) of sera react more strongly with one sequence than with the other (Egan, Chappel et al. 1995; Shi, Sayed et al. 1996), particularly when the antigens differ in the sequence of the second EGF motif (Egan, Chappel et al. 1995).
Variation in expression systems for MSP-119

The same MSP-119 sequence inserted into a vector and expressed for example in yeast, as opposed to *E. coli*, can give different amounts of reactivity, when assayed by ELISA (John, O'Donnell et al. 2004). It is thought the different expression systems give rise to antigens that differ slightly in their conformational epitopes. The range of expression systems reported for MSP-119 antigens includes, *E. coli* (most common), fused to GST or CAT, *S. cervisiae* (his-tagged) and baculovirus/insect cell culture (Riley, Allen et al. 1992; al-Yaman, Genton et al. 1996). While some studies have shown strong correlations between human antibodies to the same MSP-119 sequence, expressed in different systems (Egan, Chappel et al. 1995), others have found still significant, but comparatively weaker correlations (John, O'Donnell et al. 2004).

Variation in the design of MSP-119 constructs

Recombinant MSP-119 constructs may contain either the first, or second EGF-like domains, singly, or have a combination of both EGF-like domains from the same parasite line, or have both EGF-like domains, but with each individual one drawn from a different parasite line. For instance, an MSP-119 construct may have an EGF1-like domain based on the MAD20 parasite strain and an EGF2-like domain based on the Wellcome parasite strain (Egan, Chappel et al. 1995). In a similar vein, several MSP-119 mutants have now been engineered in which specific epitopes are modified, for instance, the removal of epitopes to which blocking mAbs are known to bind (Uthaipibull, Aufiero et al. 2001). Antibody reactivities to the mutants can then compared to those against 'wild-type' MSP-119 in standard or competition ELISAs (Corran, O'Donnell et al. 2004). Antibody reactivities to these mutants
which are also referred to as modified MSP-1\textsubscript{19} recombinant antigens or antigenic variants of MSP-1\textsubscript{19} with double or triple substitutions, have also been analyzed in studies monitoring drug resistance to anti-malarial drugs (Pinder 2006).

**Variation in the size of C-terminal MSP-1 fragments**

In general, in older studies (performed in the late 1980's and early 1990s), antibodies were measured to various sized fragments of the C terminal of MSP-1 (including MSP-1\textsubscript{42}), many of which contain antibodies to MSP-1\textsubscript{19} (Muller, Fruh et al. 1989; Fruh, Doumbo et al. 1991; Tolle, Fruh et al. 1993; Shai, Blackman et al. 1995; al-Yaman, Genton et al. 1996). The contribution of anti-MSP-1\textsubscript{19} antibodies to the overall MSP-1 or MSP-1\textsubscript{42} response in these studies cannot be ascertained.

**MSP-1\textsubscript{19} specific invasion inhibitory antibodies**

Several studies have now reported on MSP-1\textsubscript{19} specific invasion inhibitory antibodies (O'Donnell, de Koning-Ward et al. 2001; de Koning-Ward, O'Donnell et al. 2003; John, O'Donnell et al. 2004; Perraut, Marrama et al. 2005). Here, *P. falciparum* MSP-1\textsubscript{19} is replaced with the homologous region of *P. chabaudi* (most common) (O'Donnell, de Koning-Ward et al. 2001) or *P. berghei* (de Koning-Ward, O'Donnell et al. 2003). These constructs enable the estimation of invasion-inhibitory antibodies that can be accounted for by antibodies specifically targeted to MSP-1\textsubscript{19}. A recent study suggested that fetal sensitization to MSP-1, as a result of maternal malaria in pregnancy, affected the development of MSP-1\textsubscript{19} specific invasion-inhibitory antibodies (Dent, Malhotra et al. 2006). Other studies have described the fine specificity of human antibodies to various
constructs of MSP-119, and how this might relate to protection against malaria, but these will be discussed separately, below.

2.1.1.3.4.2 The prevalence of antibodies to MSP-119

Bearing these differences in mind, the reported prevalence of total IgG antibodies to MSP-119 in malaria endemic populations ranges from as low as 4% (Dodoo, Theander et al. 1999), to as high 96% (Hogh, Marbiah et al. 1995), depending on the population under study, and the specific construct of MSP-119. Apart from differences in the MSP-119 antigen already discussed, I found at least seven other distinct factors may affect, or account for this wide variability in antibody prevalence. The first four relate to the study participants, their age, whether they were healthy or acutely ill with malaria, the clinical syndrome of malaria (eg cerebral malaria versus severe anaemia) and whether they were parasitaemic or aparasitaemic at the time the serum sample was collected. The remaining three relate to malaria transmission intensity, seasonal variation and methodological issues in measuring the antibodies.

In general, the prevalence is higher when data are reported from adults than from children, and although some studies have found that antibody prevalence increased significantly with age (Egan, Chappel et al. 1995; Egan, Morris et al. 1996; Perraut, Marrama et al. 2003; Perraut, Marrama et al. 2005), many have not (Dodoo, Theander et al. 1999; Kitua, Urassa et al. 1999; Cavanagh, Dodoo et al. 2004; John, O'Donnell et al. 2004; Okech, Corran et al. 2004) (Osier 2008). Antibody prevalence is also higher at the time of an acute episode, or in convalescence, compared to samples taken when children are well. In a study on infants, Branch et al. reported a prevalence of 77%, when they were acutely ill, compared to 59%, a
month prior to the same clinical episode, McNewmar $P = 0.003$ (Branch, Udhayakumar et al. 1998). Children presenting with severe malarial anemia (SMA) or uncomplicated malaria (UM) had a lower prevalence and titre of antibodies to MSP-119 compared to those with cerebral malaria (CM), $p < 0.05$ for SMA vs CM, and $p < 0.01$ for UM vs CM, although this was also observed for other malarial antigens (Dobano, Rogerson et al. 2008). In a case-control study, the prevalence of antibodies to MSP-119 was lower in cases (severe malaria), compared to controls (uncomplicated malaria), and it differed significantly within sub-groups of severe malaria, though for this latter analysis antibodies to MSP-119 were assessed in combination with those to two other malarial antigens and the numbers in each of these sub-groups were small (TM, Elbashir et al. 2008). In yet another study where cases of severe malaria were matched to controls with mild malaria, the prevalence of antibodies to MSP-119 was comparable in both groups, in samples collected at the time of the acute episode, and three weeks later, but was higher in the severe malaria group six months later (healthy phase sample) (Kohler, Tebo et al. 2003).

As has been found with other malaria antigens, prevalence is also higher in children who are parasitized at the time the sample is collected, than in those who are not (Tolle, Fruh et al. 1993; Polley, Mwangi et al. 2004; Polley, Conway et al. 2006; Osier, Polley et al. 2007)(Osier 2008). This is important because antibody responses to MSP-119, and other malaria antigens, are known to be short-lived (Cavanagh, Elhassan et al. 1998; Kinyanjui, Conway et al. 2007; Akpogheneta, Duah et al. 2008). Antibody prevalence is predictably higher in areas with intense malaria transmission, compared with areas of lower transmission or episodic malaria (Drakeley, Corran et al. 2005),(Braga, Barros et al. 2002; John, Moormann et al. 2004; John, Moormann et al. 2005). Similarly, prevalence is also
higher when samples are collected during, or at the end of a malaria transmission season, as opposed to at the end of the dry season (minimal malaria transmission) (Cavanagh, Elhassan et al. 1998). However, in at least one study, no seasonal variation in antibody responses to MSP-119 was observed (Dodoo, Theander et al. 1999). This lack of seasonal variation in antibody prevalence has also been reported for antibodies to MSP-142 (Riley, Morris-Jones et al. 1993).

Methodological issues, or the lack of standardized protocols for measuring antibodies have been raised previously (John, Moormann et al. 2005), and also contribute to the variation in reported results. A good illustration is provided by the studies conducted by Cavanagh et al., and Dodoo et al. (Dodoo, Theander et al. 1999; Cavanagh, Dodoo et al. 2004). They both measured antibodies to exactly the same antigen, in the same set of plasma samples of children from Ghana. However, Cavanagh et al. found an MSP-119 antibody prevalence of 56%, which was significantly affected by season, while Dodoo et al. reported one of 31%, and this did not change significantly in samples collected before or after the malaria transmission season. In the study by Cavanagh et al., sera were tested at a 1/500 dilution, while in that by Dodoo et al., they were tested at a 1/1000 dilution, which may partly account for the differences.

2.1.1.3.4.3 IgG sub-class responses to MSP-119

The predominant IgG sub-classes against MSP-119 is IgG1 (Egan, Chappel et al. 1995; Egan, Morris et al. 1996; Branch, Oloo et al. 2000; Cavanagh, Dobano et al. 2001; Diallo, Spiegel et al. 2001; John, O'Donnell et al. 2004; Tongren, Drakeley et al. 2006), followed by IgG3 (Branch, Oloo et al. 2000; Cavanagh, Dobano et al. 2001; Diallo, Spiegel et al. 2002; John,
IgG2 and IgG4 have been detected in African populations in only a minority of studies (Shi, Sayed et al. 1996; John, O'Donnell et al. 2004). In studies from Brazil, the frequency and amount of IgG2 and IgG4 antibodies to MSP-119 and other malaria antigens is higher than that observed in African populations (Ferreira, Kimura et al. 1998; Scopel, Fontes et al. 2005; Scopel, Fontes et al. 2006). As reagents are not standardized across studies, it remains possible that the observed differences could be explained by the mAbs (mouse, rabbit, goat, sheep e.t.c.) used to detect the human IgG subclasses.

The MSP-119 IgG sub-class distribution does not appear to be affected by whether individuals are not infected, asymptotically infected or acutely ill with malaria (Scopel, Fontes et al. 2005). Similarly, the pattern of IgG isotype antibodies to MSP-119 does not seem to vary with age or seasonal changes. Infants, children and adults display a similar distribution of IgG isotypes to MSP-119 (Egan, Morris et al. 1996; Branch, Oloo et al. 2000; Cavanagh, Dobano et al. 2001), and the same pattern was observed when antibodies to MSP-142 were analyzed in neonates (Metenou, Suguitan et al. 2007). Although antibody levels are higher at the end of a malaria transmission season, IgG1 antibodies are still the predominant IgG isotype at this time (Diaollo, Spiegel et al. 2002).

2.1.1.3.4.4 Fine specificity of antibodies to MSP-119

Naturally-acquired antibodies to MSP-119 have different specificities, and can be functionally inhibitory, blocking, or neutral, as discussed above. Few studies have examined the fine specificity of anti-MSP-119 antibodies in immuno-epidemiological studies
The specificity is usually defined by the ability of human antibodies to compete for binding to recombinant MSP-119 with panels of mAbs of previously determined specificity (Blackman, Scott-Finnigan et al. 1994). Alternatively, or in addition, the fine specificity can be defined by testing the binding of sera to recombinant MSP-119 mutants, in which epitopes targeted by blocking mAbs have been disrupted (Uthaipibull, Aufiero et al. 2001). In a longitudinal study, the fine specificity of antibodies to MSP-119 was associated with protection from malaria infection and high-density parasitaemia. Children whose antibodies were able to compete with the blocking mAb 1E1 were less likely to become infected with malaria than those whose antibodies did not compete ($p = 0.04$) (Okech, Corran et al. 2004).

2.1.1.3.4.5 Antibodies to MSP-119 and associations with protection from malaria

Definition of ‘protection’ from malaria

Although antibodies to MSP-119 have been frequently associated with protection from malaria, this has not been a consistent finding. In order to compare the results from different studies, it is necessary to consider the definition of ‘protection’, in the context of malaria. Once an individual has been infected with malaria parasites, he/she could manifest one or more of several outcomes; asymptomatic infection, mild or severe clinical disease. The definition of protection is based on these outcomes or end-points. In addition, the absence of infection, particularly following drug treatment to clear (all) parasites at the beginning of a study (treatment re-infection studies), is also commonly used to define
protection. Meaningful comparisons can only be made when the outcome of interest is shared between the studies.

Cross-sectional studies often report protective associations that are based on presence or absence of parasitaemia, with individuals free of parasites considered to be protected (from infection). Whether in cross-sectional or longitudinal studies, when parasitaemia is used as an end-point, it can be further classified into being of low, moderate or high density (not consistent between studies), and protective associations are sought (and reported!) within these strata (Corran, O'Donnell et al. 2004; Okech, Corran et al. 2004). On the other hand, longitudinal studies generally use clinical episodes (mild or severe malaria) (Hogh, Marbiah et al. 1995; Dodoo, Theander et al. 1999; Kitua, Urassa et al. 1999; Polley, Mwangi et al. 2004) (Osier 2008), or time to re-infection (in the treatment re-infection study design) (Perraut, Marrama et al. 2003; John, O'Donnell et al. 2004; John, Moormann et al. 2005) as their study end-points. As with parasitaemia, various classifications can be employed for mild or severe malaria, for instance, protection may be reported against anaemia (Branch, Udhayakumar et al. 1998).

Some longitudinal studies have analyzed multiple endpoints, simultaneously (Perraut, Marrama et al. 2003), while others make the reasonable argument that the lack of parasites may imply that individuals were simply not challenged during the period of observation and thus, this data cannot be reliably used to identify protected individuals. This has led to protection in some longitudinal studies being defined as the presence of parasites (obvious
challenge), but the absence of clinical manifestations (Egan, Morris et al. 1996; Dodoo, Theander et al. 1999).

In an interesting concept, protection is assessed in the context of studies designed to monitor drug resistance to anti-malarial drugs. Here, it is hypothesized that recovery from uncomplicated malaria in patients carrying drug resistant parasites is a measure of functional immunity that may be antibody-mediated. Antibodies to specific antigens were more common, or present at higher levels in successfully treated children (clinical and parasitological cure) compared to those with treatment failure (Mawili-Mboumba, Borrmann et al. 2003), (Aubouy, Migot-Nabias et al. 2007), or amongst children with drug resistant parasites who nevertheless recovered (assessed at different levels of residual parasitaemia), compared to those who did not (Pinder, Sutherland et al. 2006).

Other sources of variation in studies on 'protection' from malaria

Apart from the definition of protection, the study design, and the details of the MSP-19 antigen itself, attention must also be paid to the MSP-19 immune response measured, and how it was measured. A whole range of assays have been used to detect and/or quantify antibodies to MSP-19. Simple measurements include total IgG, subclass IgG or IgM and a wide range of different reagents, in particular secondary anti-human antibodies are used to detect these. Total IgG to MSP-19 are most commonly assayed using routine standardized ELISA protocols, but can also be measured using microarrays (Sundaresh, Doolan et al. 2006; Gray, Corran et al. 2007), suspension array technology (Fouda, Leke et al. 2006), microspheres and flow cytometry (actually measured murine MSP-142 in this report) (van
der Heyde, Burns et al. 2007) and immunoblots, particularly in older studies. More sophisticated measurements reported include; MSP-1\textsubscript{19} specific invasion inhibition using transgenic parasites (O'Donnell, de Koning-Ward et al. 2001; de Koning-Ward, O'Donnell et al. 2003); fine specificity of MSP-1\textsubscript{19} antibodies (determined using competition ELISA with varying panels of MSP-1\textsubscript{19} mAbs (Blackman, Scott-Finnigan et al. 1994), competition ELISA using panels of MSP-1\textsubscript{19} mutants (Uthaipibull, Aufiero et al. 2001) and invasion-inhibition using human antibodies affinity-purified on MSP-1\textsubscript{19} (various constructs) (Egan, Burghaus et al. 1999).

As such, though many studies report protective associations, it is nearly impossible to compare them because the majority have used diverse assays, to measure diverse specificities of antibodies to a wide range of slightly differing MSP-1\textsubscript{19} antigens. Furthermore, some studies are conducted in sera drawn from adults, children, or both. Other studies are conducted in pregnant women and resulting mother-infant pairs (Hogh, Marbiah et al. 1995; Branch, Udhayakumar et al. 1998). Protective associations have been detected from particular age-groups in some studies, but not in others (Shi, Sayed et al. 1996), with particular MSP-1\textsubscript{19} constructs in certain populations, but not in others (Egan, Morris et al. 1996). Other issues that have been raised previously which are pertinent to consider in studies reporting protective associations are the different transmission settings, the timing of the sampling (wet or dry season), the status of individuals at recruitment (whether healthy and aparasitaemic, having asymptomatic parasitaemia or acutely ill) and, an important subject to discussed later on in the thesis, the analyses employed to detect protective associations.
Antibodies to MSP-119 in longitudinal studies

I identified eleven longitudinal studies including twelve distinct populations, that had clinical episodes of malaria as the outcome. All the studies were adequately powered, had sufficient levels of follow-up with individuals monitored at least once weekly, had clear methodology for the measurement of antibodies and robust statistical analyses. In these studies regardless of the differences discussed above, antibodies to MSP-119 were not significantly associated with protection against clinical episodes of malaria in eight distinct cohorts from the Gambia (Conway, Fanello et al. 2000), Ghana (Dodoo, Theander et al. 1999; Cavanagh, Dodoo et al. 2004), Sierra Leone (Egan, Morris et al. 1996), Senegal (Perraut, Marrama et al. 2003), (Roussilhon, Oeuvray et al. 2007) and Burkina Faso (Nebie, Diarra et al. 2008). In contrast, in three other studies from separate cohorts in the Gambia (Egan, Morris et al. 1996), Senegal (Perraut, Marrama et al. 2005) and Liberia (Hogh, Marbiah et al. 1995) antibodies to MSP-119, and particularly high titres (Hogh, Marbiah et al. 1995; Perraut, Marrama et al. 2005) were associated with a reduced risk of clinical malaria.

Summary

While the optimist could, with some justification decide to ignore the fine print, and conclude that overall, there was a reasonable body of evidence to suggest that on the whole, anti- MSP-119 antibodies were protective, the pessimist could equally make a strong argument that responses to MSP-119 have not been studied systematically enough for any firm conclusion to be reached regarding their role in protection against malarial disease. Perhaps more importantly, for the future, concerted efforts need to be made to harmonize
studies to allow clear evidence on protection, or the lack of it, to accumulate. Considerable resources will have gone into the studies reviewed here, and we may be none the wiser for it. Malaria researchers urgently need to improve the design of studies, standardize the antigens and assays employed, standardize the analytical approaches, agree on the end-points for different designs and then conduct adequately powered studies in multiple malaria endemic areas.

2.1.1.3.5 Vaccine trials in humans

A malaria vaccine containing MSP-142 (the 3D7 variant) has been successfully tested in multiple phase I safety and immunogenicity studies in North American (Ockenhouse, Angov et al. 2006) and Kenyan adults (Stoute, Gombe et al. 2007), and in a dose-escalation phase Ib trial in Kenyan children (Withers, McKinney et al. 2006). Concerns that vaccine-induced anti-MSP-142 antibodies may not induce antibodies to capable of reacting with multiple variants at this locus were allayed by the finding of cross-reactive responses with homologous proteins derived from the FVO and CAMP/FUP parasite 'strains' (Thera, Doumbo et al. 2006). In spite of this, anxiety persists over the possibility that an MSP-119 based vaccine may induce allele-specific antibodies, and therefore, fail to protect, if the MSP-119 variant included in the vaccine differed from the prevailing variants in the vaccine trial site (Takala, Coulibaly et al. 2007).
2.1.2 MSP-1 BLOCK 2

2.1.2.1 Antigen discovery, location and structure

A search for mAbs against MSP-1 that could inhibit parasite replication \textit{in vitro} led to the discovery of CE2 and EB2, mAbs specific for the variable tri-peptide repeat region of the N-terminal 83kDa protein that is shed at the time of erythrocyte invasion (Locher, Tam et al. 1996). It was surprising that mAbs to this region would inhibit invasion, firstly because this N-terminal fragment had been shown to be discarded at invasion (Holder, Lockyer et al. 1985), and secondly because invasion was thought to be dependent upon the cleavage of MSP-1\textsubscript{42} into MSP-1\textsubscript{19} and MSP-1\textsubscript{33} (Blackman, Ling et al. 1991). The authors (Locher, Tam et al. 1996) speculated that CE2 and EB2 interfered with merozoite release and/or binding to erythrocytes, or perhaps caused merozoite agglutination. They did not exclude the fact that the observed inhibition was an artifact of the \textit{in vitro} growth inhibition assay, especially because it could only be detected when the mAbs were used at very high concentrations (500\textmu g/ml), which were unlikely to occur in nature (Locher, Tam et al. 1996).

Although the mechanism by which CE2 and EB2 inhibited erythrocyte invasion was not clear, this effect was nonetheless dose-dependent and interestingly, the mAbs were targeted to polymorphic epitopes (Locher, Tam et al. 1996). Subsequent studies sought to determine whether allelic variation at this locus resulted in antigenic polymorphism with consequent effects on the specificity of antibody responses (Cavanagh and McBride 1997). Recombinant MSP-1 block 2 antigens were found to be immunogenic and contained conserved and type-specific epitopes, which could be distinguished by sera from humans naturally exposed to malaria (Cavanagh and McBride 1997).
At the molecular level, block 2 is the most polymorphic locus of the MSP-1 gene. Sequence variants at this locus can be classified into three main allelic families or types (K1-like, MAD20-like and RO33-like), after the prototypic parasites in which they were first described (Holder, Lockyer et al. 1985; Certa, Rotmann et al. 1987; Tanabe, Mackay et al. 1987). More recently, a fourth allelic family (MR) has been reported, and is thought to have arisen as a result of intragenic recombination between MAD20 and RO33 alleles (Takala, Branch et al. 2002). Parasites bearing MR alleles have been detected in isolates from Kenya, India, Thailand and Venezuela (Takala, Escalante et al. 2006). The K1-like and MAD20-like types contain different centrally located tri- or hexa-peptide repeat sequences, which also vary in length. These repeats are flanked by type-specific, non-repetitive sequences. The RO33-like variants do not contain any repetitive sequences and are largely conserved (Certa, Rotmann et al. 1987). Although the different repeated amino acid motifs in both K1- and MAD20-like alleles vary considerably in length, the size of the block is largely maintained (maximum of approximately 90 amino acids), suggesting that this is functionally constrained (Tetteh, Cavanagh et al. 2005). Unlike several other merozoite proteins thought to inhibit erythrocyte invasion, including MSP-119, the crystal structures of the MSP-1 block 2 antigens have not been determined (Bentley 2006).

2.1.2.2 Antigen function and effect of gene disruption

The function of MSP-1 block 2 proteins remains unknown, but the fact mAbs to this region inhibit merozoite invasion of erythrocytes suggests that they have a role to play in invasion (Locher, Tam et al. 1996). The K1- and MAD20-like variants contain repeat sequences which on the one hand are thought to impair the development of protective immunity to
malaria (Anders 1986; Schofield 1991), and on the other, have been associated with protection both from infection (Bojang, Milligan et al. 2001; Kester, McKinney et al. 2001) and clinical disease (Polley, Tetteh et al. 2003). Attempts to knock out or disrupt MSP-1 block 2 specifically, have not been reported.

2.1.2.3 Supportive evidence that antibodies play a role in protection against malaria

2.1.2.3.1 In vitro studies
The only in vitro study that suggests antibodies to MSP-1 block 2 may play a role in protection against malaria is that already described above by Locher et al. (Locher, Tam et al. 1996). In a separate study that aimed to map the epitopes of MSP-1 that were targeted by human immune antibodies, octapeptides that corresponded to the N-terminal repeats were found to give the strongest reactivity by ELISA (Lyon, Carter et al. 1997). However, in the same experiment, antibodies dissociated from immune clusters of merozoites (a potential mechanism for inhibiting merozoite dispersal) failed to recognize both the recombinant fragments and octapeptides from block 2 of MSP-1 (Lyon, Carter et al. 1997).

2.1.2.3.2 Vaccination and passive antibody transfer studies
The majority of vaccination studies in animal models of malaria have been performed using the entire MSP-1 protein, or various C-terminal portions of it, such as MSP-142 and MSP-119 (discussed above). Only one study was identified where synthetic peptides corresponding to the N-terminal 83kDa protein of MSP-1 were used to immunize Saimiri monkeys (Cheung, Leban et al. 1986). Although monkeys immunized with these peptides had very low anti-malarial antibody titres, following challenge, three of the four immunized
monkeys had lower parasitaemias than control animals and recovered without therapy (Cheung, Leban et al. 1986).

2.1.2.3.3 Population genetic analyses

Molecular population genetic studies have found evidence of balancing selection in block 2 of MSP-1, using different methods. The number of non-synonymous amino acid replacements was found to exceed that of synonymous amino acid replacements in three of eleven regions of six MSA-1 alleles suggesting that positive selection was favouring diversity at these sites, one of which was region 3 which is currently referred to as block 2 (Hughes 1992). The Ewens-Watterson test is one in which allele frequencies in a population sample are determined and used to calculate an observed $F$ statistic which is compared to that expected under neutrality. The $F$ statistic represents the homozygosity in a gene which would exist in a diploid under Hardy-Weinberg equilibrium and is equal to the sum of the squared allele frequencies. For malaria parasites this is achieved by sampling blood stage parasites which are haploid. This test was applied to three malaria antigens, MSP-1, MSP-2 and GLURP in a population sample of 100. $F$ was found to be lower than expected under neutrality for all antigens although this was only significant for MSP-2 and GLURP (Hughes 1992; Conway 1997; Conway, Cavanagh et al. 2000). The strongest evidence comes from the study by Conway et al., where ten loci within the msp-1 gene of *P. falciparum* were analyzed in large population samples from seven malaria endemic countries from East, West and South Africa. This study employed Wright’s $F$ statistic ($F_{ST}$) which is a measure of the proportion of overall diversity that is attributable to differences between populations. When allele frequencies are very similar different populations, i.e.
very low $F_{ST}$, this suggests that balancing selection is maintaining the alleles. In comparison to the rest of $msp-I$, they found strong evidence of balancing selection within Block 2, which was subsequently supported by data from immuno-epidemiological studies (Conway, Cavanagh et al. 2000).

2.1.2.3.4 Immuno-epidemiological studies

2.1.2.3.4.1 Introduction

By far the majority of immuno-epidemiological studies on MSP-1 have focused on its C-terminal regions (described above). However, early studies indicated that various N-terminal regions of the molecule were also targets of naturally acquired immune responses, and thus could play a role in protecting against malaria. These studies included children and adults, and analyzed antibodies to a variety of N-terminal fragments of MSP-1, in samples drawn both from cross-sectional surveys (Chizzolini, Dupont et al. 1988; Muller, Fruh et al. 1989; Fruh, Doumbo et al. 1991), and longitudinally monitored cohorts (Tolle, Fruh et al. 1993). The N-terminal fragments of MSP-1 analyzed were not as well characterized as they are currently, and so the studies cannot be directly compared with more recent ones. Nevertheless, antibodies to particular N-terminal fragments were relatively common, prevalence being higher in adults compared to children, and associated with lower parasitaemias (analyzed at various thresholds) (Chizzolini, Dupont et al. 1988; Muller, Fruh et al. 1989; Fruh, Doumbo et al. 1991; Tolle, Fruh et al. 1993). Antibody reactivity was higher against dimorphic, compared to conserved epitopes, and appeared to reflect the genotypes of parasites prevalent in the study location, although only a handful of parasite isolates ($n=8$) were actually genotyped (Tolle, Fruh et al. 1993).
2.1.2.3.4.2 The prevalence of antibodies to MSP-1 Block 2 antigens

With the advent of new, well characterized MSP-1 block 2 antigens (Cavanagh and McBride 1997; Polley, Tetteh et al. 2003), studies conducted in multiple locations can now be directly compared. In addition, several studies have analyzed antibody responses using synthetic peptides corresponding to the different block 2 allelic families (Jouin, Rogier et al. 2001; Ekala, Jouin et al. 2002). In general, the prevalence of antibodies to recombinant, *E. coli*-expressed MSP-1 block 2 antigens has ranged from 5 to 35% in children, depending on the specific antigen (Mawili-Mboumba, Borrmann et al. 2003; Cavanagh, Dodoo et al. 2004; Osier, Fegan et al. 2008). However, in a cross-sectional survey among school children in Cameroon, the prevalence was unexpectedly high (close to 60% for some antigens) in one of four schools (Kimbi, Tetteh et al. 2004). The reasons for this were unclear, especially since neither the prevalence of *P. falciparum* parasitaemia, nor that of multiple clone infections, was different across the schools (Kimbi, Tetteh et al. 2004). Prevalence was also unexpectedly low among children presenting to hospital with severe malaria anaemia (1.7%) or uncomplicated malaria (3.7%), compared to those with cerebral malaria (10.3%), for antibodies to the R033 type, although this is not a representative population sample (Dobano, Rogerson et al. 2008). Antibody prevalence was observed to increase with age in some (Mawili-Mboumba, Borrmann et al. 2003; Cavanagh, Dodoo et al. 2004), but not all studies (Osier, Fegan et al. 2008), and to be higher at the end, as opposed to the beginning, of a malaria transmission season (Cavanagh, Dodoo et al. 2004).
The predominant IgG isotype response to MSP-1 block 2 antigens is IgG3 followed by IgG1, in both adults and children (Cavanagh, Dobano et al. 2001; Cavanagh, Dodoo et al. 2004), as well as symptomatic and asymptomatic *P. falciparum* infections (Scopel, Fontes et al. 2005), though this pattern (IgG3>IgG1) appeared to be reversed in one study from the Brazilian Amazon (Da Silveira, Dorta et al. 1999). The prevalence of antibodies to MSP-1 block 2 was also higher in an area of higher malaria transmission (Chonyi) compared to one with lower transmission (Ngerenya) (Conway, unpublished). As with other malaria antigens, the prevalence (and levels) of antibodies to MSP-1 block 2 antigens was higher in children who were parasitaemic at the time of sampling, compared to those who had no detectable parasites (Osier 2008). Finally, like IgG antibodies to other malaria proteins (Kinyanjui, Conway et al. 2007), those to MSP-1 block 2 are typically short-lived (Cavanagh, Elhassan et al. 1998).

### 2.1.2.3.4.3 Allele- and type-specificity of antibodies to MSP-1 Block 2

Polymorphism is widely considered to be a mechanism by which parasites evade protective immune responses (Conway 1997). Given that block 2 is the most polymorphic locus of the *msp1* gene, it has been of interest to investigate the relationships between the genotypes of infecting parasites and the corresponding allele-specific antibody responses. In the first immuno-epidemiological study using MSP-1 block 2 antigens, Cavanagh *et al.* found that in general, the antibodies were type-specific, and correlated with the PCR typing of parasites present at the time of infection (Cavanagh, Elhassan et al. 1998). While a similar trend has been found in some studies (Kimbi, Tetteh et al. 2004), and, in particular
with cytophilic antibodies (Da Silveira, Dorta et al. 1999), others have found no relationship between the allele-specific antibodies detected and the genotypes present in the concurrent infection (Jouin, Rogier et al. 2001; Ekala, Jouin et al. 2002).

2.1.2.3.4.4 Antibodies to MSP-1 block 2 and associations with protection

With regards to protection, antibodies to MSP-1 block 2 have been analyzed largely in two contexts. The first is in traditional longitudinal studies, where individuals are monitored for the development of clinical disease over a defined period of time. The presence and/or levels of antibodies (measured at the start of the observation period), are then compared among 'susceptible' and 'protected' individuals. The second is the more recent approach, where antibodies are analyzed in studies primarily designed to monitor drug resistance (described above). A range of comparisons can be made from the latter studies, at multiple time points, between specific IgG and clinical and/or parasitological outcome. As such, in longitudinal studies where the outcome was defined as clinical episodes of malaria (fever plus a parasitaemia threshold), antibodies to MSP-1 block 2 were associated with protection in some (Conway, Cavanagh et al. 2000; Polley, Tetteh et al. 2003; Cavanagh, Dodoo et al. 2004), though not all studies (Osier, Fegan et al. 2008). Similarly, in a study on the efficacy of amodiaquine in the treatment of uncomplicated \textit{P. falciparum} malaria, a significantly higher proportion of children with antibodies to more than 2 variants of the K1 block 2 type was found in the cured, compared to the treatment failure group. These data are contradictory because the authors also report that the prevalence of antibodies to
block 2 antigens, in particular to K1-like antigens was similar in both groups of children (Mawili-Mboumba, Borrmann et al. 2003).

2.1.2.3.5 Vaccine trials in humans

Although no vaccine trials have been conducted (at least not published) with MSP-1 block 2 antigens, an interesting concept with regards to the design of such a vaccine has been proposed. Tetteh et al. analyzed the complex polymorphism arising as a result of repeats within the predominant K1-like variants, and constructed a composite antigen (K1-like Super Repeat), incorporating diverse deduced epitopes, and able to induce broad specificity following immunization (Tetteh, Cavanagh et al. 2005). This approach could be extended to include sequences from the other major allelic types of block 2, and thus potentially overcome the challenge of including multiple, diverse alleles into an MSP-1 block 2 based malaria vaccine.
2.2 MSP-2

2.2.1 ANTIGEN DISCOVERY, LOCATION AND STRUCTURE

MSP-2, previously referred to as MSA2, gp35-56, or GP3, appears to have been identified by several distinct groups at about the same time. The earliest report was made by Stanley, Howard and Reese (Stanley, Howard et al. 1985), who used hybridomas from mice that had been repeatedly injected with disrupted schizonts and merozoites of *P. falciparum*. They demonstrated that the antibodies secreted by these hybridomas bound to the merozoite surface, and immuno-precipitated a 56kDa molecular weight protein (Stanley, Howard et al. 1985). In subsequent studies, several methods were employed to arrive at the same antigen. In what was considered to be a novel approach at the time, Smythe *et al.* used temperature dependent phase separation with the non-ionic detergent Triton X-114 to isolate integral membrane proteins which they then blotted onto nitrocellulose. Human antibodies were subsequently affinity purified on these immobilized antigens, and used to identify cDNA clones (from a phage expression library) encoding the corresponding peptides. Thus, they isolated a 45kDa protein (Smythe, Coppel et al. 1988), and established using mAbs that it corresponded to the 55kDa antigen described by Epping *et al.* (Epping, Goldstone et al. 1988), as well as the 45kDa protein reported as GYMMSA by Ramasamy *et al.* (Ramasamy 1987), and possibly to the 56kDa protein described by Stanley *et al.* (Stanley, Howard et al. 1985), above. In a separate study, Miettinen-Baumann *et al.* isolated a 46kDa protein by extracting freshly harvested merozoites of *P. falciparum*, subjecting the extract to gel electrophoresis and electroelution. They showed that this protein was present in late ring, trophozoite, schizont and segmenter stages, and could be localized to
the surface of the merozoite at the end of schizogony (Miettinen-Baumann, Strych et al. 1988). MSP-2 was also identified by immuno-precipitating the proteins targeted by mAbs known to inhibit erythrocyte invasion (Clark, Donachie et al. 1989), or those eluted from immune clusters of merozoites (ICM) (Thomas, Carr et al. 1990).

At the molecular level, numerous alleles of MSP-2 have been described, which can be grouped into two main families, 1C-1-like and FC27-like, based on the prototype parasites in which they were described. This classification has not changed in essence, since it was first put forward, based on the analysis of far fewer alleles than have been subsequently reported (Thomas, Carr et al. 1990; Smythe, Coppel et al. 1991) and was supported by serological analyses (Fenton, Clark et al. 1991). For simplicity and consistency, IC-1-like MSP-2 alleles and antigens will be referred to as type A alleles and antigens, and similarly FC-27-like MSP-2 alleles and antigens will be referred to as type B, in a similar fashion to Fenton et al. (Fenton, Clark et al. 1991). MSP-2 sequences contain a central domain comprised of repeats that vary in number, length and sequence, flanked in turn by non-repetitive variable sequences, and by conserved N- and C-terminal domains. Dimorphic, non-repetitive sequences internal to the N- and C- termini distinguish the two main allelic families, as illustrated in Figure 2.2.1. The central repeats define individual alleles. Type B alleles have a 32 residue motif that is repeated one to four times, followed by a 7-mer residue sequence, and by one to five tandem copies of a variable 12-mer sequence. Type A alleles are characterized by shorter repeats of 3 to 10 residues. In addition, further diversity arises as a consequence of point mutations, which occur in all regions of the gene.
Structurally, MSP-2 is reported to have the characteristics of an intrinsically unstructured protein, and can form amyloid-like fibrils in its recombinant form, the latter lending support to a role in invasion (Low, Chandrashekaran et al. 2007). Knowledge of the structure of MSP-2 is relevant for vaccine development, as the monomeric form and fibrils may have different antigenic properties (Low, Chandrashekaran et al. 2007).

![Diagram of MSP-2 gene structure](image)

**Figure 2.2.1 The msp2 gene**

Schematic of the msp-2 gene illustrating the locations of the constant domains, family-specific domains and allele-specific repeats. Taken from Felger 2003.

### 2.2.2 Antigen Function and Effect of Gene Disruption

Attempts to knock out MSP-2 have so far been unsuccessful (Cowman and Crabb 2006) and its precise function has yet to be determined.
2.2.3 SUPPORTIVE EVIDENCE THAT ANTIBODIES PLAY A ROLE IN PROTECTION AGAINST MALARIA

2.2.3.1 In vitro studies

Several murine mAbs known to inhibit erythrocyte invasion have been shown to bind to epitopes within MSP-2, such as 8G10/48 and 9E3/48 (Epping, Goldstone et al. 1988), mcab 13.4 (Clark, Donachie et al. 1989), (Ramasamy 1987; Miettinen-Baumann, Strych et al. 1988). Such experiments support the idea that MSP-2 has a role to play in merozoite invasion of erythrocytes. It is not clear whether these mAbs were effective at inhibiting invasion-inhibition of multiple parasite strains, or only the strains used to generate them. In studies where actual invasion-inhibition experiments were performed, only one parasite strain was tested (Epping, Goldstone et al. 1988; Miettinen-Baumann, Strych et al. 1988). However, an early suggestion that the observed invasion-inhibition was 'strain-specific' came from the study by Stanley and colleagues. Monoclonal antibodies generated from hybridomas created using the FVO strain reacted with the same FVO strain and the Geneva strain in IFAT (Indirect Fluorescence Antibody Test) and immuno-precipitated a 51kDa protein, but failed to do the same with four other parasite strains (Stanley, Howard et al. 1985). Similar results were subsequently reported in another study but with different parasite strains (Epping, Goldstone et al. 1988).

2.2.3.2 Vaccination and passive antibody transfer studies

As MSP-2 has no homologues in other Plasmodium species, limited studies have been carried out in animal models of malaria. In one study, mice immunized with peptides from
the conserved N- and C-termini were partially protected from challenge with *P. chabaudi*, suggesting that a similar protein existed in this rodent malaria parasite (Saul, Lord et al. 1992). However, although mice immunized with such peptides produced antibodies at levels that were comparable with those generated in response to vaccination with full length MSP-2, the specificities of the two types of antibodies differed significantly (Lawrence, Stowers et al. 2000). In a separate study, MSP-2 peptides were part of a cocktail used unsuccessfully to immunize Saimiri monkeys (Pye, Edwards et al. 1991).

2.2.3.3 Population genetic analyses

Varying levels of evidence suggest that MSP-2 is under positive natural selection. Hughes and Hughes analyzed non-synonymous and synonymous mutations in eight polymorphic *P. falciparum* genes. In four out of eight genes, including *msp-2*, an excess of non-synonymous over synonymous mutations was found (Hughes and Hughes 1995). In a separate study as previously described, *msp-2* was one of three genes for which the *F* statistic from the Ewens-Wattersons test of neutrality was significantly lower than that expected under neutrality (Conway 1997). However relatively weaker evidence was found in a third study. Although significantly more synonymous than non-synonymous mutations were found in *msp-2* suggesting positive selection, two additional tests for selection, Tajima’s *D* and McDonald-Kreitman, failed to identify positive selection (Escalante, Lal et al. 1998). These additional tests of selection will be described in detail in a subsequent chapter.
2.2.3.4 Immuno-epidemiological studies

2.2.3.4.1 Variations in MSP-2 antigens

Antibodies to MSP-2 are common in populations exposed to malaria, and absent in those never exposed to malaria. However, as with antibodies to MSP-119, different studies have analyzed antibodies to different fragments of MSP-2, either as recombinant proteins, commonly expressed in *E. coli*, or, as synthetic peptides. The difficulties these differences pose in reviewing such data have already been discussed at length. Nevertheless, a few examples are given to illustrate the case with regards to MSP-2. The reported *E. coli*-expressed antigens may represent the full-length antigens (Polley, Conway et al. 2006; Sarr, Pelleau et al. 2006; Osier, Fegan et al. 2008), varying lengths of polymorphic, or conserved, parts of the gene, or both (Taylor, Smith et al. 1995; Taylor, Allen et al. 1998; Metzger, Okenu et al. 2003). Some portions of the gene may be missing, e.g. the central repeats (al-Yaman, Genton et al. 1994; al-Yaman, Genton et al. 1996; Ranford-Cartwright, Taylor et al. 1996).

On the other hand, synthetic MSP-2 peptides contain amino acids which may or may not be consistent between studies, from the conserved N- and C-termini, (Aucan, Traore et al. 2000; Kohler, Tebo et al. 2003), from the central repetitive regions (these repeats vary in length and sequence) (Zhou, Xiao et al. 2002), or from multiple locations within the gene (Ntoumi, Ekala et al. 2002). In one study, short MSP-2 peptides were synthesized from undisclosed 'antigenic determinants' of the gene, with no information on how these were determined (Ayisi, Branch et al. 2003). As such, in considering the prevalence of antibodies
to MSP-2 in malaria-endemic populations, attention needs to be paid to the nature of the antigen being tested.

2.2.3.4.2 Prevalence of anti-MSP-2 antibodies

From published reports, the prevalence of antibodies to MSP-2 from adequately sized cross-sectional surveys ranges from as low as 3% in rural Amazonians with little malaria exposure (Scopel, da Silva-Nunes et al. 2007), to as high as 100% among Kenyan (Polley, Conway et al. 2006) or Gambian adults (Taylor, Allen et al. 1998), with a wide range in between. In these three studies, this wide variation in prevalence is explained in part both by differences in the nature of the antigen tested, as well as differences in the transmission intensity of malaria (Taylor, Allen et al. 1998; Polley, Conway et al. 2006; Scopel, da Silva-Nunes et al. 2007). In particular for MSP-2, antibody prevalence is higher for polymorphic as opposed to conserved epitopes (Taylor, Smith et al. 1995; Metzger, Okenu et al. 2003), and in general, the prevalence and levels are highest when measured against the full-length antigens (Taylor, Smith et al. 1995). Other factors affecting the prevalence of antibodies are similar to those already described for antigens to MSP-119 and MSP-1 block 2.

Anti-MSP-2 antibodies are found more commonly in adults, as compared to children (Polley, Conway et al. 2006), in pregnant mothers in their third trimester compared to their infants (Riley, Wagner et al. 2000), and in parasite positive compared to parasite negative children (Polley, Conway et al. 2006) and adults (Tami, Grundmann et al. 2002). The prevalence of antibodies to MSP-2 was comparable in HIV+ and HIV- mothers and their
respective infants (Ayisi, Branch et al. 2003), and among children with the sickle trait (HbAS) compared to those with normal haemoglobin genes (HbAA). However, in one study, individuals with adaptive hemoglobin variants (HbC or HbS) have higher antibody levels to MSP-2 compared with individuals who have normal hemoglobin (Verra, Simpore et al. 2007). Higher antibody levels to an MSP-2 antigen of unspecified provenance were reported in children and adults from Sudan with uncomplicated malaria, compared to those with severe malaria, although this was significant for only one of the MSP-2 alleles tested (TM, Elbashir et al. 2008). In a different study from Malawi, antibody levels to a conserved C-terminal fragment of MSP-2 were lowest in children with severe anaemia, followed by those with uncomplicated malaria, and highest in children with cerebral malaria (Dobano, Rogerson et al. 2007). Somewhat similar findings were found in Gabon, where at three time points (acute, convalescent and healthy), the prevalence of antibodies to a peptide from the conserved N-terminal of MSP-2 was significantly higher in children with severe malaria compared to those with mild malaria (Kohler, Tebo et al. 2003). However, the epidemiology and clinical spectrum of malaria differ considerably in these three settings (Sudan, Malawi and Gabon).

Given the differences in the MSP-2 antigens, and in the design of the studies, the study participants and epidemiological contexts, it is difficult to make a simple summary of the prevalence of antibodies to MSP-2.
2.2.3.4.3 IgG isotype antibodies to MSP-2

The predominant IgG isotype responses to MSP-2 are IgG3, followed by IgG1 (Taylor, Smith et al. 1995; Taylor, Allen et al. 1998; Cavanagh, Dobano et al. 2001; Polley, Conway et al. 2006; Sarr, Pelleau et al. 2006; Tongren, Drakeley et al. 2006). This pattern is observed in infants (Riley, Wagner et al. 2000), children and adults (Taylor, Allen et al. 1998; Tongren, Drakeley et al. 2006), and in one study the prevalence of IgG3 to MSP-2 was observed to increase with age, while that of IgG1 decreased (Taylor, Allen et al. 1998). However, this age-dependent increase in IgG3, and decrease in IgG1 was not observed in the Brazilian Amazon, and was thought to depend on the pattern of cumulative exposure to malaria (Tonhosolo, Wunderlich et al. 2001). In a similar vein, antibodies induced in 20 adult travelers returning to Europe from Africa and presenting to hospital with malaria were not skewed towards IgG3; IgG1 antibodies were predominant (Eisen, Wang et al. 2007).

A few studies have reported comparable levels of non-cytophilic IgG isotypes (IgG2 and IgG4), and cytophilic IgG1 and IgG3 isotypes (Aucan, Traore et al. 2000; Ntoumi, Ekala et al. 2002; Ntoumi, Flori et al. 2005). The studies that detect predominantly cytophilic antibodies measure responses to *E. coli*-expressed recombinant antigens (full length or near full-length), while those detecting non-cytophilic isotypes measure antibodies to varying peptides of MSP-2. However, in studies conducted in the Brazilian Amazon, antibodies detected to various *E. coli*-expressed recombinant antigens of MSP-2 were of all isotypes, although the predominant response was still IgG3 (Scopel, Fontes et al. 2006).

Factors thought to affect the polarization of IgG isotype responses include the duration of exposure to malaria (Tonhosolo, Wunderlich et al. 2001; Tongren, Drakeley et al. 2006),
genetic factors (Stirnadel, Al-Yaman et al. 2000; Aucan, Traore et al. 2001) and intrinsic properties specific to MSP-2 (Garraud, Perraut et al. 2002).

2.2.3.4.4 Allele-specificity of anti-MSP-2 antibodies

Efforts to determine whether sequence polymorphisms within MSP-2 result in functionally important antigenic changes that might indicate a possible parasite strategy for immune evasion have not yielded clear-cut results. Several studies have analyzed naturally acquired antibodies to MSP-2 in relation to concurrent infecting parasite genotypes. This type of analysis can be envisaged in two directions. The first is whether the infecting parasite *msp-2* genotype determines the concurrently or subsequently detected antibody response. The second is whether allele-specific responses to MSP-2 prevent infection or clinical disease with parasites bearing the corresponding *msp-2* genotypes. The former analysis is complicated by, among other things, pre-existing antibodies from previous infections, and the lack of certainty in most if not all studies regarding the onset of current infections, and hence the timing of sampling with regards to generation of primary or secondary antibodies. This potentially leads to a misclassification bias, but this is probably minimized by the fact that sampling is random across studies. The latter analysis requires a longitudinal study design in which antibody concentrations can be measured in a healthy cohort and related to subsequent risk of developing malaria disease with parasites bearing particular *msp-2* genotypes. A similar approach has been reported from treatment-reinfection studies, where antibodies to MSP-2 were assayed at first presentation when the patient was treated and parasite evaluated later at re-infection (Weisman, Wang et al. 2001). For both types of analysis, it is hard to distinguish whether detected antibodies
were stimulated by the infecting parasite genotype, or were actually partially protective (definitely not fully protective or they would not have been detected!).

As will be discussed below, many studies have been largely descriptive, analyzing genotypes and corresponding antibodies in small numbers of patients, often in sub-group analyses, and thus lacking the power for definitive conclusions to be drawn. Other factors that appear to affect the results of such analyses are the study location (e.g. Africa versus South America), the ages of the individuals tested, and the particular proteins used (E. coli-expressed antigens versus synthetic peptides). An equally important and related question is whether antibodies are cross-reactive, allele-specific (between main allelic types) or variant-specific (within type). These issues will now be highlighted with specific examples, before general conclusions are made.

*Does the infecting parasite genotype determine the specificity of the concurrent antibody response (i.e. a type A or type B response)?* Polley et al. analyzed data from 146 children and adults from Kenya and found that sera from individuals who had a majority of parasites with type A alleles had significantly lower antibody levels to the type B antigen, $z = 3.130, P = 0.002$. Similarly, individuals with a majority of type B parasites had lower levels of the discordant antibodies, although the statistical evidence for the latter was weak, $z = -1.799, P = 0.072$ (Polley, Conway et al. 2006). Parasites were genotyped and the predominant or majority alleles were determined semi-quantitatively by viewing PCR products on agarose gels (Polley, Conway et al. 2006). Three South American studies also support the idea that parasite genotype determines the antibody specificity, although the strength of the
evidence varies (Tami, Grundmann et al. 2002; Kanunfre, Leoratti et al. 2003; Sallenave-Sales, Faria et al. 2007). The best evidence comes from Tami et al., who analyzed samples taken from two large cross-sectional malaria surveys, although parasite prevalence was low (e.g., 43/708, 6% in the survey of 1995/1996 and 12/925, 1.3% in the survey of 1997) (Tami, Grundmann et al. 2002). They genotyped parasites from both surveys, obtained from participants of all ages, and analyzed antibodies to recombinant MSP-2 antigens in all the samples. Parasite diversity was low. The majority of individuals had single clone infections. However, in both parasite-positive, and parasite-negative individuals, the proportion of sera recognizing type A or type B MSP-2 antigens corresponded with the msp-2 alleles present in the population at the time. Thus there was concordance at a population level, and whether this was also true at the individual level was not analyzed or reported (Tami, Grundmann et al. 2002). The remaining two South American studies involved considerably fewer individuals (all adults), recruited on presentation to hospital with uncomplicated malaria (Kanunfre, Leoratti et al. 2003; Sallenave-Sales, Faria et al. 2007). In both these studies, there is evidence for concordance between infecting parasite genotype and corresponding antibodies at the individual level in the majority of samples, although there is also clear evidence for the opposite scenario in a minority of samples (Kanunfre, Leoratti et al. 2003; Sallenave-Sales, Faria et al. 2007).

In a separate study, to avoid the confounding effect of pre-existing antibodies on the analysis, Felger et al. analyzed data from primary infections in 48 non-immune travelers with no previous history of malaria (Felger, Steiger et al. 2003). Sera were tested against 14 type A and 5 type B E.coli-expressed antigens based on the msp-2 alleles isolated from
the patient samples. Concordant genotype-antibody relationships were observed in the majority of paired samples, but not all. Sera from hosts of type A parasites recognised a higher number of type A antigens (5.8/14) than those with type B parasites, while those from hosts with type B parasites reacted with a mean of 1.9/5 type B antigens. Although the number of sera from hosts of type A parasites recognizing type B antigens is not reported, and vice-versa, logistic regression analysis was used to reject the null hypothesis that the ratio of numbers of type A:type B antigens recognized was independent of the infecting parasite genotype, likelihood ratio chi-squared = 4.5, 1 degree of freedom, P = 0.03 (Felger, Steiger et al. 2003). Similar findings were reported more recently, albeit in a much smaller study (n=20) (Eisen, Wang et al. 2007).

Two other studies failed to find a clear relationship between the infecting parasite genotype and the corresponding MSP-2 antibodies at the individual level (Weisman, Wang et al. 2001; Ekala, Jouin et al. 2002). Both are relatively small, and differ fundamentally in design, from the studies discussed up to this point. Weisman et al. analyzed msp-2 genotypes and antibodies to near full-length recombinant antigens, and various truncated versions in 15 teenagers from an area of Vietnam where malaria is highly endemic (Weisman, Wang et al. 2001). Serum samples were collected at the time of infection and radical drug treatment (T₀), at the first re-infection (T₁), when they were also treated, and 28 days later (T₂₈). They found conflicting results between individual patients at different time points, and between different patients apparently infected with parasites bearing similar genotypes. There was also no relationship between antibody response and time to re-infection and they reasonably concluded that "there was no clear relation between the
infecting form of MSP-2 and the ensuing antibody response" (Weisman, Wang et al. 2001). Ekala et al. on the other hand performed a sub-group analysis on 25 Gabonese residents of all ages, using a panel of 12 biotinylated MSP-2 synthetic peptides (Ekala, Jouin et al. 2002). The main study involved patients presenting to hospital with uncomplicated malaria (n=45), who were compared with asymptomatic subjects (n=45) from the same area. Although the prevalence of antibodies to MSP-2 had increased seven days post treatment, concordant genotype-antibody relationships were found in only 44% of symptomatic patients at day 7 (following treatment at presentation to hospital), and 24% in all samples at day 0 (Ekala, Jouin et al. 2002). From all the studies presented so far, the balance of evidence favours the idea that the infecting parasite genotype does determine the specificity of the concurrent antibody response, particularly in areas with lower malaria endemcity.

This leads to the next linked set of important questions. Do sequence polymorphisms result in antigenic changes that can be detected by measuring reactivity in ELISA assays? Are anti-MSP-2 antibodies cross-reactive within type, between types or both? These questions are answered most directly and clearly in the studies of Franks et al. and Ranford-Carwright et al. (Ranford-Cartwright, Taylor et al. 1996; Franks, Baton et al. 2003). Briefly, Ranford-Carwright et al. showed differential antibody recognition of type B repeats, by testing sera from Gambian children against a panel of type B recombinant antigens that contained varying numbers of repeats (Ranford-Cartwright, Taylor et al. 1996). In a study of 13 Ghanaian children, Franks et al. found evidence of cross-reactivity within but not between types (Franks, Baton et al. 2003). This was confirmed in a larger set of Gambian sera
(n=201), but counter-intuitively, cross-reactivity was most evident for type A antibodies, even though sequence diversity is most pronounced in type A compared to type B alleles (Franks, Baton et al. 2003). They also excluded the possibility that this apparent cross-reactivity could be explained by concomitant exposure to non-cross reactive proteins (distinct variant-specific antibodies) by performing competition ELISAs (Franks, Baton et al. 2003). Other studies have also demonstrated the lack of cross-reactivity between type A and B antibodies (Taylor, Smith et al. 1995) and the presence of cross-reactivity between variant-specific antibodies within either type (Tonhosolo, Wunderlich et al. 2001), although parasite genotypes from the corresponding samples were not determined.

*Are antibodies to MSP-2 associated with protection from malaria?* In all but one identified study, antibodies to MSP-2 have been associated with protection from malaria. The earliest immuno(sero)-epidemiological study that assessed protection from malaria by naturally-acquired antibodies to MSP-2 was conducted in Papua New Guinea by Al-Yaman and colleagues (al-Yaman, Genton et al. 1994). Using a cross-sectional survey, they demonstrated that antibodies to full-length or near full length recombinant MSP-2 antigens were associated with a history of fewer fever episodes and less anaemia (al-Yaman, Genton et al. 1994). Although there were clear deficiencies in the study design and particularly in case definition, this study nevertheless suggested that MSP-2 was worth investigating further. Al-Yaman and colleagues then conducted a longitudinal study which showed that antibodies to MSP-2 were indeed associated with a lower risk of subsequent clinical malaria, in a different set of children from Papua New Guinea (al-Yaman, Genton et al. 1995). Only antibodies to the two type A antigens (3d7 and d3d7), and not to the type B
antigen (FC27) were associated with protection. This no doubt contributed to the inclusion of the MSP-2 3D7 allele in the malaria vaccine that was later tested in Papua New Guinea, and will be discussed below (Genton, Anders et al. 2003).

Two other studies subsequently showed that antibodies to MSP-2, particularly of the IgG3 isotype, were associated with a lower risk of clinical episodes to malaria (Taylor, Allen et al. 1998; Metzger, Okenu et al. 2003). Although both studies were conducted in rural villages in the Gambia, with presumably similar malaria endemicities, the results were slightly different. Around Basse, antibodies to both type A and type B antigens were associated with protection (Metzger, Okenu et al. 2003), whereas around Farafenni, only antibodies to the type A antigen were associated with protection and IgG1 antibodies to the type B antigen were in fact associated with an increased risk of clinical disease (Taylor, Allen et al. 1998). In a Kenyan study, high levels of antibodies to both types of antigens were associated with a lower risk of clinical malaria in a high (Chonyi), and low (Ngerenya), malaria transmission region, although this did not reach significance for all the categories of antibody levels tested (Polley, Conway et al. 2006). The one study in which antibodies to MSP-2 were not associated with protection was that conducted in Gambian infants by Riley et al. (Riley, Wagner et al. 2000). Maternal antibodies measured in infants at birth, and monitored for 20 weeks, were not associated with resistance to malaria infection (not clinical episodes as in the other studies discussed previously) (Riley, Wagner et al. 2000).

All the studies on protection discussed so far have tested antibodies against similar full-length, or near full-length recombinant MSP-2 antigens, expressed in E. coli. In at least one
study where peptides from the N- and C- termini of MSP-2 were assayed, children were monitored for a year to detect clinical episodes (Aucan, Traore et al. 2000). The results from this study are strikingly different from those using *E. coli*-expressed full-length MSP-2. IgG2 antibodies which are rarely detected in the studies using *E. coli* antigens measured at the end of the transmission season were associated with a low risk of infection, while IgG4 antibodies were associated with an increased risk of malaria attack. The analytical strategies employed in this study, and the style of data presentation make it difficult to interpret the results (Aucan, Traore et al. 2000). Overall, it is reasonable to conclude that antibodies to MSP-2 are associated with protection from clinical episodes of malaria.

2.2.4 VACCINE TRIALS IN HUMANS

The first mention of MSP-2 in a human vaccine trial comes from a phase I safety and immunogenicity study conducted in Swiss volunteers. In a move to trial multi-component, multi-stage malaria vaccines, instead of mono-component ones, MSP-2 was included in vaccine formulation together with a CS protein (Sturchler, Berger et al. 1995). Although this vaccine was safe and immunogenic, it offered no protection against infection (Sturchler, Berger et al. 1995). In further phase I studies conducted in medical and veterinary school volunteers, the combination of MSP-1, MSP-2 and RESA was tested in two vaccine trials that aimed to address antigenic competition between the antigens, as well as provide further data on safety and immune response, as a function of both dose, and timing of vaccinations (Saul, Lawrence et al. 1999). Rather disappointingly, as had been found with other malaria antigens, vaccine-induced antibody responses to this combination were
much weaker than those obtained in experimental animals. Nevertheless, minimal antigenic competition was reported, except for one MSP-1 antigen when groups receiving mixture versus individual antigens were analyzed, and the immune response was dose-dependent (Saul, Lawrence et al. 1999). There was concern however that the vaccine could induce different responses in people already exposed to malaria, a concern the authors pointed out, could only be addressed by conducting challenge experiments in persons living in malaria endemic areas. Notably, none of the measured vaccine-induced immune responses correlated with parasite growth rates (Lawrence, Cheng et al. 2000).

Nevertheless, these studies led to the phase 1-2b combination B vaccine trial in Papua New Guinea that has already been discussed (under erythrocytic malaria vaccines) (Genton, Betuela et al. 2002; Genton, Al-Yaman et al. 2003).
2.3 MSP-3

2.3.1 ANTIGEN DISCOVERY, LOCATION AND STRUCTURE

MSP-3 was identified using different techniques by two independent groups of researchers at about the same time. McColl and colleagues described an antigen which they named SPAM (secreted polymorphic antigen associated with merozoites) (McColl, Silva et al. 1994). SPAM was a 43.2kDa protein identified by screening a *P. falciparum* cDNA library using immune serum from Papua New Guinea (McColl, Silva et al. 1994). Simultaneously and independently, Ouevray and colleagues identified a 48kDa protein that they named MSP-3, which was recognized both by non-cytophilic antibodies of individuals exposed but not protected against malaria, and by cytophilic antibodies of individuals resistant to malaria (Oeuveray, Bouharoun-Tayoun et al. 1994). Subsequently, sequence comparisons indicated that SPAM and MSP-3 were identical, and the name MSP-3 was retained (McColl and Anders 1997). The one thing that differed in the descriptions of MSP-3 from the two groups was its location. McColl *et al.* held that MSP-3 lacked the C-terminal GPI anchor signal, and a stop transfer sequence and therefore was not an integral membrane protein. However, it appeared to be secreted into the parasitophorous vacuole of the mature parasite, although a small proportion of the protein remained associated with the merozoite surface at schizont rupture (McColl, Silva et al. 1994). Ouevray *et al.* on the other hand, used several techniques to show that MSP-3 was indeed located on the merozoite surface and was not present at any other stage of the parasite’s life cycle (Oeuveray, Bouharoun-Tayoun et al. 1994). It is unclear whether these differences have been resolved, and the matter is largely un-discussed in subsequent publications on MSP-3.
Structurally, MSP-3 contains 3 blocks of four heptad repeats of the type AXXAXXX, a hydrophilic region, followed by a putative leucine zipper sequence at the C-terminus. The heptad regions have hydrophobic residues which result in helical bundles or coiled-coil structures in proteins (Mulhern, Howlett et al. 1995). At the sequence level, MSP-3 is polymorphic in its N-terminal, but largely conserved in the C-terminal. N-terminal variations arise through substitutions and deletions in non-repetitive sequences within and flanking the alanine-heptad repeat domains (McColl and Anders 1997). Huber et al. genotyped parasites from diverse geographical locations around the world at the msp-3 locus and found a distinct dimorphism, with parasite isolates falling into two major types; 3D7-like or K1-like, as shown in Figure 2.3.1 (Huber, Felger et al. 1997).
Figure 2.3.1 Amino acid sequences of *msp3* genes

An alignment of the amino acid sequences of *msp3* genes from a panel parasite isolates obtained from diverse regions of the world. Light grey areas indicate complete conservation among isolates tested. Intermediate and dark grey shading indicates the two alternative dimorphic forms. Non-shaded amino acids indicate substitutions that do not follow a dimorphic pattern. Figure taken from Huber 1997.

2.3.2 ANTIGEN FUNCTION AND EFFECT OF GENE DISRUPTION

The precise function of MSP-3 is not known, but it is thought to have a role in merozoite invasion of erythrocytes. Disruption of *msp-3* interfered with the trafficking of the protein to the parasitophorous vacuole and its interaction with the merozoite surface.
Additionally, gene disruption of *msp-3* also led to the loss of another merozoite surface protein, ABRA (acidic-basic repeat antigen), and led to reduction in invasion efficiency, supporting a role in invasion (Mills, Pearce et al. 2002).

2.3.3 SUPPORTIVE EVIDENCE THAT ANTIBODIES PLAY A ROLE IN PROTECTION AGAINST MALARIA

2.3.3.1 *In vitro* studies

The best evidence for the protective role of anti-MSP-3 antibodies has been demonstrated in the ADCI assay (described previously), which also led to its discovery (Oeuvey, Bouharoun-Tayoun et al. 1994). Pooled hyper-immune serum from adults in the Ivory Coast (n=180) that conferred passive protection when administered to Thai patients with severe malaria (Sabchareon, Burnouf et al. 1991) was affinity-purified on three synthetic peptides of MSP-3, MSP-3a, MSP-3b, and MSP-3c. These are 24 to 28 mer overlapping peptides derived from a conserved section of MSP-3. Only antibodies affinity-purified on MSP-3b, significantly inhibited the growth of cultured erythrocytic parasites. This was true both for human antibodies, as well as for sera from mice immunized with MSP-3b (Oeuvey, Bouharoun-Tayoun et al. 1994).

Subsequent studies have confirmed these findings (Badell, Oeuvey et al. 2000; Singh, Soe et al. 2004; Theisen, Soe et al. 2004). In an *in-vivo* model in which *P. falciparum* infection and growth can be monitored in immuno-deficient mice, only the combination of hyper-immune immunoglobulin and monocytes, and not either of the two components singly, was
effective in controlling and in some cases, completely clearing parasitaemia. This was observed with total hyper-immune serum, and hyper-immune serum affinity-purified on MSP-3b, but not on RESA (ring erythrocyte surface antigen). When antibodies affinity-purified on MSP-3b were used, parasite clearance was faster than that observed with total hyper-immune serum, and as fast as that induced by treatment with chloroquine, although the data for the latter were not shown (Badell, Oeuvray et al. 2000).

In a separate study, Singh et al. took pooled serum from 30 hyper-immune adults from the Ivory Coast, and affinity-purified it on a wider panel of overlapping peptides from the conserved C-terminal of MSP-3 (MSP-3a to f) (Singh, Soe et al. 2004). They confirmed the previous findings that antibodies to MSP-3b were effective in ADCI. In addition they found that antibodies to MSP-3c, d, and f were similarly effective in ADCI, were of the cytophilic IgG sub-classes, and controlled parasitaemia in-vivo in a previously described immuno-compromised mouse model (Singh, Soe et al. 2004). Murine antibodies raised against a GLURP-MSP-3 chimeric protein were also effective in ADCI (Theisen, Soe et al. 2004). In a different approach, mRNA from peripheral blood leukocytes of clinically immune individuals from Senegal (n=13) was used as a source of Fab (fragment antibody genes), which were then used to make a Fab-phage display library from which three distinct anti-MSP-3 antibodies were isolated by panning. The three antibodies thus identified were produced in CHO cells (IgG1 and IgG3), and were shown to recognize the native parasite protein, and importantly, one of them (RAM1), was effective in ADCI (Lundquist, Nielsen et al. 2006).
2.3.3.2 Vaccination and passive antibody transfer studies

Passive antibody transfer studies have only been conducted in the immuno-compromised mouse model, described above (Badell, Oeuvey et al. 2000; Singh, Soe et al. 2004). Several vaccination studies have been conducted in *Aotus* and *Saimiri* monkeys (Hisaeda, Saul et al. 2002; Carvalho, Oliveira et al. 2004; Carvalho, Alves et al. 2005). In the first trial, although full-length MSP-3 (FVO strain) was expressed in both *S. cerevisiae* and *P. pastoris*, there were significant differences in the protein yield of MSP-3 by species, as well as its electrophoretic mobility on SDS-page gels. As such, only *P. pastoris* derived MSP-3 was used to immunize New World *Aotus* monkeys because of its higher yield, purity and the fact that its electrophoretic migration more closely resembled that of native MSP-3 (Hisaeda, Saul et al. 2002). In comparison to five of seven control monkeys, only one of seven monkeys immunized with MSP-3 developed an acute infection that required treatment to control parasitaemia. Additionally, protection correlated with pre-challenge titres of anti-MSP-3 antibodies (Hisaeda, Saul et al. 2002).

In another study, six different MSP-3-adjuvant combinations were tested in a total of 15 *Saimiri scireus* monkeys. The best results were obtained in the immunization regimen with MSP-3212-380-AS02, where one of two monkeys completely controlled parasite growth, while the other showed a delay in the appearance of parasitaemia. This was contrast to the two control monkeys which showed fast rising parasitaemias that required treatment (Carvalho, Oliveira et al. 2004). In a follow-up to this study, *Saimiri scireus* were immunized with a hybrid GLURP/MSP-3 protein, with three different adjuvants with
disappointing results. All groups of immunized monkeys (5 monkeys per adjuvant group) required treatment, although this was delayed in some monkeys, in some groups. Overall, they were able to detect a statistically significant association between high antibody titres and partial protection and proposed that the induction of high antibody titres was the key to successful vaccines based on these antigens (Carvalho, Alves et al. 2005).

2.3.3.3 Population genetic analyses

The initial evidence that msp-3 was under selection was weak. In the study previously described by Escalante and colleagues, msp-3 was one of eight P. falciparum genes analyzed for evidence of positive selection. Similar to msp-2, although non-synonymous amino acid replacements significantly exceeded synonymous replacements, two additional tests failed to detect positive selection in msp-3. However, the value of the Tajima's D was close to significance (Escalante, Lal et al. 1998). Better but still relatively weak evidence for selection was subsequently obtained from later studies. MSP-3 has homologues in P. vivax (Galinski, Corredor-Medina et al. 1999), P. knowlesi (Hudson, Miller et al. 1983), P. reichenowi (Okenu, Thomas et al. 2000) and P. cynomolgi (Galinski, Ingravallo et al. 2001). In a study that analyzed five alleles of msp-3 from P. falciparum and that of its most closely related species, P. reichenowi, the ratio synonymous to non-synonymous (dN/dS) amino acid replacements was found to differ between the species, though the difference was not statistically significant. This would indicate that selection was acting to maintain alleles within one species relative to the other. This analysis was complemented with other tests for selection, the D index of Tajima and Fu and Li, both of which were positive,
suggesting balancing selection, but not reaching significance (Okenu, Thomas et al. 2000). The best evidence comes from large molecular population studies in which 100 msp-3 alleles were sampled from Nigeria and Thailand. Tajima's $D$ was significantly positive in both populations suggesting that balancing selection was acting to maintain alleles. These findings were supported by immunological assays showing that antibodies to both alleles of MSP-3 were associated with a reduced risk of clinical episodes of malaria (Polley, Tetteh et al. 2007).

2.3.3.4 Immuno-epidemiological studies

2.3.3.4.1 Variation in MSP-3 antigens

Compared to the antigens discussed up to this point, far fewer immuno-epidemiological studies have been conducted for MSP-3. In general, two types of studies can be identified in the literature; those that analyze antibodies against long or short synthetic peptides of MSP-3 (MSP-3b, discussed above), or those that have assayed antibodies to *E. coli*-expressed full-length MSP-3, or a C-terminal fragment of MSP-3 that was similarly expressed in *E. coli*. A few exceptions to this include studies that have in addition to MSP-3b, analyzed antibodies to overlapping fragments of the conserved region of MSP-3 (MSP-3a to f) (Singh, Soe et al. 2004). Reported antibodies to MSP-3b include those that have been tested against a long synthetic peptide MSP-3b$_{154-249}$ (Meraldi, Nebie et al. 2004), and a shorter peptide MSP-3b$_{184-210}$ (Singh, Soe et al. 2004; Soe, Theisen et al. 2004; Roussilhon, Oeuvray et al. 2007). Antibodies have also been measured against synthetic MSP-3 peptides that span slightly different but overlapping epitopes around MSP-3b, such as MSP-
3181-276 (Nebie, Diarra et al. 2008; Nebie, Tiono et al. 2008). Full-length MSP-3 antigens (3D7 and K1) are alternative versions of MSP-3 (Polley, Tetteh et al. 2007), which represent the dimorphism observed at this locus in world-wide isolates (Huber, Felger et al. 1997). Immunization of mice with full-length MSP-3 induced predominantly type-specific antibodies, which are also common in humans naturally exposed to malaria (Polley, Tetteh et al. 2007). The E. coli-expressed conserved C-terminal fragment described by Polley et al. (Polley, Tetteh et al. 2007) does not share epitopes with MSP-3b (Osier, Polley et al. 2007).

2.3.3.4.2 Prevalence of anti-MSP-3 antibodies

The prevalence of total IgG against MSP-3 antibodies from cross-sectional surveys conducted in malaria-endemic areas ranges from as low as 21.3% at the beginning of a malaria transmission season, in a region of low malaria-endemicity in Burkina Faso (Nebie, Tiono et al. 2008), to as high as 97.2%, in a highly endemic area of Senegal (Roussilhon, Oeuvery et al. 2007). Part of this variation in prevalence is undoubtedly accounted for by differences in the specific MSP-3 antigen being tested, as has been described. Additional factors include: the intensity of malaria transmission, with prevalence not surprisingly being higher in areas with higher, as opposed to lower malaria transmission (Nebie, Tiono et al. 2008); the age of the study participants, with prevalence being higher in older children and adults, compared to children (Meraldi, Nebie et al. 2004; Osier, Polley et al. 2007); the timing of sampling, with prevalence being higher at the end, compared to the start, of a malaria transmission season in an area of low malaria transmission, but
remaining stable over the two time points, in an area with higher malaria transmission (Nebie, Tiono et al. 2008).

In studies using E.coli-expressed MSP-3 antigens, prevalence was higher against full-length antigens (including polymorphic and conserved epitopes), compared to the conserved C-terminal fragment; and against allele-specific epitopes (determined by competition ELISA), compared to conserved epitopes (Osier, Polley et al. 2007; Polley, Tetteh et al. 2007), and in individuals who were parasite-positive, compared to those who were parasite-negative at the time of serum sampling (Osier, Polley et al. 2007). Antibodies to both the synthetic peptides and E. coli–expressed full length antigens are predominantly of the cytophilic IgG sub-classes (IgG1 and IgG3) (Meraldi, Nebie et al. 2004; Osier, Polley et al. 2007).

2.3.3.4.3 Associations of anti-MSP-3 antibodies with protection
Antibodies to both MSP-3 peptides and full-length recombinant antigens have been associated with protection to malaria in five out of six studies where it has been tested. In studies conducted in Myanmar, South East Asia (Soe, Theisen et al. 2004), Burkina Faso (Meraldi, Nebie et al. 2004), (Nebie, Tiono et al. 2008) and Senegal (Singh, Soe et al. 2004), antibodies to MSP-3, particularly those of the IgG3 sub-class were associated with reduced clinical episodes of malaria. In perhaps the longest and most detailed follow up for clinical episodes that has been conducted to date (six consecutive years), IgG3 antibodies to MSP-3b were strongly associated with protection from clinical malaria (Roussilhon, Oeuvey et al. 2007). However, in a separate longitudinal study conducted in Senegal, antibodies to
MSP-3_{181-276} (similar to MSP-3b) were not associated with a lower risk of clinical episodes (Nebie, Diarra et al. 2008). On the other hand, antibodies to full length MSP-3 have been associated with protection from clinical disease in all the studies reported to date (Osier, Polley et al. 2007; Polley, Tetteh et al. 2007; Osier, Fegan et al. 2008), although in one study protection was only observed with allele-specific responses to K1-, and not 3D7-MSP-3 (Osier, Polley et al. 2007). Sero-positivity to the C-terminal fragment of MSP-3 was associated with protection in the Gambian (Polley, Tetteh et al. 2007), but not the Kenyan study (Osier, Polley et al. 2007).

2.3.4 VACCINE TRIALS IN HUMANS

The long synthetic peptide of MSP-3, MSP-3b, has entered the phase I trial stages of vaccine development. When tested in a malaria vaccine trial in Swiss volunteers, it was found to be safe and immunogenic, although unacceptably reactogenic when combined with a Montanide adjuvant (Audran, Cachat et al. 2005). In a publication that unusually preceded that of the actual phase I trial by Audran et al., vaccine-induced antibodies to MSP-3b were shown to inhibit *P. falciparum* erythrocytic growth in a monocyte-dependent manner. This inhibition was in the majority of cases as high, or higher than that observed in hyper-immune sera from West Africa, and was still present 12 months after vaccination (Druilhe, Spertini et al. 2005). A phase Ib trial of the same antigen has since been conducted in adult male volunteers in Burkina Faso, where the vaccine was well tolerated (Sirima, Nebie et al. 2007). Although humoral responses to MSP-3 were comparable in both the vaccine and placebo groups, cellular responses appeared to increase significantly compared to the controls, following the second vaccine dose of MSP-3 (Sirima, Nebie et al. 2007).
2.4 AMA1

2.4.1 ANTIGEN DISCOVERY

AMA1 was first described in *P. knowlesi*, in experiments designed to identify protective antigens from blood-stage parasites (Deans, Alderson et al. 1982). Monoclonal antibodies specific for *P. knowlesi* were raised by fusion of rat myeloma cells with spleen cells of rats immunized with the W1 parasite strain. Of the 28 mAbs raised in this fashion, only two inhibited parasite growth when fully purified from ascetic fluid or bulk hybridoma culture supernatants (both of which had undefined factors inhibitory to parasite growth). Both of these mAbs bound to a 66kDa polypeptide, which appeared to be a minor parasite component, as it was not readily detectable on SDS page gels of total parasite antigen preparations (Deans, Alderson et al. 1982). The name AMA1 was actually coined by Peterson *et al.*, when they reported a 'novel' blood stage antigen of *P. falciparum*, that had the characteristics of an integral membrane protein, and was localized to the apical complex (Peterson, Marshall et al. 1989). It appears that Peterson and her colleagues were not aware at the time, that this 'novel' antigen was the analogue of that previously described by Deans *et al.* (Deans, Alderson et al. 1982), as no mention of the latter is made in their report (Peterson, Marshall et al. 1989). Interestingly, a separate research group had also identified *P. falciparum* AMA1 (an 83kDa protein) and presented their findings at the '3rd International Congress on Malaria and Babesiosis' in 1987, but did not publish their results (Thomas, Deans, Waters, Chulay, reported by Narum and colleagues (Narum and Thomas 1994)). In any case, sequence data subsequently revealed that *ama1* was conserved across at least three Plasmodial species, *vivax*, *falciparum* and *knowlesi* (Waters, Thomas et al. 1990). Other studies have since described homologues of *ama1* from *P. yoelii*.

2.4.2 ANTIGEN LOCATION AND PROCESSING

Early studies indicated that as was the case for *P. knowlesi*, AMA1 in *P. falciparum* was expressed in late-stage schizonts, with at least seven or eight nuclei. The 83kDa protein was then proteolytically cleaved to a 66kDa molecule, and both of these were initially localized within the merozoite apex before merozoite release. Following schizont rupture, while the 83kDa protein remained apically restricted, the 66kDa processed form spread all over the surface of the merozoite (Deans, Thomas et al. 1984; Narum and Thomas 1994). These early findings have since been confirmed and extended in several studies (Howell, Withers-Martinez et al. 2001; Healer, Crawford et al. 2002; Bannister, Hopkins et al. 2003; Howell, Well et al. 2003; Howell, Hackett et al. 2005). In particular, AMA1 was definitively located in the micronemes (Healer, Crawford et al. 2002; Bannister, Hopkins et al. 2003). The current understanding of the synthesis and processing of AMA1 is illustrated in Figure 2.4.1. AMA1 is synthesized as an 83kDa precursor protein with a signal peptide (A) that allows transport through the endoplasmic reticulum, from where it traffics to the micronemes (Healer, Crawford et al. 2002). The micronemes are translocated from a single Golgi-like cistern near the nucleus of the merozoite, to its apex, and dock close to the rhoptry tips (Bannister, Hopkins et al. 2003). The N-terminal pro-region sequence is cleaved in the micronemes (B), and the mature 66kDa peptide is translocated out of the micronemes (C), via the neck of the rhoptry to the merozoite surface before further
proteolytic processing (Healer, Crawford et al. 2002). On the merozoite surface, the 66kDa protein is cleaved (D) into two soluble fragments of 44 and 48kDa, that are released, leaving behind a ‘stub’ (Howell, Wel1 et al. 2003), which can be detected in young ring stage parasites (Howell, Hackett et al. 2005).

![Figure 2.4.1 AMA1 synthesis, translocation and processing](image)

The letters A-D indicate sites of proteolytic cleavage (Taken from Healer 2002).

### 2.4.3 Antigen Structure

The arrangement of disulphide bonds in AMA1 suggest that the ectodomain is comprised of three sub-domains (Hodder, Crewther et al. 1996), which are commonly referred to as domain I, II and III. These disulphide bonds define conformational epitopes that are essential for inducing protective immune responses (Crewther, Matthew et al. 1996; Anders, Crewther et al. 1998). The crystal structure of AMA1 for *P. vivax* has been
resolved, and domains I and II were found to contain a PAN-like motif, which defines a superfamily of protein folds that are associated with receptor binding functions (Pizarro, Vulliez-Le Normand et al. 2005). Detailed NMR spectroscopy studies have also been conducted separately for DII (Feng, Keizer et al. 2005) and DIII (Nair, Hinds et al. 2002) of *P. falciparum*, and while the details of such studies are beyond the scope of this review, it is apparent that in general, the disulphide bonds are not only key structural components of the individual domains, but also stabilize the entire ectodomain. At the sequence level, although *ama1* is highly polymorphic, it does not contain repeat sequences commonly found in other merozoite proteins. Diversity between alleles of *ama1* is characterized by numerous single point mutations scattered throughout the ectodomain, with the majority of polymorphic sites falling within domain I. In a population sample of 51 *ama1* alleles from Nigeria, although polymorphic sites were found across the entire ectodomain, 38 of them were located within domain I, while domains II and III contained only 9 polymorphic sites each (Polley and Conway 2001). An illustration of the structure of AMA1 is shown in Figure 2.4.2.
Figure 2.4.2 The ectodomain of PfAMA1

Schematic of the ectodomain of PfAMA1, showing the three separate domains I, II and III.

The location of eight di-sulphide bridges found within the molecule is shown in blue.

Residues in red represent the mutations occurring in 11 P. falciparum isolates. N and C indicate the N- and C-termini. Figure taken from Nair 2002
Although the precise function of AMA1 is unknown, several lines of evidence suggest that it plays an important role in invasion of erythrocytes and hepatocytes, by merozoites and sporozoites respectively. Attempts to disrupt the gene using 'knock-out' plasmids have been unsuccessful, suggesting that it is essential to parasite survival (Triglia, Healer et al. 2000). However, it is possible to complement the function of \textit{P. falciparum} \textit{ama1} by targeting the gene via homologous recombination with the divergent transgene from \textit{P. chabaudi}. Expression of this transgene in \textit{P. falciparum} led to more efficient invasion of murine erythrocytes, supporting a role in invasion (Triglia, Healer et al. 2000). In an extension of this approach, Healer and colleagues generated a panel of chimeric \textit{P. falciparum}/\textit{P. chabaudi} transfection constructs, where they substituted different sub-domains of \textit{ama1} across the two species in an attempt to identify those that were critical for erythrocyte invasion (Healer, Triglia et al. 2005). In invasion-inhibition assays using the parasites expressing chimeric AMA1 proteins, they demonstrated that chimeras from each domain were able to complement the function of PfAMA1 (Healer, Triglia et al. 2005).

Invasion of red blood cells involves several steps including, primary recognition, followed by reorientation, and the formation of a tight junction which moves from the apical, to the posterior pole of the merozoite, the shedding of the merozoite coat and finally invasion, via the formation of a parasitophorous vacuole (Cowman and Crabb 2006). AMA1 is thought to be required at the re-orientation step, after the initial attachment of the merozoite to the erythrocyte (Mitchell, Thomas et al. 2004). This was demonstrated by incubating \textit{P.}
knowlesi merozoites with red cells in the presence of a rat mAb raised against a known invasion-inhibitory epitope of PkAMA1, and fixing the material for ultra-structural analysis. In striking results, they found that in comparison to control cultures lacking inhibitory mAb or containing a non-inhibitory antibody, these merozoites bound to red cells normally, but failed to re-orientate, and did not invade (Mitchell, Thomas et al. 2004).

Other studies also lend support to the role of AMA1 in erythrocyte invasion. Fraser et al. expressed a range of P. yoelii AMA1 domains singly, and in varying combinations, in COS-7 cells, and tested these for binding to erythrocytes. They found that domains I and II, in combination, gave the best results in erythrocyte binding and mediated adhesion to mouse and rat erythrocytes, but not to human erythrocytes (Fraser, Kappe et al. 2001). In a similar approach, Kato and colleagues expressed domains of P. falciparum AMA1 on the surface of CHO-K1 cells and demonstrated that domain III bound to the red cell membrane protein Kx on human erythrocytes, but only following treatment with trypsin (Kato, Mayer et al. 2005). In a different approach, Urquizo et al., tested short synthetic peptides spanning the entire ectodomain of P. falciparum AMA1 for binding to human erythrocytes, and found that 8 of 31 peptides, scattered throughout the ectodomain, bound with high affinity and inhibited erythrocyte invasion (Urquiza, Suarez et al. 2000). Recent studies indicate that AMA1 is also expressed in sporozoite stages and antibodies to AMA1 inhibit sporozoite invasion of hepatocytes (Silvie, Franetich et al. 2004).
2.4.5 Supportive evidence that antibodies play a role in protection against malaria

2.4.5.1 Antibody-mediated invasion-inhibition in vitro

2.4.5.1.1 Anti-AMA1 monoclonal antibodies

In several species of malaria, a large number of mAbs to AMA1 have been identified which inhibit erythrocyte invasion, and these are presumably targeted to diverse epitopes within the molecule. Indeed, as was previously discussed, AMA1 was discovered by characterizing the targets of two rat mAbs that inhibited erythrocyte invasion of *P. knowlesi* merozoites (Deans, Alderson et al. 1982). Similar mAbs have been described for AMA1 in other plasmodial species, including *P. falciparum* (Coley, Campanale et al. 2001; Coley, Parisi et al. 2006). Invasion-inhibition has been performed with intact mAbs, as well as monovalent Fab fragments (Fab) and divalent F(ab')2 fragments. Early studies with *P. knowlesi* showed that these fragments enhanced invasion-inhibition, when compared to intact IgG (Thomas, Deans et al. 1984). These studies indicated that invasion inhibition could not be explained simply by that fact that antibodies caused merozoite agglutination, and suggested that merozoite binding to red cells was blocked via specific receptors (Thomas, Deans et al. 1984). Similar findings have been demonstrated more recently for *P. falciparum*, where purified Fab fragments of the mAb 4G2 were more efficient at invasion-inhibition, compared to the intact mAb (Collins, Withers-Martinez et al. 2007).
2.4.5.1.2 Mapping of epitopes targeted by anti-AMA1 invasion inhibitory mAbs

Several approaches have been employed to map the precise locations of the mAbs that inhibit merozoite invasion, in attempts to better understand their strain-specificity, and thus identify residues that are critical for this function. In addition, such studies help to understand the structural requirements within AMA1 that are necessary for high affinity binding to mAbs. One technique entails the use of phage display of antigen fragments to identify the location of the epitopes, followed by the use of random peptide libraries displayed on the phage to accurately identify the amino acids actually involved in the epitope. Epitopes thus identified are subsequently verified using phage display of mutant fragments, thus confirming the role of each residue in the epitope. This technique was used to show that mAb 5G8 bound to a short linear epitope within the pro-domain of *P. falciparum* AMA1, while the epitope for mAb IF9 was located within domain I (Coley, Campanale et al. 2001). The better studied mAb 4G2, binds to epitopes within domain II of AMA1 (Pizarro, 2005, Collins 2007). In more detailed studies, site-directed mutagenesis was used to define the precise single polymorphic residue which defined the strain-specificity observed in invasion-inhibition experiments with the mAb IF9 (Coley, Parisi et al. 2006). The same approach (site-directed mutagenesis) has also been used to show conclusively, that another mAb, 4G2, bound exclusively to epitopes with domain II (Collins 2007). In Figure 2.4.3 below, the locations of two well-studies mAbs, 4G2 and IF9, are shown in a three-dimensional structure.
Figure 2.4.3  X-ray structure of AMA1 domains I and II of *P. falciparum* (3D7)

The location of residue 197 (dark blue), which is critical for the binding of mAb IF9 is indicated. In light blue is the 57-residue fragment of domain I that reacts with the mAb IF9. In red are the residues required for the binding of mAb 4G2. In yellow, is a hydrophobic cleft that appears to be flanked by epitopes for both mAbs, 4G2 and IF9, supporting the idea that this is an important site for receptor–ligand interactions. Figure taken from Coley 2006.
2.4.5.1.3 Proposed mechanisms for antibody mediated invasion-inhibition

Several plausible mechanisms have been proposed to explain how antibodies, in general, mediate erythrocyte invasion by merozoites, but not all have been demonstrated specifically for anti-AMA1 antibodies. One of these is merozoite agglutination, and as previously discussed, early studies showed that this was unlikely to be the only mechanism since single Fab fragments from anti-AMA1 antibodies could inhibit invasion as well as, or more effectively than intact IgG (Thomas, Deans et al. 1984). Antibodies may also work through the direct blocking of specific receptors required for invasion both for the parasite and the host erythrocytes. This has not been demonstrated directly for any parasite antigen, including AMA1, to-date. However, anti-AMA1 antibodies have been shown to disrupt the proteolytic processing and redistribution of AMA1 on the merozoite surface. When used at high concentrations, anti-AMA1 antibodies inhibited invasion and this was accompanied by a concurrent decrease in the 48 and 44kDa products, suggesting that proteolytic cleavage had been interrupted (Dutta, Haynes et al. 2005). Furthermore, soluble AMA1 fragments were cross-linked by bivalent IgG, thereby inhibiting its circum-merozoite redistribution and shedding (Dutta, Haynes et al. 2005).

2.4.5.1.4 Small peptides (mimotopes) that bind to AMA1 and thereby inhibit invasion

Random phage display peptide libraries have been used to identify peptides (mimotopes) that bind to AMA1, and thus inhibit merozoite invasion of host erythrocytes. In these studies it is proposed that a better understanding of the molecular interactions between AMA1 and peptides thus identified, and their structures, will aid in the development of
novel malaria vaccines. Several such peptides have been described, such as R1 (Harris, Casey et al. 2005), as well as F1 and F2 (Li, Dluzewski et al. 2002). These peptides can then be used as immunogens that mimic functionally important epitopes. In one study, a mAb (4G2dc1) was used to screen a phage display library of >10^8 individual peptides. The three most reactive peptides identified were then used to immunize rabbits, and elicited antibodies that not only recognized the peptide immunogen, but also bound to recombinant and native AMA1 (Casey, Coley et al. 2004). Both human and rabbit antibodies specific for two of three peptides just mentioned were able to inhibit merozoite invasion of *P. falciparum* erythrocytes (Casey, Coley et al. 2004). In subsequent studies, the structures of these peptides have been determined using NMR spectroscopy, and analogues designed that bind to an AMA1 invasion-inhibitory mAb (4G2), with greater affinity (Sabo, Keizer et al. 2007).

2.4.5.2 Vaccination in animal models of malaria and allele-specific anti-AMA1 antibody responses

Immunization with purified native AMA1, or correctly folded recombinant AMA1, or even the passive transfer of anti-AMA1 antibodies, has been shown to confer complete or partial protection against challenge with malaria parasites, particularly of the homologous strain, in many studies (Deans, Knight et al. 1988; Collins, Pye et al. 1994; Crewther, Matthew et al. 1996; Anders, Crewther et al. 1998; Narum, Ogun et al. 2000; Stowers, Kennedy et al. 2002). However, it is well established both from *in vitro* assays (e.g., invasion inhibition) and *in vivo* experiments (challenge following vaccination, or passive transfer of anti-AMA1 antibodies) that vaccine-induced anti-AMA1 immunity is 'strain'-specific, with significantly
better invasion-inhibition, or clinical protection, observed with homologous, as opposed to heterologous parasites (Crewther, Matthew et al. 1996; Hodder, Crewther et al. 2001; Kennedy, Wang et al. 2002; Kocken, Withers-Martinez et al. 2002). *In vitro*, this is best detected using functional assays, as simple recognition of parasites containing allelic variants of AMA1 (for example by immuno-fluorescence) may appear similar, while a clear difference between the two antibodies becomes apparent when invasion-inhibition assays are performed (Kocken, Withers-Martinez et al. 2002). However, the breadth of antibody specificity can be increased, without compromising the efficacy of the antibodies by vaccination with more than one allelic variant (Kennedy, Wang et al. 2002).

More direct evidence that sequence differences between AMA1 alleles have important functional consequences has been obtained in studies using transgenic parasites (Healer, Murphy et al. 2004). Here two parasite lines (3D7 and W2mef) were selected which differed in their susceptibility to the invasion-inhibitory activity of polyclonal anti-AMA1 antibodies. The sequence of *ama1* in these two parasite 'strains' also differs, particularly in domain I. Transgenic parasites were constructed that expressed the heterologous AMA1 proteins separately, or a chimera of the two AMA1 proteins. They then compared invasion-inhibition in parasites expressing homologous versus AMA1 proteins, or the chimera and demonstrated clearly that sequence differences in AMA1 resulted in differential invasion-inhibition, lending support to the idea that polymorphisms are selected by variations in the protective immune response (Healer, Murphy et al. 2004).
2.4.5.3 Population genetic analyses

Different approaches have been used to support the idea that polymorphisms within AMA1 arise as a result of immune pressure, and that this is a strategy employed by the parasite to evade protective immune responses. While the strengths and weaknesses of these different approaches are debatable, there is little doubt from all the studies that *ama1* is under positive natural selection. In an early study, Escalante *et al.* compared the polymorphisms within 10 loci of potential *P. falciparum* vaccine candidates, to determine whether or not they were under positive selection (Escalante, Lal *et al.* 1998). From their analyses, *ama1* was one of four genes for which there was clear evidence of positive selection (Escalante, Lal *et al.* 1998). However, this was based only on the excess of non-synonymous over synonymous amino acid replacements, with no evidence from the Tajima's *D* and McDonald-Kreitman test as previously discussed. In a different approach, Verra *et al.* compared the ratio of synonymous to non-synonymous mutations in immunogenic (pre-defined T-cell epitopes) versus non-immunogenic regions of *ama1* and concluded that there was evidence of positive selection favoring genetic diversity within the T-cell epitopes (Verra and Hughes 1999).

In a separate study polymorphisms within *ama1* were compared between *P. reichenowi* and 12 laboratory isolates of *P. falciparum*, and significant evidence for selection-maintaining polymorphisms within the *P. falciparum* alleles was detected using the McDonald-Kreitman test (Kocken, Narum *et al.* 2000). Alleles of *ama1* have also been sampled from several large population studies from all over the world including, Nigeria
(51 alleles) (Polley and Conway 2001), Thailand (50 alleles) (Polley, Chokejindachai et al. 2003), Papua New Guinea (168 alleles) (Cortes, Mellombo et al. 2003), and India (157 alleles) (Garg, Alam et al. 2007), and though not all studies analyzed the entire ectodomain, it is clear overall that there is strong evidence of balancing selection maintaining polymorphisms, particularly within domains I and III.

Two other studies have analyzed \textit{ama1} alleles from smaller population samples (Escalante, Grebert et al. 2001; Rajesh, Singamsetti et al. 2008). In the study by Escalante \textit{et al.}, diversity in \textit{ama1} was 20-30\% higher in the alleles from Kenya (n=12), compared to those from SE Asia (n=10), and Venezuela (n=10) (Escalante, Grebert et al. 2001). Unlike all the other studies discussed so far, in a study of 13 \textit{ama1} alleles from India, Rajesh and colleagues found the highest degree of polymorphism within domain II, although the signature of selection was evident throughout the gene (Rajesh, Singamsetti et al. 2008).

\textbf{2.4.5.4 Immuno-epidemiological studies}

\textbf{2.4.5.4.1 Overview}

Antibodies to AMA1 in malaria-endemic regions have been studied using a variety of AMA1 antigens, including the full ectodomain, single sub-domains, combinations of domains I and II, I and III, or II and III, and a range of different mer synthetic peptides from various regions of the gene. The full-length and sub-domain constructs have been expressed in a range of systems including \textit{E. coli}, \textit{P. pastoris} and Baculovirus, using the sequence of AMA1
from different parasite lines, including 3D7, FVO, HB3, 7G8, and D10. These differences will be highlighted in the discussion on antibody prevalence below.

2.4.5.4.2 Prevalence of antibodies to full-length AMA1 antigens

Considering initially data from adequately large cross-sectional studies that were representative of the malaria-endemic population in which they were carried out, the prevalence of antibodies to full-length AMA1 (entire ectodomain) ranges between 67% and 100% (Thomas, Trape et al. 1994; Johnson, Leke et al. 2004; Polley, Mwangi et al. 2004; Cortes, Mellombo et al. 2005). In these four studies, antibodies were analyzed for full-length AMA1 expressed in baculovirus, based on the 7G8 parasite 'strain' (Thomas, Trape et al. 1994; Johnson, Leke et al. 2004), or in E. coli, based on the 3D7 strain (Polley, Mwangi et al. 2004; Cortes, Mellombo et al. 2005) or the HB3 and D10 parasite strains (Cortes, Mellombo et al. 2005), or in P. pastoris, based on the FVO strain (Polley, Mwangi et al. 2004). In the Kenyan study, a tight concordance was found between antibodies to AMA1 based on the FVO sequence and expressed in P. pastoris, and that based on the 3D7 strain and expressed in E. coli, suggesting cross-reactivity between antigens from the two 'strains' (Polley, Mwangi et al. 2004). Similarly, in the study from Papua New Guinea, very high correlation coefficients (>0.90) were found between antibodies to AMA1 based on three different allelic forms (HB3, D10 and 3D7), all expressed in E. coli (Cortes, Mellombo et al. 2005). Antibody levels increased with age in all four studies, except that by Thomas et al. (Thomas, Trape et al. 1994) in a Senegalese population, where no age relationship was observed. Cortes et al. suggest this lack of an age-dependence of antibodies may be explained at least in part, by differences in the antigen, which was highly glycosylated.
compared to native AMA1, or, differences in immuno-assay methodologies (capture versus direct ELISA, as performed in more recent studies) (Cortes, Mellombo et al. 2005). In the Cameroonian study, although the prevalence of anti-AMA1 antibodies was 100% (children under the age of 5 were not included in the study, n = 200), antibody levels were still observed to rise with age (Johnson, Leke et al. 2004). Antibodies were also more common in individuals that were parasitaemic at the time of serum sampling, compared to those who were not (Polley, Mwangi et al. 2004), and acquired earlier in an area of higher malaria transmission compared to one with lower transmission (Polley, Mwangi et al. 2004) and again more common in individuals with the HLA DRB1*1201 haplotype compared to all other haplotypes in Cameroon (Johnson, Leke et al. 2004). As is the case for several merozoite antigens, antibodies to full-length AMA1 appear to be short-lived in children (Kinyanjui, Conway et al. 2007).

Two studies have analyzed antibodies to AMA1 in cord blood from newborns, or the early neonatal period (Riley, Wagner et al. 2000; Metenou, Suguitan et al. 2007). In one study cord blood mononuclear cells from 120 Cameroonian infants were cultured and the supernatants tested for IgG antibodies against full-length AMA1, and were positive in 58% of samples, indicating that fetal lymphocytes had been primed in utero (Metenou, Suguitan et al. 2007). In the other study, the majority (>80%) of paired maternal and neonatal samples (collected at birth, n=143), contained antibodies to full-length AMA1, with antibody levels being highly correlated between the pairs, presumably due to placental transfer of maternal antibodies (Riley, Wagner et al. 2000). Amongst individuals presenting to hospital with malaria, the prevalence of antibodies to AMA1 was significantly
lower in children in severe malarial anaemia (42.9%, n = 59), compared to those with cerebral malaria (71.8%, n=126), or those with uncomplicated malaria (52.5%, n=84, difference not significant), although these finding were not unique to AMA1 (Dobano, Rogerson et al. 2008). In a study of >250 pregnant women in Malawi, the concentration of antibodies to full-length AMA1 was significantly lower among HIV-positive women, compared to those that were HIV-negative, but did not correlate with viral load or CD4 positive T cell counts, making it difficult to interpret the results (Mount, Mwapasa et al. 2004).

2.4.5.4.3 Prevalence of antibodies to sub-domains and peptides of AMA1

In general, naturally acquired antibodies appear to be more commonly directed against epitopes found within domain I, than those within either of the remaining two sub-domains. This conclusion is drawn from the fact that the antibody reactivity against the full ectodomain is much higher than that against either domain II, or domain III (Cortes, Mellombo et al. 2005). When antibodies against the combination of domains II and III were compared with those against the entire-ectodomain, it was also apparent that a considerable amount of reactivity was attributable to epitopes within domain I (Polley, Mwangi et al. 2004). Difficulties in expressing domain I, singly, have precluded the examination of antibodies to this individual domain in more direct comparison (Cortes, Mellombo et al. 2005). Although it may be expected that reactivity to domain I would be highest, simply because it is larger than the other two domains and therefore contains more epitopes, it is clear that as AMA1 is a conformation-dependent antigen, all domains of the molecule contribute significantly to the detected antibody reactivity (Lalitha, Ware et
al. 2004). In one study the highest prevalence reported for any single peptide (linear B cell epitope), out of a panel of peptides spread across the entire ectodomain of AMA1, was between 40-50% (antibodies to PL162 from domain I were analyzed at three time points, 3-4 months apart) (Udhayakumar, Kariuki et al. 2001). In a separate study, antibodies to this same peptide of AMA1 (PL162) were present in nearly 100% of individuals' tested from an urban region of Orissa, India (Biswas, Seth et al. 2008). The findings from this latter study do not seem correct as antibody responses to a panel of peptides from different blood stage antigens were significantly more common in individuals from the urban area which appears to have a lower intensity of malaria transmission, compared to the forest area where the malaria transmission intensity was higher (Biswas, Seth et al. 2008).

2.4.5.4.4 IgG isotypes of anti-AMA1 antibodies

Naturally acquired antibodies to AMA1 are predominantly of the cytophilic IgG isotypes, IgG1 and IgG3, with IgG1 antibodies being the most common. Polley et al. tested random samples (n=96) that were positive for total IgG to full-length AMA1, out of a cohort comprising both adults and children and found a predominantly IgG1 response (Polley, Mwangi et al. 2004). Similarly, culture supernatants of cord blood mononuclear cells that were positive for IgG to full-length AMA1 were found to contain predominantly IgG1, with a handful of samples (6%) being positive for IgG3, and none positive for IgG2 (Metenou, Suguitan et al. 2007). It is not clear from this study whether IgG4 antibodies were analyzed or not as no mention of it is made (Metenou, Suguitan et al. 2007). In the plasma of newborns, the prevalence of IgG1 and IgG3 antibodies to full-length AMA1 was comparable (22 and 18% respectively), followed by Ig4 (14%) and IgG2 (4%) (Riley, Wagner et al.
However, when IgG2 and IgG4 antibodies to AMA1 were detected, the levels were very low, with OD values just above cut-offs defined using non-malaria exposed sera (Riley, Wagner et al. 2000). In children aged between 6 months and fifteen years in Burkina Faso, anti-AMA1 antibodies were predominantly of the cytophilic classes, and though the exact prevalence of either IgG1 or IgG3 was not reported, it appears from the figures presented that the levels of antibody were higher for the IgG1 isotype compared to IgG3 (Nebie, Diarra et al. 2008).

2.4.5.4.5 Allele-specificity of anti-AMA1 antibodies

Although a high correlation is reported between naturally acquired antibodies to different allelic forms of AMA1, a proportion of these antibodies are directed against allele-specific epitopes. Using competition ELISAs in a sub-set of samples (n=18), Polley et al. demonstrated that the majority of sera (15/18) contained allele-specific antibodies (Polley, Mwangi et al. 2004). In a separate study using inhibition assays, Cortes et al. found that although most individuals had equivalent titres of antibodies to all three allelic forms of AMA1 tested, the majority nevertheless contained a fraction of antibodies that were directed against allele-specific epitopes (Cortes, Mellombo et al. 2005). In addition, a minority of individuals (19/262) had marked differences in binding to the different AMA1 allelic antigens, indicating allele-specificity, and the prevalence these antibodies decreased with age (Cortes, Mellombo et al. 2005).
2.4.5.4.6 Association of anti-AMA1 antibodies with protection in longitudinal studies

Naturally-acquired IgG antibodies to full-length AMA1 have been associated with protection from clinical episodes of malaria in some (Polley, Mwangi et al. 2004; Gray, Corran et al. 2007; Osier, Fegan et al. 2008), but not all (Roussilhon, Oeuvray et al. 2007; Nebie, Diarra et al. 2008), longitudinal studies. In Kenya antibodies were analyzed from study participants from two separate villages that differ in malaria transmission intensity, and were only associated with protection among individuals who were parasitaemic at the time of serum sampling, in both villages (Polley, Mwangi et al. 2004). Notably, this protection (reduced clinical episodes during a 6 month period of observation) was evident with either of the two allelic versions of AMA1 (3D7 and FVO) tested (Polley, Mwangi et al. 2004).

In a separate Kenyan study conducted amongst children aged between 1 and 5 years, high titre antibodies were associated with a lower risk of being admitted to hospital with malaria over an eight month period (Osier, Fegan et al. 2008). In this Kenyan study (Osier, Fegan et al. 2008), and in a separate study from the Gambia (Gray, Corran et al. 2007), antibodies to AMA1, in combination with those to other merozoite antigens (MSP-2 and MSP-3), were more predictive of protection from clinical episodes of malaria. However, in a study from Burkina Faso conducted in children between 6 months and fifteen years, IgG antibodies to full-length AMA1 were not associated with a reduced incidence of clinical malaria (Nebie, Diarra et al. 2008). When the IgG sub-class antibodies were analyzed, IgG1 to AMA1 was significantly associated with a reduced incidence of clinical malaria, though
the actual estimate was not remarkable (incidence rate ratio 0.87 (95% CI 0.78-0.97), \( p = 0.013 \)) (Nebie, Diarra et al. 2008). In the previously mentioned study where individuals were monitored daily for 6 years, neither total IgG antibodies to AMA1, nor the cytophilic to non-cytophilic ratio of IgG sub-class isotypes, were able to distinguish individuals who were protected from those who were susceptible to malaria attacks (Roussilhon, Oeuvray et al. 2007). Methodological differences between the studies, particularly in the statistical analyses may account for some of the differences in the results obtained. In a time-to-infection study among 68 Kenyan adults, high titre IgG antibodies to AMA1 on their own, or in combination with a panel of blood stage antigens, were not associated with a shorter time to infection following drug clearance (John, Moormann et al. 2005). Similarly, the presence of placentally transferred anti-AMA1 IgG in newborns was not associated with a lower risk of infection in the first twenty weeks of life (Riley, Wagner et al. 2000).

2.4.5.5 Vaccine trials in humans

At least a dozen phase I and II clinical trials in which at least five separate AMA1 vaccine constructs have been tested have been published to date. These vaccines are based either on the full-length sequence of one (Saul, Lawrence et al. 2005; Polhemus, Magill et al. 2007; Thera, Doumbo et al. 2008), or two (Malkin, Diemert et al. 2005), allelic forms of AMA1 or contain the conserved loop of domain III of AMA1, singly, or in combination with pre-erythrocytic stage antigens (Genton, Pluschke et al. 2007; Okitsu, Silvie et al. 2007; Thompson, Porter et al. 2008), and are delivered with a range of adjuvants, on a variety of platforms. Numerous attempts have been made to enhance the immunogenicity of AMA1 vaccines using new and old technologies and those will not be discussed here. However,
results from the vaccine trials with the AMA1 vaccine that has progressed the furthest along the vaccine development pipeline are discussed briefly, below.

The AMA1 vaccine named AMA1-C1/Alhydrogel contains an equal mixture of recombinant proteins based on sequences from the FVO and 3D7 'strains' of *P. falciparum*, expressed in *P. pastoris* and adsorbed onto the adjuvant Alhydrogel (Malkin, Diemert et al. 2005). This was the first AMA1 malaria vaccine to be tested in a malaria endemic area (Mali) in a dose-escalation study (Dicko, Diemert et al. 2007). Vaccination with AMA1-C1/Alhydrogel increased titres of pre-existing naturally acquired AMA1 antibodies in a dose-dependent fashion, but only for the first two doses (Dicko, Diemert et al. 2007). This was in contrast to what had been observed in malaria naïve volunteers, where a recall response was induced following the third dose of the same vaccine, albeit with a different immunization schedule (Malkin, Diemert et al. 2005). In Malian adults, although broad antibody specificity to diverse allelic forms of AMA1 was observed following vaccination, and indeed may have existed pre-vaccination, antibody titres did not correlate with parasite growth inhibition *in vivo* (Dicko, Diemert et al. 2007). Nevertheless, the vaccine was safe and immunogenic, and a phase I trial proceeded in Malian children (Dicko, Sagara et al. 2008). Based on the results from the adult study, Malian children were vaccinated with only two of the higher doses of AMA1-C1/Alhydrogel, in two instead of three doses (Dicko, Sagara et al. 2008). Vaccine-induced antibodies were short-lived, peaking at day 42 post-vaccination and declining to pre-vaccination levels by day 98 (Dicko, Sagara et al. 2008). Functional assays on vaccine-induced antibodies were not performed, or are yet to be published (Dicko, Sagara et al. 2008).
3 Objectives, Materials and Methods

3.1 Overall Aim

To determine whether naturally-acquired antibodies to a panel of polymorphic and conserved *P. falciparum* merozoite antigens are associated with protection from clinical episodes of malaria

3.1.1 Specific Aims

1. To determine whether there is an association between both allele specific and conserved antibody responses for Merozoite Surface Proteins-1, 2, 3 and Apical Membrane Antigen 1 and protection from clinical episodes of malaria

2. To test whether the magnitude of antibody responses and the breadth of antibody specificity are important determinants of protection

3. To compare antibodies between children developing mild or severe clinical episodes of malaria

4. In the subgroup of children who develop clinical episodes of malaria, to test whether pre-existing allele-specific antibodies protect against disease associated with parasites bearing homologous genotypes
5. To identify polymorphic sites within AMA1 under balancing selection and test whether these sites contain important B and/or T-cell epitopes.
3.2 MATERIALS AND METHODS

3.2.1 STUDY LOCATION AND POPULATION

The studies were all conducted in Kilifi, a rural district along the Kenyan coast (Figure 3.2.1). The district is sub-divided into administrative units called locations, and the study areas comprise locations both to the north, and to the south of the Kilifi creek. Kilifi town, and the Kilifi District Hospital, which serves a population of about 250,000 people, is located close to the creek. Three different sampling frameworks were used for these studies, which were based in locations both in the north and the south, and are shown in more detail in Figure 3.2.2 and Figure 3.2.3. Transmission intensity is lower in the northern part of the district with an average entomological inoculation rate (EIR) of approximately 1.5 - 8 bites/person/year (Mbogo, Snow et al. 1993), while it is higher south of the creek, with an EIR of 20 - 100 infective bites/person/year (Mbogo, Mwangangi et al. 2003). The population belongs to the 'Mjikenda' ethnic group and consists of predominantly small-scale subsistence farmers. The area typically experiences two seasonal peaks in malaria transmission (June to August, and, November to December). The majority of malaria infections are due to Plasmodium falciparum (Mwangi, Ross et al. 2003).
Figure 3.2.1 Map of Kenya showing the location of Kilifi district and the study areas.
Figure 3.2.2 Enumeration zones in the Northern study area of Kilifi

Children in the case-control study were recruited from locations throughout the Northern study area. Each number on the map represents a location.
Figure 3.2.3 Enumeration zones in the Southern study area of Kilifi

The areas shaded brown represent Chonyi while those shaded green represent Junju. Each number on the map represents a location.
3.2.1.1 Chonyi Cohort

Details of the study area and population are published (Mwangi, Ross et al. 2005), along with a description of a cohort comprising both adults and children from Chonyi village in Kilifi District. Chonyi village lies south of the creek and has an EIR of 20 – 100 infective bites/person/year (Mbogo, Mwangangi et al. 2003). Following a cross-sectional bleed at the start of a malaria transmission season in October 2000, the cohort was monitored for clinical episodes of malaria in the ensuing six months. Blood collected in October 2000 was used to prepare thick and thin blood films for malaria parasites, before separating plasma and red cell pellets for storage at -80°C. Participants with asymptomatic parasitaemias were not treated with radical anti-malarial drug therapy at the beginning of the study or during follow-up. Active detection of mild clinical malaria which was the outcome of the study was achieved through weekly visits to participants’ homes. A morbidity questionnaire which inquired about symptoms occurring in the preceding week was administered to all study participants and the presence or absence of fever (temperature > 37.5°C) was ascertained by axillary thermometer readings. Participants who were found to be unwell were referred for free treatment to a dedicated outpatient clinic at the local district hospital, where they also had open access as required at any time during the study (passive case detection). Clinical episodes of malaria were treated with sulphadoxine-pyrimethamine, as per the Kenyan national treatment guidelines at the time the study was conducted. Age-specific criteria for defining clinical episodes of malaria had been developed previously for this area as follows: children under one year, fever plus any parasitaemia; children older than one year, fever plus a parasitaemia of greater than 2500/μl (Mwangi, Ross et al. 2005). Participants were only included in the study if they
were present for at least twenty-three of the twenty-six weekly visits during the six months of follow-up. For analytical purposes, only the first clinical episode was counted, although all children continued to be monitored until the close of the study. Within the cohort, children aged ten years and less (n=280) accounted for nearly 90% of all the clinical episodes. Ethical approval was granted by the Kenya National Research Ethics Committee.

3.2.1.2 Case-control study

Children included in the case-control study were selected from a larger group of children, previously recruited into a study investigating antibodies to parasite antigens on the infected red cell surface (Bull, Lowe et al. 1998). Briefly, in May 1995 a large cross-sectional survey was conducted in a predefined area immediately surrounding Kilifi town, which had an EIR of approximately 1.5 - 8 bites/person/year (Mbogo, Snow et al. 1993). Details of the study were explained to participants and following informed consent, capillary blood samples were collected from 4783 children (aged 1-5 years old) at the start of a malaria transmission season. Serum was separated and stored (at -80°C) and microscopy was done on thick and thin blood films to detect parasites. Over the ensuing eight months of follow-up, children from this cohort who presented to Kilifi district hospital were identified (passive case detection, n = 165). Eighty-nine (89) of these children had malaria that was severe enough to require admission to the paediatric ward, while the remainder were attended to in OPD. Each case of either severe or mild malaria was randomly matched to an average of 3 controls, using a frequency based matching method that took into account age and location. The controls (n = 298) were drawn from children who took part in the
cross-sectional survey but did not present to hospital with malaria. Thus, the case-control study containing 463 individuals (89 severe malaria cases, 76 mild malaria cases and 298 healthy controls) was assembled retrospectively. Antibody assays were performed on pre-transmission season sera for all children in the case-control study (n=463). In a separate study of the antibody response of disease cases to clinical malaria additional sera that had been collected from the 165 children who presented to hospital at the time of the acute episode (acute sample), and three weeks following later (convalescent sample), were also analyzed. Parasite isolates from the acute clinical episode were frozen at the ring stages as previously described (Bull, Lowe et al. 1998). Ethical approval was granted by the Kenya National Research Ethics Committee. A summary of the study design is presented in Figure 3.2.4 below.
Figure 3.2.4 Schematic of the study design of the case-control study

* Some children (n=19) presented to hospital more than once during the follow-up period. Only data from the first episode is presented in subsequent analyses below.

3.2.1.3 Junju adults

A total of 26 semi-immune adults were recruited from Junju in December 2007, a sub-location close to Chonyi. These adults had not been recruited into the main Chonyi cohort, described above. Malaria transmission in the area had been declining steadily since the year 2000 when the original Chonyi cohort was recruited, and although the actual EIR was
not determined, parasite prevalence in a cross-sectional survey of 363 children conducted in November 2007 was as low as 17% (Kilifi immunology cohort, in Junju). Recruitment and consenting began in October - November 2007, following an explanation of the study. In December 2007, transport was provided to bring the study participants to the outpatients department of Kilifi District hospital, for sampling and treatment of any current illnesses. Fifteen mls of venous blood were drawn from each patient into 15 ml Falcon tubes containing heparin and immediately transferred to the laboratory for processing. Thick and thin blood smears were prepared to detect malaria parasites. A small volume of blood (0.5mls) was collected in EDTA containing tubes for the determination of haemoglobin levels. Microscopy was done on thick and thin blood films to detect parasites. In the laboratory, PBMCs were separated for immediate use in the ex-vivo ELISpot assay, and serum was stored at -80°C for antibody assays. Ethical approval was granted by the Kenya National Research Ethics Committee.

3.2.2 RECOMBINANT ANTIGENS

All the antigens are derived from sequences of specific loci within various 'strains' of *P. falciparum* and are denoted locus_'strain'. For example, the MSP-2 antigen, based on the sequence of the Dd2 parasite 'strain' is denoted MSP-2_Dd2. Most of the antigens were expressed in *Escherichia coli* as GST-fusion proteins for MSP-2 (MSP-2_Dd2 and MSP-2_CH150/9) (Taylor, Smith et al. 1995), for MSP-1 Block 2 (RO33, Palo Alto, 3D7, MAD20 and Wellcome) (Cavanagh and McBride 1997), and for MSP-19 (Burghaus and Holder 1994) or his-tagged for AMA1 (AMA1_3D7) (Dutta, Lalitha et al. 2002) and EBA-
175_F2_CAMP (Pandey, Singh et al. 2002), or as MBP-fusion proteins (MSP-3_K1, MSP-3_3D7 (Polley, Tetteh et al. 2007). Recombinant AMA1_FVO (Kocken, Withers-Martinez et al. 2002) was expressed in *Pichia pastoris* while EBA-175_F2_3D7 (Daugherty, Murphy et al. 1997) is a baculovirus-expressed product. The plasmids for the MSP-2 antigens and the clones for the MSP-1 block 2 antigens were kindly provided by Dr Jana McBride and Dr David Cavanagh, University of Edinburgh, and I expressed the antigens with the assistance of Dr Kevin Tetteh, London School of Hygiene and Tropical Medicine. MSP-119 was kindly provided by Dr Patrick Corran, London School of Hygiene and Tropical Medicine. The clones for the MSP-3 antigens were generously provided by Drs David Conway and Spencer Polley, and I expressed these antigens under their supervision at the London School of Hygiene and Tropical Medicine. The EBA-175 antigens, were kindly provided by Drs Chetan Chitnis and David Lanar, from the International Centre for Genetic Engineering and Biology (ICGEB), New Delhi, India and the Walter Reed Army Institute of Research (WRAIR), USA, respectively. The AMA1 antigens were kindly provided by Dr Alan Thomas, Biomedical Primate Research Centre (BPRC), The Netherlands and Dr David Lanar (WRAIR). Details on these antigens are provided in the *Table 3.2.1.*
Table 3.2.1 Details of recombinant merozoite antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Amino acid positions</th>
<th>Description</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA1_FVO</td>
<td>25-544</td>
<td>Domains I, II, III*</td>
<td>Kocken 2002</td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>83-531</td>
<td>Domains I, II, III</td>
<td>Dutta 2002</td>
</tr>
<tr>
<td>MSP-2_CH150/9</td>
<td>1-184</td>
<td>Allelic type A</td>
<td>Taylor 1995</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>22-247</td>
<td>Allelic type B</td>
<td>Taylor 1995</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>2-354</td>
<td>Full-length protein</td>
<td>Polley 2007</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>2-379</td>
<td>Full-length protein</td>
<td>Polley 2007</td>
</tr>
<tr>
<td>MSP-1_B2_RO33</td>
<td>54-144</td>
<td>RO33-like type</td>
<td>Cavanagh 1997</td>
</tr>
<tr>
<td>MSP-1_B2_3D7</td>
<td>54-144</td>
<td>K1-like type</td>
<td>Cavanagh 1997</td>
</tr>
<tr>
<td>MSP-1_B2_PaloAlto</td>
<td>54-144</td>
<td>K1-like type</td>
<td>Cavanagh 1997</td>
</tr>
<tr>
<td>MSP-1_B2_MAD20</td>
<td>54-144</td>
<td>MAD20-like type</td>
<td>Cavanagh 1997</td>
</tr>
<tr>
<td>MSP1-B2_Wellcome</td>
<td>54-144</td>
<td>MAD20-like type</td>
<td>Cavanagh 1997</td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>461-753</td>
<td>F2 sub-domain</td>
<td>Daugherty 1997</td>
</tr>
<tr>
<td>EBA-175_F2_CAMP</td>
<td>447-795</td>
<td>F2 sub-domain</td>
<td>Pandey 2002</td>
</tr>
</tbody>
</table>

Antigens are designated 'locus_P. falciparum strain'. *Includes prosequence

3.2.3 ANTIBODY ASSAYS

3.2.3.1 Total IgG Assay

ELISAs for serum IgG reactivity against each recombinant antigen and against parasite schizont extract were performed according to a standard protocol as previously described (Polley, Mwangi et al. 2004; Polley, Conway et al. 2006; Osier, Polley et al. 2007).

Individual wells of Dynex Immunolon 4HBX ELISA plates (Dynex Technologies Inc) were coated with 50ng of antigen in 100μl of carbonate coating buffer (15mM Na₂CO₃, 35mM NAHCO₃, pH 9.3). *P. falciparum* schizont extract (A4 strain for the Chonyi cohort and Wellcome strain for the case-control study) was coated onto wells in PBS according to the method of Ndungu et al. (Ndungu, Bull et al. 2002). Plates were incubated overnight at 4°C, before washing four times in PBS/Tween (Phosphate Buffered Saline/0.05% Tween 20), before washing four times in PBS/Tween (Phosphate Buffered Saline/0.05% Tween 20),
and blocking for 5 hours at room temperature with 1% skimmed milk in PBS/Tween (blocking buffer). Following this wells were washed again and incubated overnight at 4°C with 100μl of test sera (diluted 1/1000 in blocking buffer). Plates were then washed four times and incubated for 3 hours at room temperature with 100μl of HRP-conjugated rabbit anti-human IgG, (Dako Ltd.) at 1/5000 dilution in blocking buffer before final washing and detection with H2O2 and O-phenylenediamine (Sigma). The reaction was stopped with 25μl of 2M H2SO4 per well and absorbance read at 492nm. Two positive control sera drawn from adults semi-immune to malaria were tested individually in duplicate on each day of the experiment, on each plate, to allow for standardization of day-to-day and plate-to-plate variation. Single dilution serum ELISA Optical Density (OD) values were used as proxies for antibody titres as they correlate closely with full end-point antibody titrations when used at appropriate dilutions (Drakeley, Corran et al. 2005; Tongren, Drakeley et al. 2006).

3.2.3.2 IgG Sub-class Assay

This assay differed from that described for total IgG only in the secondary antibody reagents used. To detect IgG sub-classes, HRP-conjugated polyclonal sheep antibodies specific for human IgG1, IgG2, IgG3 and IgG4 (The Binding Site, Birmingham, UK) were used at a dilution of 1/3000.

3.2.3.3 Competition ELISA

Given two antigens that share some antibody epitopes and not others, competition ELISA assays can be performed to dissect out the contribution of shared or antigen-specific
epitopes to the observed reactivity. I pre-incubated test sera for 5 hours with an excess of competing antigen (1000ng, maximum amount) before following the indirect ELISA protocol for bound antigen. In separate experiments, I also added increasing concentrations of competing antigen to test sera. Any epitopes shared between the competing and plate bound antigens alleles were thus blocked in the pre-incubation step. The resulting reactivity could then be attributed to epitopes that are not shared between the two antigens. The stringency of the assay was increased by performing simultaneous ‘homologous’ and ‘heterologous’ competition assays. In the homologous assay, the competing antigen and the plate-bound antigen were identical, resulting in negligible reactivity, while in the heterolous assay, the competing antigen was different from (but shared some epitopes with) the plate bound antigen. The difference between the two assays demonstrates the reactivity to epitopes that are not shared.

3.2.4 T-CELL ASSAYS

3.2.4.1 Separation of PBMC

Fifteen ml of freshly collected, heparinized whole blood was centrifuged at 1400 x g for 7 min and the plasma separated and stored at -80°C. The cells were then reconstituted to the initial volume (15 ml) by topping up with RPMI 1640. This mixture was carefully layered onto Lymphoprep (7.5 ml of blood to 3 ml of Lymphoprep (Nycomed)) and centrifuged for 12 min at room temperature without brakes. PBMC were collected from the interface and transferred into 15 ml falcon tubes for washing. Fourteen ml of RPMI 1640 was added to each falcon tube, before spinning at 1600 x g for 10 mins at room temperature. The supernatant was discarded and the washing procedure repeated, this time spinning at

186
1400 x g for 7 min. The cells were then re-suspended in 1ml of RPMI 1640 for counting. Counting was done in a Neubauer chamber, using a 1/50 dilution of the cell suspension.

3.2.4.2 Ex-vivo ELISPOT

Ex-vivo ELISPOT assays were performed with freshly isolated PBMC using a well-established protocol (Flanagan 2003, Bejon 2006, Dunachie 2006). Briefly, 10 µl of capture antibody (anti-human IFNy mAb 1-D1K, purified, and, anti-human IL-2 mAb IL2-I, purified, both from MabTech) per ml of ELISPOT coating buffer (one carbonate-bicarbonate buffer capsule (Sigma) dissolved in 100 ml deionized water, and autoclaved), resulting in a final concentration of 10 µg/ml was coated onto Millipore MAIP S45 plates (Millipore, Massachusetts, USA) in a final volume of 50 µl/well, and incubated overnight at 4°C. Unbound catcher antibodies were flicked off and 100 µl of blocking buffer (10% heat inactivated fetal calf serum (FCS), prepared in RPMI with added penicillin and streptomycin) added to each well, before incubation for 1 hr at room temperature. Excess blocking buffer was then flicked off prior to the addition of PBMCs. 3 x 10^5 PBMC's suspended in 10% human AB serum diluted in RPMI were then added to each well and incubated for 18-20 hrs at 37°C in 5% CO₂ with either: i) test AMA1 peptides (25 µg/ml diluted in neat dimethylsulfoxide (DMSO)) in test wells, ii) media alone in a negative control well, and iii) Staphylococcal Enterotoxin B (SEB) (1 µg/ml) in a positive control well. Plates were washed with PBS/Tween and 1µg/ml biotinylated anti-human detector antibody (7-B6-1-Biotin for IFNy (Mabtech) or IL-2 (BD Biosciences)) added for 2 hrs. The plates were then washed 6 times in PBS/Tween. Streptavidin-ALP (MabTech) was added
next and the plates incubated for a further 1 – 2 hrs at room temperature. To develop the spots, 50 µl of development buffer (prepared from the AP conjugate substrate kit (BioRad)) was added to each well for 3 – 7 min. The reaction was stopped by rinsing the plates thoroughly in tap water. Plates were then soaked in tap water overnight, air-dried for 24 hours, before counting with an ELISPOT reader (AID EliSpot Reader System, Straßberg, Germany). The number of antigen-specific cells per 300,000 PBMC or spot forming units (SFU) were calculated by subtracting spot numbers in wells containing media only from spot numbers in peptide-containing wells (Keating 2005, Webster 2005, McConkey 2003, Vuola 2005).

### 3.2.5 PARASITE GENOTYPING

Frozen parasite isolates were thawed and cultured using standard techniques from the ring to the late-trophozoite or early-schizont stages, to bulk up parasite DNA for extraction and subsequent genotyping. Parasite DNA was extracted using DNA Qiamp mini-kits (Qiagen, UK). Parasites were genotyped by polymerase chain reaction (PCR) at three loci, MSP-1 block 2 (K1-like, MAD20-like, R033-like), MSP-2 (IC1-like or type A and FC27-like or type B), and MSP-3 (K1-like and 3D7-like). For MSP-1 block 2 and MSP-2, a nested PCR was used in which outer PCR primers were used to identify products at the locus, followed by nested family-specific primers at each locus (Snounou 2002). Only one reaction was required for MSP-3, using the outer primers of a previously described semi-nested PCR (Osier, Polley et al. 2007). The primer sequences are listed in the Table 3.2.2, below. All PCR reactions were performed using BioMix Red (ready-to-go 2x reaction mix containing BIOTAQ Red
DNA Polymerase, 1.5mM MgCl2, and dNTPs (Bioline)), in final volumes of 20μl, in 96 well plates, with 100nM primers. Following initial template denaturation at 95°C for 5 minutes, cycling conditions were as follows for MSP-1 block 2 and MSP-2; outer PCR, 30 cycles of 58°C for 2 minutes, 72°C for 2 minutes, 94°C for 1 minute, with final annealing at 58°C for 2 minutes, and final extension at 72°C for 5 mins; nested PCR, as for outer PCR, except that the initial annealing temperature was at 61°C. For MSP-3, initial template denaturation was at 94°C for 2 minutes, and cycling conditions were as follows: 44 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes, with a final extension of 72°C for 5 minutes. PCR products were visualized on 2% agarose gels for MSP-1 block 2 and MSP-2, while 3% agarose gels allowed for a clear distinction of alternative alleles of MSP-3.
Table 3.2.2 Primers used for PCR

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sets</th>
<th>Primer name</th>
<th>Primer sequence</th>
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</thead>
<tbody>
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<td>MSP-1</td>
<td>Outer PCR</td>
<td>M1-OF</td>
<td>5'_CTAGAAGCTTCTTGAAGATGCAGTATTG_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1-OR</td>
<td>3'_CTTAAATAGTTATTCTAATTCAAGTGGATCA_5'</td>
</tr>
<tr>
<td>Block2</td>
<td>K1-specific</td>
<td>M1-KF</td>
<td>5'_AAATGAAGAAGAAATTACTACAAAAGGTGC_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1-KR</td>
<td>3'_GCTTGCATCAGCTGGAGGCTTGGACCA_5'</td>
</tr>
<tr>
<td></td>
<td>MAD20-specific</td>
<td>M1-MF</td>
<td>5'_AAATGAGGAACAGTAGGAAAGTGGAGA_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1-MR</td>
<td>3'_ATCTGAAGATTTGTACGTTGAATTCC_5'</td>
</tr>
<tr>
<td></td>
<td>RO33-specific</td>
<td>M1-RF</td>
<td>5'_TAAAGGATGGAGCAATCTCAAGTTGTG_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1-RR</td>
<td>5'_CATCTGAAGATTTCACCTGGAGAC_3'</td>
</tr>
<tr>
<td>MSP-2</td>
<td>Outer PCR</td>
<td>M2 - OF</td>
<td>5'_ATGAAGGTAATAAACATTTCTATTATA_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2 - OF</td>
<td>3'_CTTTGTACATCGGTACATTCTT_5'</td>
</tr>
<tr>
<td>IC1-specific</td>
<td>M2-ICF</td>
<td></td>
<td>5'_GAAGATTGGAGCAAGAAGTAAKCCYTACT_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2-ICR</td>
<td>3'_GATTGTAATTCCGGGATGTTGGTTCG_5'</td>
</tr>
<tr>
<td>FC27-specific</td>
<td>M2-FCF</td>
<td></td>
<td>5'_AATACTAAGGTAGGTGCAARATGTCTCCA_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2-FCR</td>
<td>3'_TTTTTATTGGTACATTGCCAATTTC_5'</td>
</tr>
<tr>
<td>MSP-3</td>
<td>Standard</td>
<td>370F</td>
<td>5'_TGTAACAGCTGGTTCAAAAGG_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>586R</td>
<td>5'_CTCCTCCAATTCACACC_3'</td>
</tr>
</tbody>
</table>

The primers used in this study are published, for MSP-1 block 2 and MSP-2 (Snounou 2002) and for MSP-3 (Osier, Polley et al. 2007)

3.2.6 AMA1 SEQUENCING

AMA1 was sequenced from parasite DNA extracted from blood samples taken at the October 2000 cross-sectional survey in the Chonyi cohort and from children presenting to hospital in the case-control study. Sequence data on AMA1 from the Chonyi cohort were used for population genetic analyses as described below, while those obtained from the
case-control study were used in analyses of allele-specificity of antibody responses, and compared between children presenting with mild or severe malaria.

3.2.6.1 PCR amplification

A 1311bp segment of the \textit{P. falciparum} AMA1 gene encoding the surface-accessible ectodomain of the protein was amplified using a nested PCR approach, and sequencing performed using three overlapping pairs of primers. All PCR reactions were performed using BioMix Red with primer sequences as shown in Table 3.2.3. In the first round, 1µl of genomic DNA was amplified using primers 428F and 1799R. The cycling conditions were 94°C for 2 min, 94°C for 45 s, 62°C for 45 s, 72°C for 2.5 min x 44 cycles, then 72°C for 5 min. In the second round, the PCR product was amplified in two overlapping fragments, using two pairs of primers. For the first fragment, 1 µl of PCR product was amplified using the primers 428F and 1477R, and similarly for the second fragment, 1 µl of PCR product was amplified using the primers 1030F and 1799R. The cycling conditions for both the second round reactions were 94°C for 2 min, 94°C for 45 s, 60°C for 45 s, 72°C for 1 min 30 s x 39 cycles, then 72°C for 5 mins. The resulting 1045bp and 769bp amplification products were then purified using QIAquick PCR Purification Kit Protocol (QIAGEN, Crawley, UK) in preparation for sequencing.

3.2.6.2 DNA sequencing

The sequencing strategy using overlapping fragments was adapted from one that had been previously described for the same gene (Polley and Conway 2001; Polley, Chokejindachai et
al. 2003). Briefly, three pairs of primers were used to sequence two DNA templates separately and the resulting sequence electropherograms subsequently assembled. The primers were chosen to allow overlap between these three fragments to avoid gaps in the sequence when it was assembled. The primer sequences are detailed in Table 3.2.3. Two pairs of primers, 428F and 1138R, as well as 936F and 1477R, were used to sequence the 1045bp fragment while the 769bp fragment was sequenced using one pair of primers, 1030F and 1799R. For each fragment, forward and reverse primers were used for sequencing employing BIG DYE v. 3.1 terminator technology (Applied Biosystems, Warrington, UK). Sequencing products were run on an ABI Prism 3730 capillary DNA sequencer (Applied Biosystems). Forward and reverse reactions from each fragment were aligned and the three fragments for each allele united into a contiguous sequence using SeqMan II (DNASTAR). For population genetic analyses isolates were excluded if the electropherogram of any of the three fragments showed evidence of there being more than one allele of AMA-1 (34 of the Chonyi cohort isolates). Thus samples that contained only one clear allele of AMA-1 were included in the analysis (49 of the Chonyi cohort isolates).
### Table 3.2.3 Primers used for PCR amplification and sequencing of *P. falciparum* ama1

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sets</th>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA1 PCR</td>
<td>Outer PCR</td>
<td>428F</td>
<td>5'GACTTCCATCATCGGAAATGTCC_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1799R</td>
<td>5'GCCTCAGATCTAATCATTTCATC_3'</td>
</tr>
<tr>
<td></td>
<td>Nested PCR 1</td>
<td>428F</td>
<td>5'GACTTCCATCATCGGAAATGTCC_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1477R</td>
<td>5'CACATGGGCGATTTAAACTGTC_3'</td>
</tr>
<tr>
<td></td>
<td>Nested PCR 2</td>
<td>1030F</td>
<td>5'TTGAGTGCTCCGATCAACCTAA_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1799R</td>
<td>5'GCCTCAGGATCTAATCATTTCATC_3'</td>
</tr>
<tr>
<td>AMA1 Sequencing</td>
<td>Primer Pair 1</td>
<td>428F</td>
<td>5'ATGAAGGTATTAAAAACACTTGCTATTATA_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1138F</td>
<td>5'GACTTCCATCATCGGAAATGTCC_3'</td>
</tr>
<tr>
<td></td>
<td>Primer Pair 2</td>
<td>936F</td>
<td>5'CTGCTTTAAAAGCCACCAGTGGAAG_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1477R</td>
<td>5'CACATGGGCGATTTAAACTGTC_3'</td>
</tr>
<tr>
<td></td>
<td>Primer Pair 3</td>
<td>1030F</td>
<td>5'TTGAGTGCTCCGATCAACCTAA_3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1799R</td>
<td>5'GCCTCAGGATCTAATCATTTCATC_3'</td>
</tr>
</tbody>
</table>

### 3.2.7 POPULATION GENETIC ANALYSES OF THE AMA1 GENE

To detect whether polymorphic loci within ama1 were under positive selection, molecular population genetic analyses were performed on 49 full ectodomain sequences from Chonyi, Kenya using the DnaSP4.50 program (Rozas, Sanchez-DelBarrio et al. 2003). These analyses are explained in detail below: (i) a description of the sequence diversity within the population, (ii) analyses of recombination and linkage disequilibrium, (iii) tests of neutrality (Tajima's *D* and Fu and Li's *D* and *F*), including coalescent simulations assuming...
varying levels of recombination, (iv) comparisons of the ratios of non-synonymous to synonymous amino acid changes within and between species (McDonald Kreitman test, comparing with *P. reichenowi*), and (v) comparisons of between-population divergence (comparing with data from Thailand and Nigeria).

### 3.2.7.1 Population sequence diversity

AMA1 gene sequences covering the ectodomain were aligned without any gaps using the CLUSTAL program of MEGALIGN (DNA Star) and the data transferred into DnaSP 4.0 software for detailed analysis. This included identifying polymorphic sites within the whole ectodomain, and singly for each of the sub-domains I, II and III, as well as detection of di-morphic and tri-morphic sites containing two or three amino acids variants, respectively. In addition, calculations of average pair-wise nucleotide diversity per site (π) were performed separately for each domain and for the entire ectodomain. The number of AMA1 'haplotypes' covering the whole of the sequenced ectodomain was also determined.

### 3.2.7.2 Linkage disequilibrium and recombination

To explore the magnitude of linkage disequilibrium (LD) within the locus two widely used indices, D' and R² were determined (Lewontin 1964; Hill and Robertson 1968). The magnitude of LD is conventionally expressed as D, and corresponds to the difference between the expected and observed haplotype frequency. However, this value is affected by allele frequencies, and requires normalization. The index D', is the value that normalizes for different underlying allele frequencies. In a population sample D' = 1 or -1 indicates complete LD, while D' = 0, corresponds to no LD. The other measure of LD analyzed in this
study is $R^2$, the square of the correlation coefficient (R) between two alleles at two distinct loci. The measure $R^2$ is thought to provide a more accurate estimate of LD than $D'$, particularly when allele frequencies are low (Carlson, Eberle et al. 2004). As with $D'$, when $R^2 = 1$, this indicates complete or ‘perfect’ LD, although this value is more easily given for $D'$ in any case where one of the four di-allelic haplotypes is missing. For both indices, the relationship between LD and physical distance between pairs of nucleotide sites, which affects LD, was displayed graphically, and assessed in regression analyses. Physical distance is calculated as the nucleotide count from one nucleotide site to another, adjacent nucleotides having a distance of 1. The two tailed Fisher’s exact test was used to determine the statistical significance of LD between polymorphic sites, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Decline of LD with increasing distance between nucleotides is usually seen when meiotic recombination occurs within the region analysed, indicating that genetic exchange between alleles has occurred. At meiosis, the likelihood of intra-allelic recombination is proportional to the distance between loci.

To assess the effects of recombination on the tests of neutrality, two measures of recombination were estimated. The first calculates the minimum number of recombination events ($R_M$), that must have occurred to produce the alleles sampled (Hudson and Kaplan 1985). The four gamete test (FGT) was used to calculate $R_M$, and examines pairs of single nucleotide polymorphisms (SNPs) or other bi-allelic polymorphisms: for two bi-allelic loci with ancestral and derived alleles $A/B$ and $a/b$, respectively, the potential haplotypes are $AB, Ab, aB, ab$. All these combinations can only be observed in a sample due to identical recurrent mutation (very unlikely), or recombination (the FGT scores a recombination
event if all four possible haplotypes occur). The second measure of recombination estimates recombination rate based on a probabilistic (stochastic) model of an ideal population, assuming a constant population size with random mating, neutrality, and uniformity of recombination rates across the genome. This is because historic recombination events cannot be counted with precision, nor can the number of preceding generations which are required to estimate the per-generation recombination rate be determined with certainty. It is expressed as the recombination parameter $C$, which is the product of the the effective populations size ($N$), and the per-generation recombination rate ($r$), in the formula $C = 4Nr$ (Hudson 1987). The effective population size is the size of the ideal population, which remains constant, and in which the effects of random drift would be the same as those seen in the actual population.

3.2.7.3 Tests of neutrality

Several methods can be employed to detect evidence of past natural selection using DNA polymorphism data. Many of these methods work by rejecting the neutrality model for a given set of loci when the observed data are different from expectations under a neutral model of evolution. Two such tests, Tajima's $D$, and Fu and Li's $D$ and $F$, were used for this analysis. Tajima's $D$ is based on the differences between the number of polymorphic sites and the average number of pairwise nucleotide differences within the sample (Tajima 1989). In a constant-size population in mutation-drift equilibrium, the expected Tajima's $D$ value is close to zero, because both estimates have the same expected value. If the value of $D$ is too large, or too small, the neutral 'null' hypothesis is rejected. In general, large positive values imply selection is maintaining alleles in the population or that there has
been a temporary marked reduction in population size (i.e. a bottleneck). Large negative values imply population expansion or directional selection.

Fu and Li's $D$ and $F$ tests use a different approach which compares the observed number of singleton nucleotides in the $ama1$ sequences with those expected under neutrality, based on the average number of nucleotide differences between pairs of alleles (Fu and Li 1993). For between-species comparisons, an AMA1 allele from a closely related species, *Plasmodium reichenowi* (EMBL number AJ252087) was used as the outgroup for *P. falciparum*.

3.2.7.3.1 Coalescent simulations

Tajima's test was repeated assuming varying levels of recombination using coalescent simulations, to determine the effect that high levels of recombination would have on the estimates of departure from neutrality, in comparison with the observed estimates. Ten thousand coalescent simulations were performed and the 95% confidence intervals for the estimates of Tajima's $D$ compared with the values observed in the data. Observed estimates that did not fall within the confidence limits were considered statistically significant ($p < 0.05$).

3.2.7.4 McDonald-Kreitman test

A simple statistical test of the neutral protein evolution hypothesis compares the number of amino acid replacement substitutions (non-synonymous) to synonymous (silent) substitutions within and between species (McDonald and Kreitman 1991). A nucleotide
site fixed between species is a site where all sequences sampled from one species contain a nucleotide not found in the other species. Under neutral expectations, the ratio for fixed differences between species should be equal to the same ratio within species. The presence of significantly more fixed differences between species than within species, suggests the adaptive fixation of selectively advantageous mutations (McDonald and Kreitman 1991). The sequence of AMA1 from *P. reichenowi* (EMBL number AJ252087) was used for interspecific comparison with *P. falciparum* (Kocken, Narum et al. 2000), and significant differences were detected by the Fisher’s exact test. Radical amino acid substitutions are those that alter the charge of the amino acid, and are presumed to have more functional consequence, in comparison to conservative amino acid substitutions where the amino acid is altered, but charge is maintained. The proportions of radical to conservative amino acid substitutions across the ectodomain were compared.

3.2.7.5 Inter-population fixation indices

AMA1 diversity was compared between populations using previously published equivalent datasets from Nigeria (Polley and Conway 2001) and Thailand (Polley, Chokejindachai et al. 2003). Wright’s *F*<sub>ST</sub> (Wright 1950), a measure of between-population variation, was calculated for pairwise comparisons between the three populations. Loci with exceptionally low *F*<sub>ST</sub> can be an indication of balancing selection maintaining similar allele frequencies between populations. Analyses were carried out using FSTAT version 1.2. For this analysis, polymorphic sites were only included in the minor allele had a frequency of >0.15 across the three populations.
3.3 DESCRIPTION OF COHORTS

A brief description of the characteristics of individuals in each of the three cohorts is provided below.

3.3.1 CHONYI COHORT

For the work presented in this thesis, data were analyzed only for the sub-group of children who were parasitaemic at the time the serum sample was collected, and this is explained in more detail in a chapter 4. As shown in Table 3.3.1, the mean age between both groups of children was comparable (p > 0.05 for all comparisons, Students t test). The mean parasitaemia in the 119 children who were asymptotically parasitized was 10956.64 parasites/µl and ranged from a minimum of 40 – 270,000 parasites/µl.

<table>
<thead>
<tr>
<th>Parasitaemic</th>
<th>Aparasitaemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (119)</td>
<td>mean age (yrs)</td>
</tr>
<tr>
<td>10</td>
<td>1.7</td>
</tr>
<tr>
<td>24</td>
<td>3.6</td>
</tr>
<tr>
<td>27</td>
<td>5.6</td>
</tr>
<tr>
<td>21</td>
<td>7.6</td>
</tr>
<tr>
<td>37</td>
<td>9.6</td>
</tr>
</tbody>
</table>

Similar age distribution of children who were parasitaemic (included in the analysis) and those who were not parasitaemic (NOT included in the analysis) at the time the serum sample was collected.
3.3.2 CASE-CONTROL STUDY

The characteristics of the children included in this study are shown in Table 3.3.2.

Children who developed severe malaria were significantly younger than those who developed mild malaria, mean age 31.6 versus 37.3 months, p = 0.003 (Students t test).

Bed net use was comparable in cases and controls. The prevalence of parasites in the pre-season sample as well as the actual parasite counts, were comparable in cases and controls. However, the mean levels of antibodies to *P. falciparum* parasite schizont extract (a proxy measure of exposure to malaria) were lower in children who subsequently developed severe malaria, compared to those who developed mild malaria and healthy controls, though this was only statistically significant for the latter (severe versus mild malaria, p = 0.05, severe malaria versus controls, p = 0.02, (Students t test)).

<table>
<thead>
<tr>
<th>Table 3.3.2 Characteristics of individuals in the case-control study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (mths)</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>12 - 24</td>
</tr>
<tr>
<td>25 - 36</td>
</tr>
<tr>
<td>37 - 48</td>
</tr>
<tr>
<td>&gt;48</td>
</tr>
<tr>
<td>Bednet use</td>
</tr>
<tr>
<td>Slide positive¹</td>
</tr>
<tr>
<td>Mean parasitaemia¹</td>
</tr>
<tr>
<td>Schizont antibodies²</td>
</tr>
</tbody>
</table>

¹ Parasitaemia detected in the pre-season sample. ² Reactivity to *P. falciparum* parasite schizont extract, mean OD levels. * Reactivity to schizont extract was significantly lower among children with severe malaria compared to controls.
3.3.3 JUNJU COHORT

The baseline characteristics of the Junju adults are shown in Table 3.3.3. There were more females than males, and although the mean haemoglobin was 1.5g/dl lower in females than it was in males (p = 0.01, Students t test), the average PBMC count in both groups was comparable. Only one individual had parasites detectable by microscopy at the time the sample was collected.

Table 3.3.3 Characteristics of Junju adults recruited into the study

<table>
<thead>
<tr>
<th></th>
<th>Females (n = 18)</th>
<th>Males (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yrs)</td>
<td>26.6 (23.4 – 29.9)</td>
<td>23.8 (16.6 – 30.4)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>11.6 (11.1 – 12.3)</td>
<td>13.1 (11.9 – 14.4)</td>
</tr>
<tr>
<td>Leukocyte count (/μl)</td>
<td>5140 (4280 – 6000)</td>
<td>5125 (4240 – 6250)</td>
</tr>
<tr>
<td>PBMC count</td>
<td>$1.46 \times 10^7$</td>
<td>$1.48 \times 10^7$</td>
</tr>
<tr>
<td>Prevalence of parasites</td>
<td>1/18 (5.5%)</td>
<td>0/8</td>
</tr>
</tbody>
</table>

1 Mean haemoglobin was significantly lower in females than in males (p = 0.01, Students t test). 2 Peripheral blood mononuclear cells isolated from 15 ml of whole blood.
4 Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria

4.1 Introduction

While large populations of the world are at risk of malaria (Snow, Guerra et al. 2005; Hay and Snow 2006) the brunt of mortality caused by *Plasmodium falciparum* continues to be borne by children in sub-Saharan Africa. It is estimated that in this region alone, nearly one million children under the age of five years died as a direct consequence of malaria in the year 2000 (Rowe, Rowe et al. 2006). An effective vaccine is urgently needed but has proved challenging to obtain. In endemic areas, older children and adults develop naturally-acquired immunity (NAI) to severe and life-threatening malaria but remain susceptible to infection (Marsh 1992). Classical experiments in which passively transferred antibodies from immune adults were successfully used to treat children (Cohen, McGregor et al. 1961; McGregor and Carrington 1963) with severe *P. falciparum* malaria provide the strongest evidence that antibodies are important mediators of NAI. Clinical symptoms of malaria result from the asexual blood stage of the infection where potential antibody targets include merozoite antigens involved in invasion (Cowman and Crabb 2006) and parasite-derived surface antigens on infected erythrocytes (Bull and Marsh 2002).
Studies on protective immunity to malaria involve monitoring of subjects in endemic communities for variable durations of time to measure the incidence of infection or clinical disease. Associations between the presence of a specific immune response to a target antigen and outcome determine whether an immune response to the specific antigen appears to be "protective". These immuno-epidemiological studies have often provided conflicting data, with responses to the same antigen appearing to be protective in some studies but not in others (Riley, Allen et al. 1992; Hogh, Marbiah et al. 1995; al-Yaman, Genton et al. 1996; Egan, Morris et al. 1996; Branch, Udhayakumar et al. 1998; Dodoo, Theander et al. 1999; Conway, Cavanagh et al. 2000; Cavanagh, Dodoo et al. 2004; Perraut, Marrama et al. 2005). Most antibody-based analyses of protection are tethered on sero-positivity (usually defined as the mean plus three standard deviations of non-malaria exposed sera) and do not take into account the continuous, quantitative nature of antibody responses. Furthermore, the majority of studies have concentrated on associations between responses to single, or a limited number of antigens and protection from clinical malaria, despite the fact that individuals living in endemic areas are simultaneously and repeatedly challenged with numerous malaria antigens. Few studies have examined the interactions between specific antibody responses against multiple malaria antigens (Meraldi, Nebie et al. 2004; John, Moormann et al. 2005), and whether these might be synergistic or antagonistic or neither with regards to protection.

To test whether either the number of important target antigens to which antibodies are made, and/or the levels of such antibodies in serum, are associated with protection from malaria, we analyzed naturally-acquired antibodies to five leading *P. falciparum* merozoite
stage vaccine candidate antigens (apical membrane antigen 1 (AMA1), merozoite surface proteins- 1, 2 and 3, (MSP-1, MSP-2, MSP-3), and erythrocyte binding antigen (EBA-175)), as well as *P. falciparum* schizont extract, in a cohort of Kenyan children who were monitored longitudinally for mild (uncomplicated) clinical malaria (Chonyi cohort). We also examined combinations of, and interactions between, antigen-specific antibodies to determine the combination(s) that predicted the strongest protection from clinical malaria. These antigens were selected for study because of the cumulative evidence that the presence of antibodies to these antigens may be associated with protection (Taylor, Smith et al. 1995; Taylor, Allen et al. 1998; Conway, Cavanagh et al. 2000; Metzger, Okenu et al. 2003; Polley, Tetteh et al. 2003; Cavanagh, Dodoo et al. 2004; Polley, Mwangi et al. 2004; Polley, Conway et al. 2006; Osier, Polley et al. 2007; Polley, Tetteh et al. 2007), backed by evidence that polymorphisms in their sequences are maintained by natural selection (Conway and Polley 2002), and their biological plausibility (Deans, Alderson et al. 1982; Epping, Goldstone et al. 1988; Clark, Donachie et al. 1989; Blackman, Heidrich et al. 1990; Sim, Orlandi et al. 1990; Oeuvery, Bouharoun-Tayoun et al. 1994; Locher, Tam et al. 1996). The analytical approaches were developed using data from the Chonyi cohort and the methods subsequently validated in an independent case-control study whose end-point was malaria severe enough to require admission to hospital.
4.2 **SPECIFIC AIMS**

1. To determine whether either the number of important target antigens to which antibodies are made, and/or the levels of such antibodies in serum, are associated with protection from clinical episodes of malaria in the Chonyi cohort.

2. To identify the combination(s) of antibodies that predicted the strongest protection from clinical malaria.

3. To validate the findings from the Chonyi cohort in the case-control study of severe malaria.

4.3 **MATERIALS AND METHODS**

The Chonyi cohort and case-control studies have already been described, as have been the recombinant antigens, and ELISA assay protocol, see materials and methods, sections 3.2.1-3. For both studies, IgG antibodies to AMA1, MSP-2, MSP-3, EBA-175, MSP-1 block 2, MSP-119 and *P. falciparum* schizont extract were analyzed. Data on antibody responses to AMA1, MSP-2 and MSP-3 from the Chonyi cohort had been previously published (Polley, Mwangi et al. 2004; Polley, Conway et al. 2006; Osier, Polley et al. 2007), but in this study were completely re-analysed from the raw data, using only the data from children under ten years old. For the case-control study, antibody data for all the antigens were generated specifically for this thesis.
4.3.1 STATISTICAL ANALYSIS

4.3.1.1 Overview

All data analyses were performed in STATA version 9.2 (Statcorp, Texas, USA). Models were firstly developed using data from the Chonyi cohort and subsequently validated in the case-control study with some modifications (below). The primary analysis was on the sub-group of 119 children from the Chonyi cohort (n=280) who were asymptotically parasitized at the time of serum collection in October 2000 because in previous analyses, *P. falciparum* parasitaemia at the time of serum collection modified the effects of antibodies to both variant red cell surface (Bull, Lowe et al. 2002) and merozoite (Polley, Mwangi et al. 2004; Polley, Conway et al. 2006; Osier, Polley et al. 2007) antigens on the risk of disease. The confounding effects of exposure on antibody responses were controlled for by adjusting both for age, as well as antibody reactivity to parasite schizont extract in multi-factorial analyses.

4.3.1.2 Antibodies and the probability of a clinical episode

The probability of a clinical episode for each antigen (and each allelic form), for given antibody levels was estimated by logistic regression fitting ELISA OD values for the antigen as a linear covariate, and adjusting for age (in 2 year categories). The logits from these models were converted into probabilities (Mitchell and Chen 2005), to give estimates of risk (Figure 4.4.1). These analyses established that for most antigens and antibodies, higher antibody levels were associated with a lower risk of disease and that allelic versions of the same antigen (or the same allelic family for MSP-1 Block 2) generally gave similar patterns of protection. The probability plots were used to define a threshold (cut-off) for
high versus low/undetectable antibodies as the OD level above which the risk of disease was lower than the population's risk of 33.6% (i.e. the risk of disease assuming no role for any antibodies) (Figure 4.4.1 and Table 4.4.1). The suitability of the logistic model was checked by examining the residuals when the OD data were fitted in quintiles. The individual effects of high levels of each antibody on the risk of disease were then re-analyzed fitting antibody level as a factor rather than as a linear covariate (Table 4.4.2) for ease of interpretation, and to facilitate analyses of breadth and the interactions between antibodies. To avoid the lack of convergence commonly encountered in conventional binomial regression analyses, data were fitted to a modified Poisson regression model with robust error variance, which tends to provide conservative results (Zou 2004).

4.3.1.3 Correlations between allelic versions of antigens
Antibodies to different allelic forms of most antigens (AMA1, MSP-2, MSP-3 and the F2 sub-domain of EBA-175) and to the main allelic types of MSP-1 Block 2 (K1- and MAD20-types) were highly correlated (Table 4.4.3) and generally gave similar patterns of protection (Figure 4.4.1). Consequently, high level antibodies of one allelic form of each antigen was selected for the analysis of antibodies to multiple antigens, using the Bayesian Information Criteria (BIC) to identify the allelic form with the best model fit. Antibodies to MSP-1 Block 2 (MSP-1_B2) were highly correlated only within the main allelic families, and so for this antigen, antibodies to one antigen from each of the three main allelic families (MSP-1_B2_Wellcome, MSP-1_B2_3D7 or MSP-1_B2_RO33).
4.3.1.4 Breadth of antibody specificity

Breadth was analyzed in an age and schizont extract-adjusted modified Poisson regression model, that compared the risk of disease among children who had high level antibodies (fitted as a factor) to between one and six antigens to those who had low/undetectable antibodies to all six antigens. The combination of antibodies that was associated with the lowest risk of clinical malaria was determined by analyzing all pair-wise combinations, investigating interactions between antigens by fitting a model with two main effects and an interaction term. Interaction as presented here, refers to statistical interaction where the estimate of risk obtained for antibodies to two antigens is significantly lower than expected (ie than the product of the individual risk ratios). It does not exclude biological interaction. To make certain that we were not simply measuring correlated antibodies arising from shared exposure, we separately included antibodies to all antigens in a single regression model, together with age and reactivity to schizont extract, dropping each out sequentially in decreasing order of their P-values. Antigens that remained significant in this model at the P <0.10 level were MSP-2, MSP-3 and AMA1.

4.3.1.5 Case-control study

Data on children from the hospital cohort were analyzed essentially as described above with minor modifications. Models were fitted to data from the entire hospital cohort (not only the sub-group that were parasitaemic at the time of serum collection) because it appeared that frequency matching of cases and controls for location (and therefore exposure) successfully eliminated the interaction between the antibody's protective effect and parasite infection status.
4.4 RESULTS

4.4.1 MAGNITUDE OF ANTIBODY RESPONSE AND PROTECTION

The probability of developing an episode of clinical malaria for a given value of measured antibody level (OD) was estimated for each antigen. We found that the levels of serum antibodies to some, but not all vaccine candidate antigens, were inversely related to the probability of developing malaria (Figure 4.4.1). Increasing OD levels to MSP-2, MSP-3, AMA1 and the *MAD20-like* antigens of MSP-1 Block 2 (denoted Wellcome and MAD20) were associated with reduced probability of malaria morbidity while those to MSP-119, EBA-175, or the *K1* - and *R033-like* antigens of MSP-1 Block 2, had little effect. Within these loci (and within the main allelic families for MSP-1 Block 2), the patterns were similar for the different allelic forms. Increasing antibody titres to whole parasite schizont extract were also associated with a reduced probability of clinical malaria.
Figure 4.4.1 Antibody levels and the risk of clinical episodes

The predicted probability of an episode of malaria in children decreases with increasing antibody titre for most antigens in the Chonyi cohort (n=119). Each panel represents the allelic antigens tested at each locus, as well as parasite schizont extract. The red horizontal line represents the risk of an episode without taking antibody responses to any antigen into account. The final panel combines antibodies to one allelic form of each antigen (and one antigen from each of the three main allelic families of MSP-1 Block 2). The lines from top to bottom represent: MSP-1_B2_Wellcome, MSP-3_K1, MSP-2_Dd2, Schizont extract, AMA1_3D7, EBA-175_F2_3D7, MSP-1_B2_3D7, MSP-1_B2_RO33 and MSP-1_{19}. 

Optical Density

Probability of clinical episode
4.4.2 PROTECTIVE EFFICACY OF INDIVIDUAL HIGH TITRE ANTIBODIES

The probability plots (Figure 4.4.1) were used to define a threshold (cut-off) for high versus low/undetectable antibodies for each antigen. This threshold varied both by antigen and by population (the Chonyi cohort and the case-control study), ranging from relatively low OD values for the MSP-1 Block 2 antigens to high values for MSP-2 (Table 4.4.1). The individual effects of high levels of each antibody on the risk of disease were re-analyzed fitting antibody level as a factor rather than as a linear covariate (Table 4.4.2) for ease of interpretation, and to facilitate analyses of breadth and the interactions between antibodies.
Table 4.4.1 ELISA OD cut-off values

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Chonyi cohort</th>
<th>Case-control study</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA1_3D7</td>
<td>1.2990</td>
<td>0.9360</td>
</tr>
<tr>
<td>AMA1_FVO</td>
<td>1.1820</td>
<td>1.0194</td>
</tr>
<tr>
<td>AMA1_HB3</td>
<td>ND</td>
<td>0.9477</td>
</tr>
<tr>
<td>MSP-2_CH150/9</td>
<td>1.3742</td>
<td>0.6200</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>1.3751</td>
<td>0.7122</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.5914</td>
<td>0.2442</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>0.7553</td>
<td>0.3421</td>
</tr>
<tr>
<td>EBA-175_F2_CAMP</td>
<td>0.8800</td>
<td>0.1584</td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>1.2534</td>
<td>0.5331</td>
</tr>
<tr>
<td>MSP19</td>
<td>0.9015</td>
<td>0.5872</td>
</tr>
<tr>
<td>MSP-1_B2_3D7</td>
<td>0.2473</td>
<td>0.2014</td>
</tr>
<tr>
<td>MSP-1_B2_Palo Alto</td>
<td>0.2660</td>
<td>ND</td>
</tr>
<tr>
<td>MSP-1_B2_Wellcome</td>
<td>0.0160</td>
<td>ND</td>
</tr>
<tr>
<td>MSP-1_B2_MAD20</td>
<td>0.0606</td>
<td>0.3421</td>
</tr>
<tr>
<td>MSP-1_B2_RO33</td>
<td>0.0586</td>
<td>0.1066</td>
</tr>
</tbody>
</table>

ELISA OD levels (cut-offs) used to define high versus low responders to merozoite antigens in the Chonyi cohort and the case-control study. Cut-offs were predicted from the probability plots for each antigen (Figure 4.4.1) as the ELISA OD value which corresponded to the population's average risk of an episode. ND: Not done.
<table>
<thead>
<tr>
<th>Antigens</th>
<th>n%</th>
<th>Risk Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Univariate analysis</td>
</tr>
<tr>
<td>AMA1_FVO</td>
<td>49</td>
<td>0.45(0.25-0.80)</td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>51</td>
<td>0.40(0.22-0.72)</td>
</tr>
<tr>
<td>MSP-2_CH150/9</td>
<td>54</td>
<td>0.32(0.17-0.59)</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>54</td>
<td>0.28(0.15-0.53)</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>39</td>
<td>0.39(0.20-0.78)</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>40</td>
<td>0.58(0.32-1.04)</td>
</tr>
<tr>
<td>EBA-175_F2_CAMP</td>
<td>34</td>
<td>0.72(0.40-1.29)</td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>41</td>
<td>0.41(0.21-0.79)</td>
</tr>
<tr>
<td>MSP-1_B2_3D7</td>
<td>20</td>
<td>0.43(0.12-1.12)</td>
</tr>
<tr>
<td>MSP-1_B2_PaloAlto</td>
<td>18</td>
<td>0.93(0.47-1.83)</td>
</tr>
<tr>
<td>MSP-1_B2_Welcome</td>
<td>19</td>
<td>0.33(0.11-1.00)</td>
</tr>
<tr>
<td>MSP-1_B2_MAD20</td>
<td>23</td>
<td>0.48(0.21-1.12)</td>
</tr>
<tr>
<td>MSP-1_B2_RO33</td>
<td>15</td>
<td>1.40(0.77-2.53)</td>
</tr>
<tr>
<td><em>MSP-1</em></td>
<td>36</td>
<td>1.44(0.87-2.38)</td>
</tr>
</tbody>
</table>

Risk of developing clinical malaria with high compared to low/undetectable antibodies to individual antigens in a subset of the Chonyi cohort (n=119). Antigens are designated 'locus_P.falciparum strain'. n% is the proportion of children with high titre antibodies (n=119). Risk ratios (95% confidence interval) are presented for univariate and multivariate analyses (adjusted initially for age, and subsequently both for age and reactivity to _P. falciparum_ parasite schizont extract as a proxy for exposure). *p <0.05

### 4.4.3 CORRELATIONS BETWEEN ALLELIC VERSIONS OF ANTIGENS

Antibodies to different allelic forms of most antigens (AMA1, MSP-2, MSP-3 and the F2 subdomain of EBA-175) and to the main allelic types of MSP-1 Block 2 (K1- and MAD20-types) were highly correlated (Table 4.4.3). The protective effects of antibodies to allelic versions of antigens were therefore analyzed in separate models, examining both their...
individual effects as well as their interactions, to identify the alleles that best fitted the data, using the BIC (Table 4.4.4). For AMA1 and F2 sub-domain of EBA-175, no improvement in model fit was obtained when responses to both alleles versus one allele were included in the model, and so only the best-fitting allele was taken forward into further analyses. For antibody responses to MSP-2 and MSP-3, there was a modest improvement in model fit when antibodies to both allelic forms were included in the model. However, to keep the models as simple as possible, BIC criteria were employed as above to select the single allelic form that best fitted the data (MSP-3_K1 and MSP-2_Dd2). Antibodies to MSP-1 block 2 were correlated within, but not across the main allelic families (these sequences do not contain any conserved epitopes). Antibodies that gave the best fit to the data within each allelic family were included in an overall MSP-1 Block 2 response, which was retained for further analysis (MSP-1_B2_Wellcome, MSP-1_B2_3D7 and MSP-1_B2_RO33). Thus, a total of six genetically (& structurally) unrelated antigens (one allelic form representing each antigen locus, and an overall MSP-1 Block 2 response) were retained for analyses of breadth and combined antibody responses on the risk of clinical episodes, and included AMA1_3D7, MSP-2_Dd2, MSP-3_K1, EBA-175_3D7, MSP-1_19 and MSP-1 Block 2 (overall response).
Table 4.4.3 Correlations between antibodies to the panel of merozoite antigens

<table>
<thead>
<tr>
<th>Schizont</th>
<th>AMA1_1</th>
<th>AMA1_2</th>
<th>MSP2_1</th>
<th>MSP2_2</th>
<th>MSP3_1</th>
<th>MSP3_2</th>
<th>EBA_1</th>
<th>EBA_2</th>
<th>MSP1_1</th>
<th>MSP1_2</th>
<th>MSP1_3</th>
<th>MSP1_4</th>
<th>MSP1_5</th>
<th>MSP1_6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizont</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>AMA1_1</td>
<td>0.47</td>
<td>0.48</td>
<td>0.54</td>
<td>0.58</td>
<td>0.29</td>
<td>0.41</td>
<td>0.50</td>
<td>0.51</td>
<td>0.41</td>
<td>0.22</td>
<td>0.22</td>
<td>0.15</td>
<td>0.14</td>
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<tr>
<td>AMA1_2</td>
<td>0.48</td>
<td>0.98</td>
<td>0.64</td>
<td>0.61</td>
<td>0.40</td>
<td>0.45</td>
<td>0.56</td>
<td>0.65</td>
<td>0.14</td>
<td>0.18</td>
<td>0.18</td>
<td>0.24</td>
<td>0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>MSP2_1</td>
<td>0.54</td>
<td>0.64</td>
<td>0.62</td>
<td>0.43</td>
<td>0.49</td>
<td>0.46</td>
<td>0.50</td>
<td>0.56</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.20</td>
<td>0.21</td>
<td>0.28</td>
</tr>
<tr>
<td>MSP2_2</td>
<td>0.58</td>
<td>0.61</td>
<td>0.16</td>
<td>0.14</td>
<td>0.22</td>
<td>0.49</td>
<td>0.54</td>
<td>0.56</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.24</td>
<td>0.24</td>
<td>0.06</td>
</tr>
<tr>
<td>MSP3_1</td>
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<td>0.40</td>
<td>0.16</td>
<td>0.14</td>
<td>0.22</td>
<td>0.51</td>
<td>0.47</td>
<td>0.50</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.21</td>
<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>MSP3_2</td>
<td>0.41</td>
<td>0.45</td>
<td>0.17</td>
<td>0.17</td>
<td>0.22</td>
<td>0.52</td>
<td>0.54</td>
<td>0.55</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.23</td>
<td>0.23</td>
<td>0.17</td>
</tr>
<tr>
<td>EBA_1</td>
<td>0.50</td>
<td>0.56</td>
<td>0.50</td>
<td>0.47</td>
<td>0.47</td>
<td>0.39</td>
<td>0.42</td>
<td>0.51</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0.20</td>
<td>0.20</td>
<td>0.17</td>
</tr>
<tr>
<td>EBA_2</td>
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<td>0.65</td>
<td>0.55</td>
<td>0.54</td>
<td>0.54</td>
<td>0.39</td>
<td>0.42</td>
<td>0.65</td>
<td>0.19</td>
<td>0.19</td>
<td>0.19</td>
<td>0.24</td>
<td>0.24</td>
<td>0.19</td>
</tr>
<tr>
<td>MSP1_1</td>
<td>0.41</td>
<td>0.14</td>
<td>0.14</td>
<td>0.22</td>
<td>0.24</td>
<td>0.11</td>
<td>0.08</td>
<td>0.24</td>
<td>0.19</td>
<td>0.19</td>
<td>0.19</td>
<td>0.21</td>
<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>MSP1_2</td>
<td>0.22</td>
<td>0.19</td>
<td>0.20</td>
<td>0.28</td>
<td>0.25</td>
<td>0.16</td>
<td>0.11</td>
<td>0.24</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>0.17</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>MSP1_3</td>
<td>0.22</td>
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<td>0.17</td>
<td>0.25</td>
<td>0.22</td>
<td>0.14</td>
<td>0.09</td>
<td>0.24</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0.20</td>
<td>0.20</td>
<td>0.17</td>
</tr>
<tr>
<td>MSP1_4</td>
<td>0.14</td>
<td>0.16</td>
<td>0.15</td>
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<td>0.17</td>
<td>0.19</td>
<td>0.20</td>
<td>0.14</td>
<td>0.15</td>
<td>0.14</td>
<td>0.15</td>
<td>0.19</td>
<td>0.19</td>
<td>0.15</td>
</tr>
<tr>
<td>MSP1_5</td>
<td>0.15</td>
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<td>0.22</td>
<td>0.28</td>
<td>0.24</td>
<td>0.20</td>
<td>0.21</td>
<td>0.17</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.21</td>
<td>0.21</td>
<td>0.20</td>
</tr>
<tr>
<td>MSP1_6</td>
<td>0.20</td>
<td>0.03</td>
<td>0.05</td>
<td>0.17</td>
<td>0.19</td>
<td>0.06</td>
<td>0.11</td>
<td>0.15</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
<td>0.05</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Figures are pair-wise correlation coefficients (Chonyi cohort). Antibodies to the main allelic forms of most antigens were highly correlated. Antigens abbreviated as follows: AMA1_1, AMA1_3, AMA1_FV0; MSP2_1, MSP-2_CH150/9; MSP2_2, MSP-2_Dd2; MSP3_1, MSP-3_K1; MSP3_2, MSP-3_D7; EBA_1, EBA-175_F2_CAMP; EBA_2, EBA-175_F2_D7; MSP1_1, MSP-1_19; MSP1_2, MSP-1_Block 2_3D7; MSP1_3, MSP-1_Block 2_Palo Alto; MSP1_4, MSP-1_Block 2_Wellcome; MSP1_5, MSP-1_Block 2_MAD20; MSP1_6, MSP-1_Block 2_RO33.
Table 4.4.4 Effects of high titre antibodies to allelic combinations of antigens on the probability of a clinical episode in the Chonyi cohort

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Main effects &amp; interactions</th>
<th>p value</th>
<th>Combination effects</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA1_FVO</td>
<td>2.46(0.71-8.47)</td>
<td>0.151</td>
<td>0.67(0.36-1.22)</td>
<td>0.196</td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>0.23(0.07-0.77)</td>
<td>0.017*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP-2_CH150/9</td>
<td>0.57(0.30-1.05)</td>
<td>0.074</td>
<td>0.30(0.13-0.67)</td>
<td>0.003*</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>0.48(0.24-0.96)</td>
<td>0.038*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.55(0.28-1.07)</td>
<td>0.078</td>
<td>0.37(0.12-1.11)</td>
<td>0.077</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>0.80(0.47-1.38)</td>
<td>0.435</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBA-175_F2_CAMP</td>
<td>1.61(0.97-2.68)</td>
<td>0.062</td>
<td>0.74(0.33-1.65)</td>
<td>0.747</td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>0.48(0.24-0.96)</td>
<td>0.039*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M MSP-1_B2_Wellcome</td>
<td>0.55(0.16-1.87)</td>
<td>0.346</td>
<td>0.55(0.14-2.15)</td>
<td>0.399</td>
</tr>
<tr>
<td>M MSP-1_B2_MAD20</td>
<td>0.97(0.38-2.44)</td>
<td>0.951</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M MSP-1_B2_3D7</td>
<td>0.48(0.19-1.16)</td>
<td>0.105</td>
<td>0.87(0.31-2.41)</td>
<td>0.794</td>
</tr>
<tr>
<td>M MSP-1_B2_PaloAlto</td>
<td>1.38(0.93-2.06)</td>
<td>0.102</td>
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<td></td>
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</tbody>
</table>

Risk of developing clinical malaria with high compared to low/undetectable antibodies to combinations of allelic antigens in a subset of the Chonyi cohort (n=119). Figures are risk ratios (95% confidence intervals) and p values. 1Main effects of antibodies to each antigen are adjusted for each other. 2Interaction (over and above main effects). 3Effects of combinations of high titre responses (combines main effects as well as interactions). "X" represents the estimates for the interaction between the two preceding antigens. No significant evidence of statistical interaction between allelic antigens was found and one antigen from each locus was used in subsequent analyses of the effects of high titre antibodies to combinations of unrelated (non-allelic) antigens on clinical episodes. ∞: unreliable estimate due to sample size limitations *p <0.05
4.4.4 BREADTH AND PROTECTIVE EFFICACY OF ANTIBODY RESPONSE

Children who had high levels of antibodies to one, two, three, four, five or six unrelated (non-allelic) antigens were compared with those who did not have high levels to any antigen to test the hypothesis that the breadth of specificities for unrelated antigens in the antibody response is important for protection. The risk of malaria was inversely associated with increasing breadth of antibody specificities in both study groups (Figure 4.4.2). None of the children in the Chonyi cohort who made high titre antibody responses to five or more antigens (17/119, 15%) experienced a clinical episode (P=0.0006 by Fisher’s Exact 2 tailed test). Similarly, in the case control study, none of the children who had high titre responses to five or more antigens (23/298, 7.7%) was admitted to hospital with severe malaria (P= 0.004 Fisher’s Exact 2 tailed test).
Figure 4.4.2 Protective efficacy of high titre antibodies and breadth of specificity

Protective efficacy increases with increasing breadth of specificity in children from the a) Chonyi (parasitaemic children, n=119), and b) hospital cohorts (all children, n=387). Each bar represents the comparison between individuals making high titre responses to ‘n’ number of antigens with those who make no responses to any antigen, 12% for the Chonyi cohort and 34% for the hospital cohort. Proportions above each bar are the percentage of individuals making high titre responses to ‘n’ antigens. The effect of high titre responses to P. falciparum schizont extract is also shown. The effect of high titre responses to P. falciparum schizont extract is also shown.
4.4.5 BREADTH OF ANTIBODY SPECIFICITY INCREASES WITH AGE AND CONCURRENT PARASITAEMIA

The breadth of high titre antigen-specific responses increased with age in both groups of children (Figure 4.4.3). Parasite positivity at the time of serum collection significantly increased the breadth of the response. In the Chonyi cohort, nearly three times as many children who were parasitaemic at the time of serum collection had high antibody titres to three or more antigens, compared to those who were aparasitaemic (47% (56/119) versus 17.3% (28/161), Pearson's chi-square 28.67, P < 0.001). This difference was more marked in the case-control study with over five times as many children who were parasitaemic at serum sampling having high titre responses to three or more antigens compared to those who were not (57% (102/176) versus 10.4% (30/287) Pearson's chi-square 120.77, P<0.001).
Figure 4.4.3 Breadth of antibody specificity and age

The breadth of antibody specificity increases with age in both the a) Chonyi cohort (n=119) and; b) case-control study (n=387). Older children make high titre antibody responses to an increasing number of antigens while younger children generally make high titre responses to fewer antigens.

4.4.6 COMBINATIONS OF ANTIBODIES AND PROTECTION

Interactions between antibodies were investigated to determine which combination(s) were associated with the lowest risk of clinical episodes in the Chonyi cohort. High levels of antibodies to combinations that included MSP-2, MSP-3 and AMA1 were associated with a lower risk of disease compared to their individual effects (Table 4.4.5). While the combined effect of antibodies was always greater than each of the individual effects, there was no statistical evidence of synergism or antagonism, i.e. more or less protection, respectively, than expected from the combination of the two antigens acting additively.

The strongest protection was associated with high levels of antibodies to both MSP-2 and
MSP-3. Thirty three children (33/119) had high antibody levels to both MSP-2 and MSP-3 and none of them experienced any episodes of disease (P<0.001 by Fisher's Exact 2 tailed test), still highly significant after a Bonferroni correction (Bland and Altman 1995) allowing for multiple comparisons (P=0.003). This finding was validated in the case-control study where admission to hospital with malaria was the end-point. Children who had high levels of antibodies to both MSP-2 and MSP-3 were significantly less likely to be admitted to hospital with malaria, odds ratio 0.26 (95% confidence interval 0.08 – 0.81), p = 0.020, Table 4.4.6.
### Table 4.4.5 Protective effects of combinations of high titre antibodies

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Main effects¹ &amp; Interactions² (X)</th>
<th>p value</th>
<th>Combination effects³</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA1_3D7</td>
<td>0.59(0.32-1.08)</td>
<td>0.091</td>
<td>0.21(0.05-0.88)</td>
<td>0.033*</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.56(0.29-1.08)</td>
<td>0.085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>0.26(0.06-1.29)</td>
<td>0.104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>0.66(0.35-1.25)</td>
<td>0.211</td>
<td>0.24(0.09-0.64)</td>
<td>0.004*</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>0.40(0.20-0.78)</td>
<td>0.008*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>0.33(0.09-1.17)</td>
<td>0.087</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>0.62(0.32-1.18)</td>
<td>0.147</td>
<td>0.61(0.29-1.29)</td>
<td>0.199</td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>0.69(0.33-1.42)</td>
<td>0.320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>1.60(0.34-10.46)</td>
<td>0.459</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>0.58(0.31-1.08)</td>
<td>0.088</td>
<td>0.48(0.17-1.38)</td>
<td>0.178</td>
</tr>
<tr>
<td>MSP-1_g</td>
<td>1.44(0.82-2.54)</td>
<td>0.199</td>
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</tr>
<tr>
<td>X</td>
<td>0.39(0.12-1.30)</td>
<td>0.129</td>
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<td></td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>0.40(0.20-0.78)</td>
<td>0.008*</td>
<td>total protection</td>
<td>n = 33</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.62(0.34-1.13)</td>
<td>0.121</td>
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<tr>
<td>X</td>
<td>0.26(0.05-1.27)</td>
<td>0.098</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>0.36(0.18-0.71)</td>
<td>0.003*</td>
<td>0.52(0.21-1.30)</td>
<td>0.166</td>
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<tr>
<td>MSP-1_g</td>
<td>1.55(0.92-2.61)</td>
<td>0.097</td>
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</tr>
<tr>
<td>X</td>
<td>0.62(0.20-1.88)</td>
<td>0.402</td>
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</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.57(0.29-1.11)</td>
<td>0.101</td>
<td>0.39(0.12-1.20)</td>
<td>0.103</td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>0.66(0.33-1.31)</td>
<td>0.240</td>
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<tr>
<td>X</td>
<td>0.71(0.17-2.97)</td>
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<tr>
<td>MSP-3_K1</td>
<td>0.48(0.25-0.93)</td>
<td>0.030*</td>
<td>0.57(0.25-1.31)</td>
<td>0.191</td>
</tr>
<tr>
<td>MSP-1_g</td>
<td>1.74(1.07-2.84)</td>
<td>0.024</td>
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<td></td>
</tr>
<tr>
<td>X</td>
<td>0.65(0.19-2.27)</td>
<td>0.509</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>0.56(0.28-1.11)</td>
<td>0.101</td>
<td>0.69(0.26-1.78)</td>
<td>0.445</td>
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<tr>
<td>MSP-1_g</td>
<td>1.61(0.97-2.68)</td>
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<td>X</td>
<td>0.70(0.19-2.52)</td>
<td>0.586</td>
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<tr>
<td>AMA1_3D7</td>
<td>0.55(0.30-1.01)</td>
<td>0.055</td>
<td>0.66(0.29-1.52)</td>
<td>0.336</td>
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<tr>
<td>MSP-1_B2</td>
<td>0.87(0.51-1.50)</td>
<td>0.636</td>
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</tr>
<tr>
<td>X</td>
<td>1.13(0.36-3.56)</td>
<td>0.829</td>
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</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>0.36(0.18-0.70)</td>
<td>0.008*</td>
<td>0.40(0.15-1.05)</td>
<td>0.064</td>
</tr>
<tr>
<td>MSP-1_B2</td>
<td>1.04(0.62-1.72)</td>
<td>0.875</td>
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<tr>
<td>X</td>
<td>0.57(0.17-1.90)</td>
<td>0.365</td>
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</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.52(0.27-1.02)</td>
<td>0.059</td>
<td>0.16(0.02-1.13)</td>
<td>0.067</td>
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<tr>
<td>MSP-1_B2</td>
<td>0.87(0.51-1.49)</td>
<td>0.634</td>
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<tr>
<td>X</td>
<td>0.15(0.02-1.14)</td>
<td>0.068</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP-1_g</td>
<td>1.61(0.95-2.74)</td>
<td>0.073</td>
<td>0.98(0.48-2.01)</td>
<td>0.972</td>
</tr>
<tr>
<td>MSP-1_B2</td>
<td>0.80(0.46-1.41)</td>
<td>0.456</td>
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</tr>
<tr>
<td>X</td>
<td>0.83(0.27-2.46)</td>
<td>0.737</td>
<td></td>
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</tr>
<tr>
<td>EBA-175_F2_3D7</td>
<td>0.58(0.30-1.14)</td>
<td>0.119</td>
<td>0.85(0.36-2.00)</td>
<td>0.720</td>
</tr>
<tr>
<td>MSP-1_B2</td>
<td>0.88(0.51-1.51)</td>
<td>0.656</td>
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</tr>
<tr>
<td>X</td>
<td>2.11(0.56-7.94)</td>
<td>0.265</td>
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</tr>
</tbody>
</table>
Protective effects of combinations of high titre antibodies. Risk of developing clinical malaria with combinations of high compared to low/undetectable antibodies to individual antigens in a subset of the Chonyi cohort (n=119). Figures are risk ratios (95% confidence intervals) and p values obtained from multivariate analyses (adjusting for both age and reactivity to *P. falciparum* schizont extract. ¹Main effects of antibodies to each antigen are adjusted for each other. ²Interaction (over and above main effects). ³Effects of combinations of high titre responses (combines main effects as well as interactions). "X" represents the estimates for the interaction between the two preceding antigens. In the majority of cases significantly more protection is obtained with high level antibodies to pairs of antigens, compared to single antigens (*Table 4.4.2*). No strong evidence of statistical interaction between pairs of antibodies is observed. *p <0.05*
### Table 4.4.6 Effects of combinations of high titre antibodies on admission to hospital with malaria

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Main effects &amp; interactions</th>
<th>p value</th>
<th>Combination effects</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMA1_3D7</strong></td>
<td>0.38(0.19-0.75)</td>
<td>0.006*</td>
<td>0.33(0.12-0.91)</td>
<td>0.034**</td>
</tr>
<tr>
<td><strong>MSP-3_3D7</strong></td>
<td>0.52(0.29-0.95)</td>
<td>0.035*</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>X</strong></td>
<td>0.81(0.22-2.91)</td>
<td>0.757</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AMA1_3D7</strong></td>
<td>0.38(0.19-0.77)</td>
<td>0.008*</td>
<td>0.20(0.08-0.52)</td>
<td>0.001*</td>
</tr>
<tr>
<td><strong>MSP-2_Dd2</strong></td>
<td>0.49(0.28-0.87)</td>
<td>0.015*</td>
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</tr>
<tr>
<td><strong>X</strong></td>
<td>0.37(0.11-1.15)</td>
<td>0.088</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AMA1_3D7</strong></td>
<td>0.38(0.19-0.73)</td>
<td>0.004*</td>
<td>0.47(0.16-1.35)</td>
<td>0.163</td>
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<tr>
<td><strong>EBA-175_F2_CAMP</strong></td>
<td>0.73(0.34-1.57)</td>
<td>0.429</td>
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</tr>
<tr>
<td><strong>X</strong></td>
<td>0.72(0.16-3.12)</td>
<td>0.666</td>
<td></td>
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</tr>
<tr>
<td><strong>AMA1_3D7</strong></td>
<td>0.36(0.18-0.72)</td>
<td>0.004*</td>
<td>0.38(0.10-1.42)</td>
<td>0.152</td>
</tr>
<tr>
<td><strong>MSP-1_B2_RO33</strong></td>
<td>1.14(0.60-2.14)</td>
<td>0.681</td>
<td>0.28(0.17-0.48)</td>
<td>0.470</td>
</tr>
<tr>
<td><strong>X</strong></td>
<td>0.34(0.07-1.58)</td>
<td>0.173</td>
<td>0.23(0.11-0.47)</td>
<td>0.552</td>
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<tr>
<td><strong>AMA1_3D7</strong></td>
<td>0.37(0.19-0.72)</td>
<td>0.004*</td>
<td>0.64(0.28-1.45)</td>
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<tr>
<td><strong>MSP-1_19</strong></td>
<td>0.79(0.42-1.50)</td>
<td>0.488</td>
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<tr>
<td><strong>X</strong></td>
<td>1.30(0.38-4.43)</td>
<td>0.668</td>
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<tr>
<td><strong>MSP-2_Dd2</strong></td>
<td>0.46(0.27-0.85)</td>
<td>0.013*</td>
<td>0.26(0.06-0.81)</td>
<td>0.020*</td>
</tr>
<tr>
<td><strong>MSP-3_3D7</strong></td>
<td>0.51(0.28-0.94)</td>
<td>0.033*</td>
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<tr>
<td><strong>X</strong></td>
<td>0.55(0.14-2.10)</td>
<td>0.387</td>
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<tr>
<td><strong>MSP-2_Dd2</strong></td>
<td>0.47(0.27-0.82)</td>
<td>0.008*</td>
<td>0.12(0.01-0.92)</td>
<td>0.042*</td>
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<tr>
<td><strong>EBA-175_F2_CAMP</strong></td>
<td>0.67(0.32-1.41)</td>
<td>0.296</td>
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</tr>
<tr>
<td><strong>X</strong></td>
<td>0.15(0.01-1.24)</td>
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<tr>
<td><strong>MSP-2_Dd2</strong></td>
<td>0.47(0.26-0.82)</td>
<td>0.009*</td>
<td>0.63(0.27-1.48)</td>
<td>0.295</td>
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<tr>
<td><strong>MSP-1_19</strong></td>
<td>0.76(0.39-1.48)</td>
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<tr>
<td><strong>X</strong></td>
<td>1.38(0.43-4.38)</td>
<td>0.578</td>
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<tr>
<td><strong>MSP-2_Dd2</strong></td>
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<td>0.008*</td>
<td>0.23(0.03-1.72)</td>
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<td><strong>MSP-1_B2_RO33</strong></td>
<td>0.99(0.54-1.83)</td>
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<td><strong>X</strong></td>
<td>0.24(0.03-2.00)</td>
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<tr>
<td><strong>MSP-3_3D7</strong></td>
<td>0.50(0.27-0.91)</td>
<td>0.024*</td>
<td>0.43(0.10-1.78)</td>
<td>0.250</td>
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<tr>
<td><strong>EBA-175_F2_CAMP</strong></td>
<td>0.68(0.32-1.44)</td>
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</tr>
<tr>
<td><strong>X</strong></td>
<td>0.95(0.18-4.86)</td>
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<td><strong>MSP-3_3D7</strong></td>
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<td>0.45(0.11-1.74)</td>
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<tr>
<td><strong>MSP-1_19</strong></td>
<td>0.68(0.35-1.30)</td>
<td>0.249</td>
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</tr>
<tr>
<td><strong>X</strong></td>
<td>0.95(0.20-4.39)</td>
<td>0.950</td>
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<tr>
<td><strong>MSP-3_3D7</strong></td>
<td>0.49(0.27-0.90)</td>
<td>0.022*</td>
<td>total protection</td>
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</tr>
<tr>
<td><strong>MSP-1_B2_RO33</strong></td>
<td>0.91(0.48-1.70)</td>
<td>0.775</td>
<td>total protection</td>
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</tr>
<tr>
<td><strong>X</strong></td>
<td>complete protection</td>
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<td></td>
<td>n= 13</td>
</tr>
<tr>
<td><strong>EBA-175_F2_CAMP</strong></td>
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<td>0.21(0.03-1.44)</td>
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<tr>
<td><strong>EBA-175_F2_CAMP</strong></td>
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<td>0.291</td>
<td>total protection</td>
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<td>0.98(0.52-1.83)</td>
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<td>total protection</td>
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<td>n= 14</td>
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<td><strong>MSP-1_19</strong></td>
<td>0.76(0.41-1.41)</td>
<td>0.392</td>
<td>0.81(0.21-3.04)</td>
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<td>0.98(0.52-1.66)</td>
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<tr>
<td><strong>X</strong></td>
<td>0.96(0.20-4.40)</td>
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</tbody>
</table>
Effects of combinations of high titre antibodies on admission to hospital with malaria. Risk of hospital admission with malaria with combinations of high compared to low/undetectable antibodies to pairs of antigens in the case-control study (n=387). Figures are risk ratios (95% confidence intervals) and p values. ¹Main effects of antibodies to each antigen are adjusted for each other. ²Interaction (over and above main effects). ³Effects of combinations of high titre responses (combines main effects as well as interactions). “X” represents the estimates for the interaction between the two preceding antigens. In the majority of cases significantly more protection is obtained with high titre responses to pairs of antigens, compared to individual antigens. No strong evidence of statistical interaction between antigens is observed. *p <0.05

4.5 DISCUSSION

We found that in two independent studies conducted in both high (chonyi cohort) and low transmission settings (case-control study) at different times, both the breadth of specificity for distinct merozoite antigens and the magnitude of antibody responses to these antigens provide robust predictors of immune status of children. High titre antibodies to combinations of three merozoite antigens in particular (AMA1, MSP-2 and MSP-3) were more strongly predictive of protection from clinical episodes of malaria compared to other putative “protective” merozoite antigens (MSP-1, EBA-175).
Out of the panel of malaria vaccine candidate antigens studied here, high levels of antibodies to combinations including AMA1, MSP-2 and MSP-3 were the most strongly associated with protection. This is consistent with other studies in which naturally-acquired antibodies to each of the three antigens individually have been associated with protection from clinical malaria in this, and other populations (al-Yaman, Genton et al. 1995; Taylor, Smith et al. 1995; Metzger, Okenu et al. 2003; Meraldi, Nebie et al. 2004; Polley, Mwangi et al. 2004; Singh, Soe et al. 2004; Soe, Theisen et al. 2004; Polley, Conway et al. 2006; Polley, Tetteh et al. 2007). Recently, long-term clinical protection was associated with IgG3 isotype antibodies to MSP-3 in Senegalese children (Roussilhon, Oeuveray et al. 2007). In contrast, antibodies to MSP-1 block 2, which have been associated with protection in two cohorts in West Africa (Conway, Cavanagh et al. 2000; Polley, Tetteh et al. 2003; Cavanagh, Dodoo et al. 2004) were not similarly protective in the two cohorts we studied from Kilifi, Kenya. Antibodies to MSP-119 have been associated with protection from clinical malaria in some studies, but not in others (Riley, Allen et al. 1992; Hogh, Marbiah et al. 1995; al-Yaman, Genton et al. 1996; Egan, Morris et al. 1996; Branch, Udhayakumar et al. 1998; Dodoo, Theander et al. 1999; Conway, Cavanagh et al. 2000; Cavanagh, Dodoo et al. 2004; Perraut, Marrama et al. 2005). This may be explained in part by the finding that the fine-specificity of anti- MSP-119 antibodies appears to be more important with regards to protection (Corran, O'Donnell et al. 2004; Okech, Corran et al. 2004). A separate study found that individuals with high titre anti- MSP-119 specific invasion-inhibitory antibodies were protected from infection (John, O'Donnell et al. 2004) and underscores the importance of developing robust functional assays for malaria. Antibodies to the F2 sub-domain of EBA-175 were not associated with protection from
clinical disease in our studies, as has been found in other parts of Africa where this (Okenu, Riley et al. 2000) and other sub-domains of EBA-175 have been studied (Okenu, Riley et al. 2000; Ohas, Adams et al. 2004; John, Moormann et al. 2005). To-date, only one study has reported significantly higher antibody levels to EBA-175 peptide 4 (1062 – 1103, within region V) in children protected from clinical attacks of malaria compared to susceptible children (Toure, Deloron et al. 2006).

The importance of allele-specific immunity was highlighted in the Combination B malaria vaccine trial in Papua New Guinea. Children who received this vaccine (containing a combination of *P. falciparum* ring-infected erythrocyte surface antigen (RESA), MSP-1 and the 3D7-allele of MSP-2) were less likely to be infected with parasites bearing the homologous allele of MSP-2 (Genton, Betuela et al. 2002), suggesting (as was later confirmed) that the vaccine had induced primarily allele-specific MSP-2 antibodies (Fluck, Smith et al. 2004). In the context of naturally-acquired infections, while some data suggest that parasites bearing specific genotypes induce allele-specific antibodies (Cavanagh, Elhassan et al. 1998; Kimbi, Tetteh et al. 2004; Polley, Conway et al. 2006), to our knowledge no studies have examined the protective effects of pre-existing allele-specific antibodies on subsequent disease caused by parasites bearing homologous alleles. We found that for most antigens tested, responses to allelic forms of each antigen had similar effects on the probability of mild or more severe malaria, suggesting the possibility that there may be significant cross-allele protection to clinical episodes. This issue is explored in detail in chapter 6, below.
In a study conducted in the Gambia, Gray et al. (Gray, Corran et al. 2007) found that while antibodies to a similar panel of individual antigens were only weakly correlated with protection, those to the combinations of AMA1 and MSP-2 were significantly associated with protection from clinical malaria. There are two important differences between this Gambian study and the ones reported here from Kenya. First, k-means clustering and phylogenetic networks were used to investigate associations between antibody reactivity profiles and clinical status in the Gambian cohort. These methods independently identified the group of children who were asymptomatic (asymptomatic parasitaemia, splenomegaly, or both) at the end of the study and had not apparently experienced clinical disease. This end-point differs from that of the studies reported here where outcome was simply defined as mild (Chonyi cohort) or severe (case-control study) malaria during the period of observation. Second, the magnitude of responses was not taken into account mainly because this generates increased individual differences, impairing cluster analysis. One other longitudinal study, carried out among children in Burkina Faso, examined antibodies to a different set of blood-stage malaria antigens (glutamate-rich protein (GLURP), P. falciparum exported protein-1 (PfExp-1), and MSP-3), and like our studies, they found that the simultaneous presence of antibodies to more than one antigen was associated with a lower frequency of malaria episodes (Meraldi, Nebie et al. 2004). However, in a separate study on protection from malaria infection as opposed to clinical episodes in Kenyan adults, John et al. (John, Moormann et al. 2005) found that high antibody titres to multiple blood-stage antigens were not protective (though there was evidence of protection for responses to pre-erythrocytic antigens). Our data suggest that the combination of blood-stage antigens analyzed in these Kenyan adults (AMA1, EBA-175 and MSP-1,9) may not...
have been optimal. While these studies are difficult to compare directly due to differences in study design, study populations and end-points, antigens tested and analytical methodologies, the picture that nevertheless emerges clearly is that antibodies to key combinations of multiple parasite targets are more strongly associated with protection from clinical malaria than those to individual antigens.

With the completion of the *P. falciparum* genome, numerous new (and old) antigens of the parasite have been identified and are being characterized. High throughput assays employing suspension array technology (Fouda, Leke et al. 2006) or micro-arrays (Sundaresh, Doolan et al. 2006; Gray, Corran et al. 2007) now allow for simultaneous analysis of antibodies to multiple antigens using minimal amounts of sera. This technology has not been matched with equivalently efficient tools for identifying protective immune responses. Robust concurrent analyses of numerous responses in relatively small studies, where children have been monitored longitudinally over a limited time-period for disease episodes remain challenging. The pair-wise analyses of combinations of high titre antibodies as presented here have obvious limitations when numerous antibodies are to be analyzed. Other analytical techniques such as clustering and the use of phylogenetic networks (Gray, Corran et al. 2007) while attractive for screening of potential vaccine candidates, similarly become more complex when increasing numbers of responses are analyzed and may well obscure genuinely ‘protective’ responses. New strategies to identify protective responses in humans are urgently needed.

Studies of associations between immune responses and clinical malaria need to take account of the possibility that any given response is merely a marker of cumulative
exposure (which is itself necessary to induce immunity) or of a response to an as yet unidentified antigen(s) that elicits strongly protective immunity. In our study, the fact that antibodies to specific antigens were more strongly predictive of protection than those to whole schizont extract (containing all the specific antigens and many other blood stage antigens) (Figure 4.4.1) suggest that specific responses do not merely reflect exposure. The finding that protective efficacy increased with increasing breadth of antibody specificity indicates that the effect of any one apparently protective response does not simply result from correlation with responses to other antigens (Figure 4.4.2), and argues for the interpretation that these are truly protective responses. Ultimately, the critical test of any such hypotheses will be to achieve equivalent protection through vaccination. Our demonstration of strong protection against malaria associated with high antibody levels to AMA1, MSP-2 and MSP-3 lends support to the development of vaccines based on combinations of these key malaria antigens.
5 COMPARISON OF ANTIBODIES IN CHILDREN PRESENTING WITH EITHER MILD OR SEVERE MALARIA

5.1 RATIONALE

In malaria endemic areas, the spectrum of infection with *Plasmodium falciparum* ranges from asymptomatic parasitaemia, through mild clinical episodes, to severe, and life-threatening disease. The reasons why some, but not all, children develop severe malaria are not well elucidated, but doubtlessly involve a complex interplay between host, parasite and environmental factors (Greenwood, Marsh et al. 1991; Lines and Armstrong 1992; Marsh 1992). More recently, the appreciation that severe *falciparum* malaria is a complex multi-system disorder, comprising much more than simply severe anaemia or cerebral malaria has increased, accompanied by the realization that the pathological processes leading to severe clinical manifestations are no less complex (Mackintosh, Beeson et al. 2004). However, regardless of the underlying mechanisms, early and more recent studies clearly demonstrated the protective role of anti-malarial antibodies against severe malaria, in both children and adults (Cohen, McGregor et al. 1961), (Sabchareon, Burnouf et al. 1991).

If antibodies play an important role in controlling or resolving acute infections with malaria, it might be expected that there would be consistent qualitative and/or quantitative differences in the humoral immune responses of children experiencing diverse clinical syndromes of malaria. However, several studies have reported conflicting data on the levels of antibodies among children with severe, as opposed to milder clinical
manifestations of malaria. Children with severe malaria have been reported to have higher (Tharavanij, Warrell et al. 1984; Cissoko, Daou et al. 2006)), lower (de Souza, Todd et al. 2002; Perraut, Diatta et al. 2005; Okech, Mujuzi et al. 2006; Dobano, Rogerson et al. 2008) or equivalent (Perraut, Diatta et al. 2005; Okech, Mujuzi et al. 2006) antibodies compared to controls with asymptomatic infections or mild malaria, and this appears to vary for different antigens. A potential limitation of these studies is the fact that at the time of an acute clinical event, the immune responses are not in steady state. On the other hand, it can also be argued that the acute clinical episode is in fact the ideal time point, as it captures children responding differentially to challenge, and therefore has greater potential to distinguish differences of direct clinical significance. Although it cannot be presumed that all malaria infections progress from being asymptomatic, to mild febrile episodes, before finally manifesting as severe malaria, it is reasonable to hypothesize that children (or adults) responding to challenge without displaying any clinical symptoms are making better immune responses than those who succumb to mild clinical symptoms, who in turn are making better responses than those presenting with severe and life-threatening malaria. In the previous chapter on multiple antigen responses, I found that the breadth and magnitude of antibody responses distinguished children who would go on to develop clinical episodes of malaria, from those that remained free of disease. In this study, I extend the previous work by exploring whether qualitative and/or quantitative differences in antibodies could distinguish children responding to natural challenge by developing either severe or mild malaria. I compare the evolution of total and sub-class IgG to the same panel of previously analyzed merozoite antigens, among children with the two
clinical syndromes in samples collected during the acute clinical episode and at convalescence.

5.2 AIMS

To determine whether there are quantitative and/or qualitative differences in antibodies to MSP-1 block 2, MSP-1t9, MSP-2, MSP-3, AMA1 and EBA-175 in the acute and convalescence samples of children experiencing either mild or severe clinical malaria.

5.3 METHODS

The study population has been previously described (section 3.2.1.2) and comprised the cases included in the case-control study, 89 of whom presented with malaria severe enough to warrant hospital admission, and 76 of whom were treated for mild malaria in the outpatients' department. The recombinant antigens, and antibody assays (total IgG and IgG sub-class) have also been described (sections 3.2.2 and 3.2.3).

5.4 RESULTS: COMPARISONS OF SEVERE VERSUS MILD MALARIA

5.4.1 STUDY POPULATION

As previously described (chapter 3.3.2), children with severe malaria were significantly younger than those presenting with mild malaria. As antibodies to the majority of antigens tested increased with age, this was taken into consideration by incorporating an age-stratified analysis, when comparing the antibodies between severe and mild malaria.
5.4.2 PREVALENCE OF HIGH TITRE IGG ANTIBODIES

The prevalence of high titre antibodies was compared amongst children presenting with either severe or mild malaria. In both groups of children, high titre antibodies to AMA1, MSP-1, and MSP-2 were the most common, followed by those to MSP-3, EBA-175 and finally MSP-1 block 2. For most antigens, the prevalence of high titre antibodies did not differ between the two groups, either in the acute (Figure 5.4.1), or the convalescence (Figure 5.4.2) samples. Although, high titre antibodies to the FVO and HB3 alleles of AMA1, as well as the 3D7 allele of the F2 sub-region of EBA-175, were significantly more common in children presenting with, mild as opposed to severe malaria (Figure 5.4.1 and Figure 5.4.2), this difference was not significant when age was taken into account in a regression analysis.
Figure 5.4.1 Antibody prevalence in children presenting with severe or mild malaria

Prevalence of high titre antibodies to a panel of merozoite antigens in samples collected during the acute clinical episode of children presenting with severe (n = 89) or mild (n = 76) malaria.
Figure 5.4.2 Antibody prevalence in children convalescing from severe or mild malaria

Prevalence of high titre antibodies to a panel of merozoite antigens in samples collected during convalescence in children presenting with severe (n = 89) or mild (n = 76) malaria.

5.4.3 IgG antibody levels

Overall, children with severe malaria had significantly lower antibody levels to most antigens, when compared to those with mild malaria. However, when age was taken into account this difference did not reach statistical significance (Table 5.4.1). However, these sub-group analyses are limited by the small sample numbers in each age category, as
illustrated using antibodies to AMA1_HB3 (Table 5.4.2). Although antibodies to AMA1_HB3 were significantly lower in children with severe as opposed to mild malaria overall, this difference did not reach significance when an age-stratified analysis was conducted (Table 5.4.2). Similar results were obtained at the convalescence time point (Table 5.4.1).

Table 5.4.1 Antibody levels in children presenting with severe or mild malaria

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Acute Mean antibody levels</th>
<th>Age-adjusted Mean antibody levels</th>
<th>Convalescence Mean antibody levels</th>
<th>Age adjusted Mean antibody levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe</td>
<td>Mild</td>
<td>p value</td>
<td>Severe</td>
</tr>
<tr>
<td>MSP-2_CHO150</td>
<td>0.68</td>
<td>0.98</td>
<td>0.042*</td>
<td>0.66</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>0.97</td>
<td>1.06</td>
<td>0.97</td>
<td>0.96</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>0.13</td>
<td>0.23</td>
<td>0.093</td>
<td>0.19</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>0.28</td>
<td>0.38</td>
<td>0.156</td>
<td>0.51</td>
</tr>
<tr>
<td>AMA1_HB3</td>
<td>0.96</td>
<td>1.32</td>
<td>0.014*</td>
<td>0.09</td>
</tr>
<tr>
<td>AMA1_FVO</td>
<td>0.82</td>
<td>1.11</td>
<td>0.009*</td>
<td>0.07</td>
</tr>
<tr>
<td>AMA1_3D7</td>
<td>0.96</td>
<td>1.18</td>
<td>0.119</td>
<td>0.421</td>
</tr>
<tr>
<td>EBA-175_3D7</td>
<td>0.11</td>
<td>0.12</td>
<td>0.697</td>
<td>0.864</td>
</tr>
<tr>
<td>EBA-175_CAMP</td>
<td>0.41</td>
<td>0.64</td>
<td>0.005*</td>
<td>0.047*</td>
</tr>
<tr>
<td>MSP-1_B2_K1</td>
<td>0.1</td>
<td>0.11</td>
<td>0.922</td>
<td>0.815</td>
</tr>
<tr>
<td>MSP-1_B2_MAD20</td>
<td>0.22</td>
<td>0.28</td>
<td>0.42</td>
<td>0.428</td>
</tr>
<tr>
<td>MSP-1_B2_RO33</td>
<td>0.05</td>
<td>0.11</td>
<td>0.265</td>
<td>0.238</td>
</tr>
<tr>
<td>MSP-1_19</td>
<td>1.16</td>
<td>1.27</td>
<td>0.534</td>
<td>0.726</td>
</tr>
</tbody>
</table>

Mean antibody levels were compared between children presenting with severe or mild malaria, in a univariate analysis, and then adjusted for age.
Table 5.4.2 Antibody levels to AMA1_HB3 in children with severe or mild malaria

<table>
<thead>
<tr>
<th>Number of children</th>
<th>Mean antibody levels</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Severe</td>
<td>Mild</td>
</tr>
<tr>
<td>1-2 yrs</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>2-3 yrs</td>
<td>35</td>
<td>19</td>
</tr>
<tr>
<td>3-4 yrs</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>&gt;4 yrs</td>
<td>11</td>
<td>18</td>
</tr>
</tbody>
</table>

Antibodies to AMA1_HB3 were significantly higher in children with severe malaria than in those with mild malaria, but these differences did not reach significance when an age-stratified analysis was performed.

5.4.4 CORRELATIONS BETWEEN ALLELE-SPECIFIC ANTIBODIES

The distribution of allele-specific antibodies was compared between children presenting with either severe or mild malaria, to determine whether antibodies to particular alleles were more commonly associated with either of the clinical phenotypes. Figure 5.4.3 and Figure 5.4.4 show that at all the loci tested, there was no apparent difference in the patterns of allele-specific antibodies generated, with children with both severe or mild malaria making similar responses to allelic versions of the same antigen.
Figure 5.4.3 Correlation between allele-specific antibodies at three loci in children with severe or mild malaria.
Figure 5.4.4 Correlation between allele-specific antibodies at two loci in children with severe or mild malaria

5.4.5 IgG SUB-CLASS ANTIBODIES

IgG subclass assays were performed on the acute and convalescence samples for antibodies to MSP-2, MSP-3 and EBA-175. The ratio of IgG1 to IgG3 sub-class antibodies was compared for these antigens between children presenting with severe or mild malaria, and no significant differences were observed, Table 5.4.3.
Table 5.4.3 IgG1:IgG3 ratios in children with severe or mild malaria

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Acute G1/G3 Ratio</th>
<th>Convalescence G1/G3 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe Mild p</td>
<td>Severe Mild p</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>1.74 1.93 0.609</td>
<td>N/D N/D</td>
</tr>
<tr>
<td>MSP-2_CHO150</td>
<td>4 2.9 0.367</td>
<td></td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>2.4 14.4 0.084</td>
<td>3.1 3.7 0.577</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>4.5 3.6 0.523</td>
<td>2.3 4.4 0.437</td>
</tr>
<tr>
<td>EBA-175_F2_CAMP</td>
<td>2.1 2.4 0.213</td>
<td>2.4 3 0.3427</td>
</tr>
</tbody>
</table>

Ratios of IgG1:IgG3 sub-class antibodies to merozoite antigens were compared among children presenting with severe or mild malaria, at the time of the acute episode and at convalescence.

5.5 DISCUSSION

In the previous analysis on antibodies to multiple merozoite antigens we found that the breadth and magnitude of the antigen response was a strong predictor of children who were protected from hospital admission with clinical malaria (chapter 4). This is in agreement with earlier studies that demonstrated the protective efficacy of malaria-immune antibodies in resolving both clinical symptoms, as well as high parasitaemias, in children and adults hospitalized with severe malaria (Cohen, McGregor et al. 1961; Sabchareon, Burnouf et al. 1991). Here, I explored a role for antibodies in preventing the progression from mild to severe disease, by comparing specific anti-malarial antibodies in children presenting with either clinical syndrome. I measured total IgG and IgG sub-class antibodies to the same panel of merozoite antigens already described (Chapter 4). Children presenting with severe or mild malaria did not differ significantly for any of the variables tested, including the prevalence and titre of IgG antibodies, the distribution of allele-
specific antibodies at particular loci, and the ratio of cytophilic antibodies (IgG1:IgG3). However, when the data were adjusted for age, although the difference was not statistically significant, children with severe malaria consistently had lower mean antibody levels than those with mild malaria.

In previous studies where antibodies were compared amongst children with severe, or mild malaria, conflicting results have been reported. For antibodies to GPI for example, three different outcomes were obtained when children with severe malaria were compared to those with mild or uncomplicated malaria. De souza et al. found no differences in the prevalence or levels of anti-GPI antibodies in Gambian children with severe or uncomplicated malaria (de Souza, Todd et al. 2002), while Perraut et al. (Perraut, Diatta et al. 2005) and Cissoko et al. (Cissoko, Daou et al. 2006) found that children with severe malaria had lower, and higher levels, respectively, of anti-GPI antibodies compared to those with uncomplicated malaria. Other studies are difficult to compare directly because antibodies have been assayed to different antigens, at different time points, and the definitions for severe and uncomplicated malaria are not standardized (Tharavanij, Warrell et al. 1984; Brasseur, Ballet et al. 1990; Al-Yaman, Genton et al. 1997; Luty, Ulbert et al. 2000; Perraut, Diatta et al. 2005; Okech, Mujuzi et al. 2006; Dobano, Rogerson et al. 2008). Nevertheless, no clear picture emerges, and different results are obtained for different antigens in the same study, and different antigens in different studies, and range from findings that antibody levels are comparable in severe versus mild malaria, to being higher in the former compared to the latter, and vice-versa. What appears to be consistent, is that the presence of parasites, regardless of the accompanying clinical phenotype, is an
important determinant of anti-malarial antibodies, with parasitaemic individuals often having higher titres of antibodies to most antigens, compared to age-matched, healthy, aparasitaemic controls (Luty, Ulbert et al. 2000; de Souza, Todd et al. 2002; Cissoko, Daou et al. 2006).

The results of my study were limited mainly by the small sample size, which neither allowed sufficient power for an age-stratified analysis of antibody responses, nor a sub-group analysis of the specific syndromes that comprise severe malaria, such as cerebral malaria, severe anaemia or respiratory distress. A further limitation was that "hospital admission with malaria" was the criterion used to define severe malaria, and this may not have allowed for a clear distinction between the two clinical phenotypes. However, even in the studies where the syndromes of severe malaria were reasonably well-defined, for instance, cerebral malaria, or severe anaemia, conflicting results were still obtained with regards to antibodies between these children and those with uncomplicated malaria (de Souza, Todd et al. 2002; Perraut, Diatta et al. 2005; Cissoko, Daou et al. 2006; Okech, Mujuzi et al. 2006; Dobano, Rogerson et al. 2008).

In conclusion, I found no evidence of a quantitative, and/or qualitative differences in antibodies drawn from children experiencing severe, or mild clinical episodes of malaria.
LACK OF EVIDENCE OF ALLELE-SPECIFIC PROTECTION AGAINST CLINICAL EPISODES OF MALARIA ASSOCIATED WITH PARASITES BEARING HOMOLOGOUS ALLELES.

6.1 INTRODUCTION

Many candidates for sub-unit malaria vaccines are polymorphic, posing considerable challenges for vaccine development. It is not clear how many alleles of a particular candidate will need to be included in a vaccine, to induce antibodies with specificity broad enough to counter the antigenic diversity present in malaria-endemic populations, and how this will change with time. Natural populations of *P. falciparum* have high recombination rates (Conway, Roper et al. 1999), and the ability to readily generate further diversity with every meiotic recombination (Walliker, Quakyi et al. 1987; Wellems, Panton et al. 1990; Rosenberg, Rungsiwongse et al. 1992; Babiker, Ranford-Cartwright et al. 1994). This situation is exemplified by AMA1, which exhibits numerous distinct haplotypes, particularly among isolates from areas of high malaria transmission (Polley and Conway 2001; Cortes, Mellombo et al. 2003; Polley, Chokejindachai et al. 2003; Garg, Alam et al. 2007). This is a genuine concern, as it has been shown in animal models of malaria, that immunization with AMA1 (Crewther, Matthew et al. 1996; Hodder, Crewther et al. 2001), or MSP-119 (Renia, Ling et al. 1997; Rotman, Daly et al. 1999) confers significantly better protection against challenge with parasites bearing homologous, as opposed to heterologous AMA1 or MSP-119 alleles, respectively. The implications of this for vaccine development are obvious. Furthermore, there are concerns that vaccination will select for
parasites bearing alternative alleles, as seemed to be the case in the phase IIb combination B malaria vaccine trial, where immunization with the 3D7-like allele of MSP-2, resulted in a preponderance of parasites bearing FC27-like alleles in a sub-group of vaccinees (Genton, Betuela et al. 2002). Although this was a small study, it nevertheless provides the best 'proof-of-principle' of vaccine-induced selection of malaria parasites in a human study, and highlights the need for molecular monitoring of malaria vaccines which has been implemented in sites where malaria vaccines are being tested (Takala, Coulibaly et al. 2007).

Evidence from both experimentally induced malaria and epidemiological observations in endemic areas has often been said to support the idea that immunity to malaria is to an important extent "strain specific" (Covell and Nicol 1951; Contamin, Fandeur et al. 1996; Daubersies, Sallenave-Sales et al. 1996; Ofosu-Okyere, Mackinnon et al. 2001; Magesa, Mdira et al. 2002). However the concept of a malaria 'strain' is poorly defined and has been contentious (for an excellent historical review, see (McKenzie, Smith et al. 2008)). Allele-specificity on the other hand, is more readily defined, and refers to the genotype of a parasite isolate at a precise molecular locus. Allele-specific immune responses are thus those stimulated by parasites bearing a particular allele at a given locus, and have been demonstrated for antibodies to AMA1 (Polley, Mwangi et al. 2004), MSP1-block 2 (Cavanagh, Elhassan et al. 1998), MSP-2 (Ranford-Cartwright, Taylor et al. 1996), MSP-3 (Polley, Tetteh et al. 2007), among others. We investigated whether pre-existing naturally-acquired allele-specific antibodies to four polymorphic malaria vaccine candidate antigens (AMA1, MSP-2, MSP-3, MSP-1 block 2), prevented disease associated with parasites bearing
homologous alleles, in Kenyan children. We analyzed antibodies to proteins representing the main allelic families of these candidates, MSP-2 (IC1-like and FC27-like), MSP-3 (K1-like and 3D7-like), and MSP-1 block 2 (K1-like, MAD20-like, RO33-like), and genotyped disease-associated parasites at the corresponding loci. For AMA1, antibodies were analyzed to proteins representing three available allelic versions of AMA1 (FVO, 3D7 and HB3), and sequencing was performed for parasite isolates. As alleles of AMA1 cannot be readily grouped into main allelic families, a different analysis was performed for antibodies to AMA1 and is presented separately in chapter 7.

6.2 AIMS

1. To determine whether pre-existing naturally-acquired allele-specific antibodies to MSP-2, MSP-3 and MSP-1 block 2, protect against clinical episodes of malaria associated with parasites bearing homologous alleles at the corresponding loci.

2. To determine whether infections with parasites bearing specific genotypes induce the corresponding allele-specific antibody responses in the acute and convalescence samples of patients presenting with malaria.

6.3 METHODS

The study population consisted of the cases from the case-control study (Section 3.2.1.2). The PCR based methods for parasite genotyping and ama1 sequencing, as well as the protocols for the ELISA antibody assays, including Competition ELISA have also been described (Sections 3.2.5, 3.2.6 and 3.2.3, respectively).
6.3.1 **Statistical Analysis**

For MSP-1 block 2, MSP-2 and MSP-3, three sets of analyses were performed, in a similar fashion for the pre-season, acute and convalescence samples. To determine whether pre-existing allele-specific antibodies protected against clinical episodes with parasites bearing homologous alleles, the proportions of acute clinical episodes with parasites bearing specific alleles were compared in the pre-season sample of children with, or without high titre antibodies to the corresponding homologous antigen using chi squared analyses. To determine whether parasite genotypes induced the corresponding allele-specific antibodies, the proportion of antibodies detected (defined as sero-positivity) was compared between children who had, or did not have infections with parasites bearing the homologous genotype in samples taken at the acute clinical episode and convalescence. Similar sub-group analyses were performed among children with severe or mild malaria, and among children in one year age group categories.

6.4 **Results**

6.4.1 **Study Patients.**

The characteristics of all study patients recruited into the case-control study have been described (Section 3.3.2). Parasite DNA was available for 138/165 (84%) individuals, from the first clinical episode of malaria in the eight month follow up period. Missing parasite isolates had either been used up in other unrelated experiments, or there had been difficulties in obtaining the sample when the patient presented to hospital. Antibody data were missing for 20 convalescence samples as the patients did not keep their follow-up appointments. The baseline characteristics were comparable between children for whom
there were no parasite isolates (n=27), compared to those for whom parasite DNA was available (n = 138) (data not shown). For the analyses of MSP-1 block 2, MSP-2 and MSP-3, data are presented for these 138 individuals.

6.4.2 Antibody prevalence and prevalence of allelic types at specific loci.

The prevalence of high titre antibodies to all the antigens tested at three time points is shown in Table 6.4.1. For most antigens, the prevalence of high titre antibodies was higher in the acute samples as compared to the pre-season samples. In samples taken at convalescence (three weeks after the acute episode), the prevalence of high titre antibodies had not significantly declined, and was comparable to that in the acute samples (Table 6.4.1). For all antigens except MSP-1 block 2, antibody prevalence was increased with age at all three time points (data not shown). The prevalence of allelic types at each locus is shown in Table 6.4.2. The majority of infections contained parasites bearing IC-1-like alleles at the msp2 locus (94.2%), and the K1-like allele at the msp1 block 2 locus (91.3%). At the msp3 locus 70.3% and 59.4% of infections bore the 3D7- and K1-like alleles respectively. The highest proportion of mixed infections (containing alternative allelic types) was observed at the msp2 locus. Although the study was not designed to detect the absolute number of parasite clones detected in the samples, for msp2, multiple bands were commonly observed within each allelic type (ie multiple variants of the same allelic type). At a population level, overall antibody levels to allelic versions of MSP-3 reflected the prevailing genetic allelic frequencies in the parasite population at this locus. However, this was not observed for either MSP-2 or MSP-1 block 2. Although 94.2% of typed infections contained an IC-1-like allele at the msp2 locus, compared to 67.4% with FC-27-like alleles,
antibody levels were higher against the FC-27-like alleles, at all the sampling times.

Similarly, at the *msp1 block 2* locus, the K1-like alleles were the most commonly detected, followed by RO33-like and MAD20-like, whilst the highest antibody levels were observed against the MAD20 antigen, followed by the K1, and the RO33 antigens, at all time points.

**Table 6.4.1 Prevalence of high titre antibodies at three time points**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Pre-season (n = 138)</th>
<th>Acute (n = 138)</th>
<th>Convalescence (n = 122)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-2_CHO150</td>
<td>19.57</td>
<td>43.48*</td>
<td>44.26</td>
</tr>
<tr>
<td>MSP-2_Dd2</td>
<td>26.02</td>
<td>49.28*</td>
<td>42.62</td>
</tr>
<tr>
<td>MSP-3_3D7</td>
<td>21.74</td>
<td>31.88</td>
<td>45.08</td>
</tr>
<tr>
<td>MSP-3_K1</td>
<td>21.01</td>
<td>21.01</td>
<td>27.87</td>
</tr>
<tr>
<td>MSP-1_B2_3D7</td>
<td>13.04</td>
<td>7.97</td>
<td>6.56</td>
</tr>
<tr>
<td>MSP-1_B2_MAD20</td>
<td>16.67</td>
<td>14.49</td>
<td>8.20</td>
</tr>
<tr>
<td>MSP-1_B2_RO33</td>
<td>10.14</td>
<td>12.32</td>
<td>8.20</td>
</tr>
</tbody>
</table>

* p <0.05, for comparisons between pre-season and acute.
Table 6.4.2 Prevalence of allelic types at three *P. falciparum* loci

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Prevalence% (n/138)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>msp-2</em></td>
<td><em>IC1</em>-like</td>
<td>94.2 (130)</td>
</tr>
<tr>
<td></td>
<td><em>FC-27</em>-like</td>
<td>67.4 (93)</td>
</tr>
<tr>
<td></td>
<td>mixed <em>IC1</em>- &amp; <em>FC-27</em>-like</td>
<td>61.6 (85)</td>
</tr>
<tr>
<td><em>msp-3</em></td>
<td>3D7*-like</td>
<td>70.3 (97)</td>
</tr>
<tr>
<td></td>
<td><em>K1</em>-like</td>
<td>59.4 (82)</td>
</tr>
<tr>
<td></td>
<td>mixed 3D7*- &amp; <em>K1</em>-like</td>
<td>29.7 (41)</td>
</tr>
<tr>
<td><em>msp-1</em> block 2</td>
<td><em>K1</em>-like</td>
<td>91.3 (126)</td>
</tr>
<tr>
<td></td>
<td><em>MAD20</em>-like</td>
<td>58.7 (81)</td>
</tr>
<tr>
<td></td>
<td><em>RO33</em>-like</td>
<td>65.2 (90)</td>
</tr>
<tr>
<td></td>
<td>all three block 2</td>
<td>38.4 (53)</td>
</tr>
</tbody>
</table>

6.4.3 HIGH TITRE ALLELE-SPECIFIC ANTIBODIES DO NOT PROTECT AGAINST DISEASE ASSOCIATED WITH PARASITES BEARING HOMOLOGOUS ALLELES

To determine whether pre-existing allele-specific antibodies were protective against clinical episodes associated with parasites bearing homologous genotypes, the proportions of specific parasite genotypes were compared amongst individuals with high or low titres of homologous antibodies in the pre-season serum sample. As shown in Table 6.4.3, at each of the three loci tested (*msp-1 block 2, msp-2* and *msp-3*), there was no difference in the prevalence of parasites of a specific genotype, among individuals who had, or did not have, high titres of pre-existing homologous antibodies (chi squared test, *p* > 0.05, all pairwise comparisons). Similar findings were obtained when the analysis was repeated for children developing severe, or mild malaria, and for children in different age groups (one year categories from 1 to 4 and over). However, the sample sizes for these sub-group
analyses were small, and for the \textit{msp-1 block 2} locus, this was further compounded by the low number of high titre responses to these antigens.

Table 6.4.3 Pre-existing antibodies and clinical disease with parasites bearing homologous genotypes

<table>
<thead>
<tr>
<th>Locus</th>
<th>Alleles</th>
<th>Antibodies</th>
<th>Preseason^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{msp-2}</td>
<td>\textit{IC1-like}</td>
<td>MSP-2_CHO150_low</td>
<td>94.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-2_CHO150_high</td>
<td>92.6</td>
</tr>
<tr>
<td>\textit{FC-27-like}</td>
<td></td>
<td>MSP-2_Dd2_low</td>
<td>69.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-2_Dd2_high</td>
<td>61.1</td>
</tr>
<tr>
<td>\textit{msp-3}</td>
<td>\textit{3D7-like}</td>
<td>MSP-3_3D7_low</td>
<td>67.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-3_3D7_high</td>
<td>80.0</td>
</tr>
<tr>
<td></td>
<td>\textit{K1-like}</td>
<td>MSP-3_K1_low</td>
<td>57.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-3_K1_high</td>
<td>65.5</td>
</tr>
<tr>
<td>\textit{msp-1_B2}</td>
<td>\textit{K1-like}</td>
<td>MSP-1_B2_K1_low</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-1_B2_K1_high</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td>\textit{MAD20-like}</td>
<td>MSP-1_B2_MAD20_low</td>
<td>58.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-1_B2_MAD20_high</td>
<td>60.9</td>
</tr>
<tr>
<td></td>
<td>\textit{RO33-like}</td>
<td>MSP-1_B2_RO33_low</td>
<td>63.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSP-1_B2_RO33_high</td>
<td>78.6</td>
</tr>
</tbody>
</table>

The proportions of individuals developing clinical episodes associated with parasites bearing specific allelic types at three loci were compared among individuals with pre-existing high versus low titres of the homologous allele-specific antibodies. ^1 Samples collected at the beginning of the malaria transmission season.
6.4.4 Antibodies to allele-specific epitopes determined by competition ELISA do not appear to protect against disease associated with parasites bearing homologous alleles.

To confirm that the lack of protection observed with high titre antibodies was due to antibodies targeted specifically to allele-specific as opposed to conserved epitopes, competition assays were performed for allelic versions of MSP-2 and MSP-3 antigens, both of which have shared or conserved epitopes between alleles. For MSP-2_CHO150, 25 children were identified who had high titre antibodies against this allele, but nevertheless experienced disease episodes with parasites bearing the homologous IC1-like alleles. Of these, 12 children had low titre antibodies to the alternative MSP-2_Dd2 antigen (and could be presumed to have mainly allele-specific antibodies to MSP-2_CHO150), while 13 had high titre antibodies to antigens of both allelic types. Competition assays (competing out antibodies to conserved epitopes by pre-incubating these sera with an excess of MSP-2_Dd2) indicated that the majority of children with high titre antibodies to MSP-2_CHO150 (10/12 of those with low antibodies to the alternative allelic antigen, and 10/13 of those with high titre antibodies to both antigens) did in fact have antibodies directed against allele-specific epitopes. Similarly for children with high titre antibodies against MSP-2_Dd2, 22 children were identified, 13 of whom had low titre antibodies to the alternative MSP-2_CHO antigen, while 9 of whom had high titre antibodies to both. Competition assays (this time pre-incubation with an excess of the alternative MSP-2_CHO150 antigen) likewise indicated that the majority of these (9/13 of those low titre antibodies to the allelic antigen, and 5/9 of those with high titre antibodies to both antigens) contained antibodies directed against allele-specific and not conserved epitopes. These results
confirm that in these children antibodies to allele-specific epitopes did not protect against parasites bearing those genotypes. For MSP-3 there was little evidence of reactivity to allele-specific epitopes among the selected samples (2/43, for both allelic types). This may have been due to the fact that for most of these samples the ELISA OD values were close to the threshold for high antibody titres for both allelic types, making it difficult to discriminate antibodies to allele-specific epitopes. Additionally, a difference of at least 0.3 ELISA OD units between heterologous and homologous competition assays was counted as substantial evidence of allele-specific reactivity.

**6.4.5 ALLELE-SPECIFIC ANTIBODIES AS A RESULT OF CONCURRENT INFECTIONS**

We determined whether allele-specific antibodies were generated by parasites bearing homologous genotypes in concurrent infections (acute clinical episode), and at convalescence (ie concordant genotype-antibody relationships). We compared the proportions of individuals who were sero-positive between individuals infected with, and without, parasites bearing homologous alleles, at the acute and convalescence time points. Allele-specific antibodies to MSP-3_K1 and MSP-2_Dd2 were significantly more common amongst individuals presenting with clinical episodes associated with parasites bearing homologous alleles at both the acute and convalescence time points (Table 6.4.4). The same was not observed for the alternative versions of these two antigens (the alleles of which also occur at higher frequencies in this population), and neither was it found for antibodies against MSP-1 block 2. No differences were observed between children with severe and mild malaria, or between various age categories.
Table 6.4.4 Parasite genotypes and homologous allele-specific antibodies

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Acute episode</th>
<th>Convalescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele neg.</td>
<td>Allele pos.</td>
<td>p</td>
</tr>
<tr>
<td>msp-2</td>
<td>IC1-like</td>
<td>87.5</td>
<td>85.5</td>
</tr>
<tr>
<td></td>
<td>FC-27-like</td>
<td>71.1</td>
<td>92.5</td>
</tr>
<tr>
<td>msp-3</td>
<td>3D7-like</td>
<td>80.5</td>
<td>70.1</td>
</tr>
<tr>
<td></td>
<td>K1-like</td>
<td>23.2</td>
<td>39.0</td>
</tr>
<tr>
<td>msp-1_B2</td>
<td>K1-like</td>
<td>8.3</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>MAD20-like</td>
<td>15.8</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>RO33-like</td>
<td>8.3</td>
<td>14.4</td>
</tr>
</tbody>
</table>

The proportions of children sero-positive for homologous antibodies were compared between those with, and without the corresponding parasite genotypes, in the acute and convalescence samples. * p < 0.05

6.5 DISCUSSION

We have previously shown that high titre antibodies to some putative vaccine antigens are associated with protection from clinical episodes of malaria (Osier, Fegan et al. 2008). These antigens are polymorphic and there is strong evidence that antigenic diversity has been driven by immune pressure (Conway and Polley 2002). In the current study we sought evidence that such protection is allele specific. For three antigens that exist in a limited number of major allelic forms, MSP-1 block 2, MSP-2, and MSP-3, we found that pre-existing high titre allele-specific antibodies did not differentially protect children against clinical episodes associated with parasites bearing homologous alleles. We obtained the
same results when the data were analyzed against sero-positivity, as conventionally defined.

These data are striking in the light of the evidence for immune selection on parasite diversity (Conway and Polley 2002) and the paradigm of "strain" specific immunity being important in protection from malaria. In malaria-endemic areas, particularly where the transmission intensity is high, both children and adults harbor asymptomatic infections, comprising complex mixtures of distinct parasite clones, which fluctuate over a matter of months, weeks, and even, days (Daubersies, Sallenave-Sales et al. 1996; Farnert, Snounou et al. 1997). These longitudinal studies have also shown that clinical episodes are often associated with an abrupt increase in parasite densities (Contamin, Fandeur et al. 1996), in which novel parasite clones are commonly detected (Contamin, Fandeur et al. 1996; Ofosu-Okyere, Mackinnon et al. 2001; Magesa, Mdira et al. 2002), consistent with the idea of 'strain'-specific immunity, limiting the growth of some, but not all parasites. While the loci used to detect the presence of distinct parasite clones or 'strains', are often identical to those that encode for antigens that are considered as targets of protective immunity against clinical episodes of malaria (MSP-1 block 2, MSP-2, MSP-3), 'strain'-specific immunity is not synonymous with allele-specific immunity, the latter at best being a 'subset' of the former. None the less, when protective antibody responses are directed to polymorphic antigens, it might be expected that the polymorphism is driven by immune pressure and that protection would be greater against disease episodes caused by homologous parasites.
Previous studies in which both the infecting parasites were genotyped, and the naturally-acquired antibody specificities to MSP-1 block 2 or MSP-2 monitored longitudinally, did not address the question of whether allele-specific antibodies protected against (re-)infection or clinical episodes with parasites bearing homologous alleles (Cavanagh, Elhassan et al. 1998; Jouin, Rogier et al. 2001; Weisman, Wang et al. 2001). In a sub-group analysis (n=5), Jouin et al. analyzed genotype-antibody relationships over a period of 15 months, and found that for all individuals, (ages 7 – 50 years), allele-specific antibodies were unrelated to the genotypes of previous, concurrent or subsequent infections (Jouin, Rogier et al. 2001). In this study, antibodies were tested to a panel of overlapping 15-mer peptides (n=82), including allelic variants of MSP-1 block 2 (Jouin, Rogier et al. 2001), and not to recombinant E. coli expressed antigens, as in our study.

Most studies in which both information on the infecting parasite genotype and corresponding allele-specific antibodies is available, have analyzed genotype-antibody relationships in concurrent samples (Da Silveira, Dorta et al. 1999; Tami, Grundmann et al. 2002; Kanunfre, Leoratti et al. 2003; Kimbi, Tetteh et al. 2004; Polley, Conway et al. 2006; Osier, Polley et al. 2007), or in acute-convalescent pairs of samples (Weisman, Wang et al. 2001; Ekala, Jouin et al. 2002; Ekala, Jouin et al. 2002), with mixed results. In general, it appears that at a population level, the prevalence of allele-specific antibodies reflects the genetic allele-frequencies of the parasites in that population (Da Silveira, Dorta et al. 1999; Tami, Grundmann et al. 2002; Kanunfre, Leoratti et al. 2003; Osier, Polley et al. 2007) and this is most readily observed in areas of comparatively low malaria endemicity, such as Venezuela (Tami, Grundmann et al. 2002) and Brazil (Kanunfre, Leoratti et al. 2003). At an
individual level, concordant genotype-antibody relationships seem to be more readily detected in areas with lower (Cavanagh, Elhassan et al. 1998; Tami, Grundmann et al. 2002), as opposed to those with higher malaria transmission (Polley, Conway et al. 2006). Thus, in studies conducted with primary malaria infections in returned travellers, there was a strong, though not absolute, concordance between the infecting parasite genotype and the corresponding allele-specific antibodies (Felger, Steiger et al. 2003; Eisen, Wang et al. 2007). Other studies have failed to find relationships between the infecting parasite genotypes and the corresponding allele-specific antibodies at the individual level (Jouin, Rogier et al. 2001; Weisman, Wang et al. 2001; Ekala, Jouin et al. 2002; Ekala, Jouin et al. 2002; Osier, Polley et al. 2007). In one study, the main determinant of the observed high prevalence of allele-specific antibodies appeared to be the high incidence of past infections, as opposed to the presence of, or genotypes contained within, current infections (Kimbi, Tetteh et al. 2004). In our study, the levels of antibodies to MSP-2 and MSP-3 largely reflected the allele-frequencies of parasites in the population at the time the acute and convalescence samples were collected, providing some evidence of the induction of allele-specific antibodies following infection. This was particularly true for the corresponding allelic types present at a lower frequency in this population. For the more common allelic types at these loci, such correlations are likely to be masked by a high incidence of previous infections with parasites bearing those alleles, as has been observed in the study from Cameroon (Kimbi, Tetteh et al. 2004).

The allele frequencies of the 3D7- and K1-like alleles at the msp3 locus were different to those obtained approximately 5 years later in the same area (Osier, Polley et al. 2007), with
a lower allele-frequency of K1-like types observed in the later study. Temporal variation in
the distribution of alleles has also been observed at the *msp1* block 2 locus in isolates from
the Brazilian Amazon, taken nearly ten years apart (Da Silveira, Dorta et al. 1999). This may
not be surprising, as high recombination rates have been reported for natural populations
of *P. falciparum*, particularly in areas of high malaria endemicity (Conway, Roper et al.
1999), and for *msp-1* in particular, recent studies show that frequent recombination events
generate novel alleles in high transmission areas (Tanabe, Sakihama et al. 2007; Tanabe,
Sakihama et al. 2007). It is not clear whether recombination would similarly contribute to
the altered allele frequency at the *msp-3* locus, or whether there is some other explanation.

A limitation of our study was the inability to determine the dominant parasite clone. This
relates to the PCR method of genotyping, which is semi-quantitative, with the intensity of
the band on the gel (PCR product) approximately correlating with the amount of starting
parasite DNA. This ability of PCR to be semi-quantitative is generally lost when nested PCRs
are performed, as in our study (Contamin, Fandeur et al. 1995; Contamin, Fandeur et al.
1996; Mercereau-Puijalon 1996). As such, in mixed infections, we were not able to
determine which parasite clone was dominant, and thus more likely to be causally
responsible for the observed clinical episode.

Overall the most striking result from our studies is the apparent absence of evidence for
allele specific protection for antibodies directed against several candidate vaccine antigens
despite the fact that overall responses to at least two of these antigens (MSP-2, MSP-3)
were shown in same population to be strongly protective against malaria. It is noteworthy
that we have previously shown that the protective effect of these antigen specific responses did not depend on the allelic form of the antigen used to measure the responses. This in itself is not, of course, evidence against the importance of allele specific protection because responses to one allelic form are highly correlated with another due to sharing of conserved sequences. Thus in a situation where individuals have experienced several infections with parasites bearing different allelic versions of a key antigen, the individual may have high titres of antibody detected against any variant but protection in any one instance may still be due to allele specific effects not detectable at the gross level of amount of antibody. Such effects could only be detected if one had a functional assay directly related to the mechanism of protection. However, it is perhaps more surprising that in individuals with high titre antibodies to a given allele, there was no evidence of differential protection against parasites bearing the homologous allele. This is in marked contrast to the situation in relation to antibody responses to variant surface antigens on the infected red cell surface (Bull and Marsh 2002). One possible interpretation is that allele diversity is not in fact driven by immune pressure but by other functional differences. However there is no evidence for this and perhaps more likely is the possibility that the differential protection necessary to drive diversity need only be marginal and could not be detected in this kind of study. If this were the case it might carry a hopeful message for vaccine development in that diversity may not in practice be as limiting as usually assumed.
7 HAPLOTYPES OF AMA1 AND CLINICAL PHENOTYPES

7.1 INTRODUCTION

The severity of a clinical episode of malaria in an individual child is determined by a complex interplay of multiple factors, including those specific to the host, the parasite, as well as environmental factors (Marsh 1992). With regards to the parasite factors, there is conflicting evidence that specific allelic types of various merozoite antigens, including MSP-1, MSP-2 and AMA-1 are found more commonly amongst individuals with more severe clinical manifestations of malaria compared to those with milder presentations of malaria (Engelbrecht, Felger et al. 1995; Robert, Ntoumi et al. 1996; Kun, Schmidt-Ott et al. 1998; Ofosu-Okyere, Mackinnon et al. 2001; Cortes, Mellombo et al. 2004). For some antigens, such as MSP-1 or MSP-2, allelic variants can usually be classified into between two and four major allelic types or families. For AMA1, the situation is not as straightforward, as alleles of AMA1 do not cluster into major allelic types, making it difficult to assess the question whether specific alleles of AMA1 are over-represented amongst different clinical phenotypes of malaria. 'Haplotypes' of AMA1 can be defined at multiple levels including, the entire ectodomain sequence (Polley and Conway 2001; Polley, Chokejindachai et al. 2003), sub-domain sequences (I, II, or III) (Cortes, Mellombo et al. 2003; Garg, Alam et al. 2007), clusters of antibody (Dutta, Lee et al. 2007) or T-cell (Lal, Hughes et al. 1996) epitopes, and even as polymorphic residues at single amino acid positions (Cortes, Mellombo et al. 2003).
In a study from Papua New Guinea, Cortes et al. compared the frequency of particular amino acids at specific sites within domain I, in symptomatic and asymptomatic infections, and found a strong imbalance, with particular residues being over-represented in alleles from the former, compared to those from the latter, particularly in children less than 10 years old (Cortes, Mellombo et al. 2003). More recently, Dutta et al. analyzed the contribution of 24 polymorphic sites across the three sub-domains of AMA1 to invasion-inhibition, and defined specific clusters of residues that had the highest inhibitory contribution (Dutta, Lee et al. 2007). They hypothesized that the polymorphic sites within these inhibitory epitopes functioned as antigenic escape residues (AER) and proposed the genotyping of high impact AER as a means of monitoring the allelic effects of AMA1 vaccines (Dutta, Lee et al. 2007). In this work, I have explored multiple definitions of AMA1 haplotypes in isolates collected from children presenting with severe or mild malaria, and tested whether any of these definitions distinguished these two groups of children. I have also attempted to correlate antibody reactivity as detected by ELISA to sequence differences between AMA1 alleles in infecting parasite isolates and three AMA1 allelic antigens.

7.2 Specific Aims

1. To genotype parasites from single clone infections at the AMA1 locus using three definitions of haplotypes, and explore whether particular haplotypes were more commonly found in children experiencing more severe malaria compared to those with mild malaria. Haplotypes were defined as follows: i) haplotypes of the entire
ectodomain sequences, ii) haplotypes of the three sub-domains, individually, iii) haplotypes of two high impact AER as described by Dutta et al. (Dutta, Lee et al. 2007)

2. To determine whether antigenic differences in parasite AMA1 alleles in individual infections correlated with allele-specific antibody reactivity in the individuals as detected by ELISA.

7.3 METHODS

A detailed description of the methods has been given in the main chapter on 'Materials and Methods'. Key points are mentioned again here very briefly.

7.3.1 STUDY POPULATION

The children recruited into the case-control study have already been described (Section 3.2.1.2). Severe malaria was defined as malaria severe enough to warrant hospital admission. Mild malaria was defined as fever and a parasitaemia of > 2500/μl. Antibody data are presented for assays performed on the serum sample stored from the cross-sectional survey at the beginning of the malaria transmission season in May 1995 (pre-season sample), and for children presenting to hospital at the time of the acute episode (acute sample) and three weeks later (convalescence sample). The entire ectodomain of AMA1 was sequenced from parasite isolates drawn from children presenting to hospital with mild or severe malaria.
7.3.1.1 Sequence analysis

A total of 158 DNA samples out of 184 detected clinical episodes were amplified by PCR and sequenced, and contiguous AMA1 sequences spanning the ectodomain were aligned using MegAlign software (DNASTAR lasergene 7). Sequence data were analyzed only from samples in which it was clear from the electropherogram that they contained a single parasite clone, or one clear dominant clone, judged visually by the magnitude of individual peaks at points of conflict on the sequence trace.

7.3.1.1.1 Haplotype analyses

Entire ectodomain and sub-domain (domains I, II and III) haplotypes were defined in MegAlign software (DNASTAR lasergene 7), using the sequence positions previously described by Hodder et al. (Hodder, Crewther et al. 1996). AER were similarly defined in MegAlign software (DNASTAR lasergene 7), and included the non-variant amino acids between the polymorphic residues described by Dutta et al. (Dutta, Lee et al. 2007). Phylogenetic trees were used to visualize the distribution of haplotypes between children with mild or severe malaria, and were constructed using MEGA version 4 software (Tamura, Dudley et al. 2007). The MEGA software employs the Neighbor-Joining tree-building method (Saitou and Nei 1987). Distinct haplotypes based on the three definitions were identified using DnaSP version 4.5 (Rozas and Rozas 1995).

7.3.1.1.2 Sequence distance analyses

Sequence distances were computed in a pair-wise fashion between each AMA1 allele obtained from patient samples, and those of the 3D7, HB3 and FVO AMA1 alleles for which
antibody assays were performed. Distance was calculated as number of nucleotide differences between pairs of AMA1 alleles using a combination of sequence alignment software programs, including MegAlign software (DNASTAR), DnaSP version 4.5 (Rozas and Rozas 1995) and Bioedit (Hall 1999). Pair-wise correlations between number of nucleotide differences and antibody reactivity (ELISA OD) to the corresponding AMA1 allele were then analyzed for each of the AMA1 alleles assayed in standard statistical packages.

7.3.2 SEQUENCING RESULTS

Samples were processed as shown in Figure 7.3.1 below. High quality sequence data were obtained for 151/158 DNA samples. Of these, 58 (38%) samples contained more than one clone, whereas 93 (62%) samples contained single clone infections. For the analyses presented below, only samples from the first clinical episode of either severe (n = 39, single clone infections (n=38) plus a clear dominant clone in a mixed infection (n=1)) or mild (n = 46, single clone infections (n=42) plus a clear dominant clone in mixed infections (n=4)) malaria were included.
**Figure 7.3.1 Results of sequencing in the case-control study**

Numbers indicate the number of samples at each stage. Dominant clones were defined by visual assessment of individual peaks on the electropherograms.
7.3.3 Comparing AMA1 haplotypes between severe and mild malaria

7.3.3.1 Haplotypes spanning the entire ectodomain

A total of 57 distinct haplotypes (H) were identified from a total of 85 AMA1 alleles, including 39 from children with severe malaria, and 46 from those with mild malaria. The number of haplotypes observed in children with severe malaria (H = 33) was similar to that observed in those with mild malaria (H = 32). The average pair-wise nucleotide diversity per site (π) for all 85 alleles was 0.01641, and was similar for alleles from the samples of children with severe or mild malaria, as shown in Figure 7.3.2, below. There were 71 polymorphic sites across the entire ectodomain and a total of 79 mutations. Eight sites had multiple alleles. There were no fixed differences at any of the polymorphic sites between the two populations (ie no nucleotide sites at which all the sequences from children with severe malaria were different from those from children with mild malaria). Sixty five mutations were shared between samples from both severe and mild malaria. A dendrogram was constructed to visualize the distribution of distinct haplotypes between children with severe or mild malaria. Figure 7.3.3 shows that there was no clustering of given alleles of AMA1 within either group of children.
Figure 7.3.2 Average pair-wise nucleotide diversity per site

Average pair-wise nucleotide diversity per site ($\pi$) for AMA1 alleles sequenced from children with mild (Pi1) and severe (Pi2) malaria.
Figure 7.3.3 Cluster dendrogram of AMA1 alleles

Cluster dendrogram of sequence dissimilarity of AMA1 alleles sequenced from children with severe and mild malaria. No clustering of haplotypes within either group of children was observed.
7.3.3.2 Haplotypes defined at the sub-domain level

Table 7.3.1 shows the distribution of haplotypes within the three sub-domains of AMA1 in isolates sequenced from children with severe or mild malaria. For each sub-domain, the number of distinct haplotypes was comparable in sequences from severe and mild malaria. The distribution of distinct haplotypes for each domain, among children with severe, or mild malaria, are displayed in Figure 7.3.4.

Table 7.3.1 Distribution of haplotypes among sub-domains of AMA1

<table>
<thead>
<tr>
<th>Sub-domains</th>
<th>Haplotypes Total (n =85)</th>
<th>Haplotypes Severe (n=39)</th>
<th>Haplotypes Mild (n= 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI</td>
<td>47</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>DII</td>
<td>22</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>DIII</td>
<td>15</td>
<td>13</td>
<td>11</td>
</tr>
</tbody>
</table>
Mild malaria

Severe malaria

Figure 7.3.4 Cluster dendrograms of domain I, II and III haplotypes
Cluster dendrograms of haplotypes of AMA1 alleles from children with severe or mild malaria. Figures are a) domain I, b) domain II, c) domain III. In all three domains, the majority of haplotypes are equally distributed among children with either severe or mild malaria.

7.3.3.3 Haplotypes defined as Antigenic Escape Residues (AER)

Two regions of high impact AER as described by Dutta et al. were identified and AMA1 alleles genotyped at these sites (Dutta, Lee et al. 2007). The first high impact AER region lies within domain I and is referred to as C1-L, while the second lies within domain 2 and is referred to as D2. A total of 17 C1-L haplotypes were defined, 15 being present in samples from children with severe malaria, and 14 being found in samples from children with mild malaria. For D2, a total of 19 distinct haplotypes were identified, 13 being from children with severe malaria, and 17 from those with mild malaria. As previously, dendrograms were used to visualize the sequence distances among alleles genotyped at the high impact AER, for children with severe or mild malaria. Figure 7.3.5 shows that both high impact AER are equally distributed among children with severe or mild malaria, with no evidence of clustering.
Figure 7.3.5 Cluster dendrograms of C1-L and D2 haplotypes

Cluster dendrograms of a) C1-L and b) D2 (AER) haplotypes of AMA1 alleles from children with severe and mild malaria. For both AER the majority of haplotypes are equally distributed among children with either severe or mild malaria.
7.3.4 GENETIC DISTANCE AND STRENGTH OF ANTI-AMA1 ALLELE-SPECIFIC RESPONSE

Antibody reactivity in the preseason, acute and convalescence samples was analyzed in relation to the AMA1 sequences of the infecting parasite isolates associated with the clinical episodes. Sequence distances (similarity) were computed in a pair-wise fashion between each AMA1 allele obtained from patient samples, and those of the 3D7, HB3 and FVO AMA1 alleles. Correlations between number of nucleotide differences and antibody reactivity (ELISA OD) are shown for the 85 samples for which this paired data was available, at three time points. For all alleles tested, at all time points, there was no correlation between number of nucleotide differences and antibody reactivity as detected by ELISA, Figure 7.3.6.
**Figure 7.3.6** Correlations between antigenic differences and antibody reactivity detected by ELISA

### 7.4 DISCUSSION

Unlike other merozoite antigens thought to have a role in invasion of erythrocytes (such as MSP-1, MSP-2 and MSP-3), and coded by single locus genes, alleles of AMA1 do not group into major families or types. To determine whether particular alleles of AMA1 were
commonly found in children experiencing more severe presentations of malaria, compared to those with mild malaria, I defined 'haplotypes' of AMA1, based on sequences from the entire ectodomain, the sub-domains, and the recently described AERs (Dutta, Lee et al. 2007). Using these definitions, I found no evidence of a clustering of haplotypes within either disease phenotype, with the majority of haplotypes being evenly distributed among children with either severe, or mild malaria.

Previous studies have compared the prevalence of parasites bearing specific allelic types of MSP-2 and MSP-1 block 2, in different categories of malaria, with mixed results. In a case-control study from Papua New Guinea (n=227), children presenting to hospital with symptomatic malaria were more likely than those with asymptomatic infections in the community, to have parasites bearing FC-27-like alleles at the msp2 locus (Engelbrecht, Felger et al. 1995). Similar findings had been previously reported from a longitudinal study in Ghana, where children developing symptomatic malaria were more likely than those with asymptomatic infections to have parasites bearing FC-27-like msp-2 alleles (Ofosu-Okyere, Mackinnon et al. 2001). However, in a separate case-control study conducted in Gabon where 100 children presenting to hospital with severe malaria were matched to those presenting with mild malaria (n = 100), no difference in the distribution of msp-2 alleles between the two groups of children was found (Kun, Schmidt-Ott et al. 1998). Likewise, no differences in the distribution of msp-2 alleles were found among severe malaria patients (n = 56) admitted to hospital in Dakar, compared to those presenting at the same hospital with mild malaria (n=30) (Robert, Ntoumi et al. 1996), or, between symptomatic (n=63) and asymptomatic (n= 306) infections in a cross-sectional survey in
Papua New Guinea (Cortes, Mellombo et al. 2004). In a small study (n = 34), where children with asymptomatic parasitaemias were monitored daily for 31 days, the development of clinical symptoms was associated with parasites bearing the alternative \textit{IC1-like} allele of \textit{msp-2} (Magesa, Mdira et al. 2002).

The situation is no clearer for alleles at the MSP-1 block 2 locus, where no single allele has been consistently associated with a specific disease phenotype, albeit in small studies (Kun, Schmidt-Ott et al. 1998; Ariey, Hommel et al. 2001; Ofosu-Okyere, Mackinnon et al. 2001; Magesa, Mdira et al. 2002; Legrand, Volney et al. 2005). For reasons already alluded to, this kind of analysis is challenging for alleles of \textit{AMA1}. Cortes and colleagues attempted to address these difficulties by comparing the frequency of particular amino acids at specific sites within domain I, in symptomatic and asymptomatic infections (Cortes, Mellombo et al. 2003). They found a strong imbalance, with particular residues being over-represented in alleles from symptomatic, compared to those with asymptomatic infections, particularly in children less than 10 years old (Cortes, Mellombo et al. 2003). One disadvantage of their approach however, is the large number of comparisons made, for each polymorphic site, with a consequent increase in type I errors, and, does not take into account any linkage that may be present between particular residues. Furthermore, they found that the diversity within the \textit{AMA1} locus in their population from PNG was lower than that reported from Nigeria (Polley and Conway 2001), perhaps as a result of differences in malaria transmission. This analytical approach would probably be less robust in areas of high malaria transmission, where diversity within the \textit{ama1} locus is higher.
It was more difficult to assess protective allele-specific antibodies to AMA1 because alleles at this locus cannot be readily grouped into main allelic families or types. Vaccination studies in animal models of malaria indicate that allele-specific antibodies provide significantly better protection against challenge with parasites bearing homologous, as opposed to heterologous AMA1 alleles (Crewther, Matthew et al. 1996; Hodder, Crewther et al. 2001). However, such studies are usually conducted in 'naïve' animals, with no previous experience of malaria, and it is not clear how this would apply to individuals in malaria endemic areas, who will have been repeatedly exposed to many parasite 'strains'.

In a phase I AMA1 malaria vaccine trial conducted in Malian adults, immunization with two allelic versions of AMA1 (3D7 and FVO) resulted in a significant boosting of pre-existing anti-AMA1 antibodies, for both the vaccine alleles and a non-vaccine allele (AMA1-L32). However, these increases in antibody levels were not associated with significant changes in in vitro growth inhibition of *P. falciparum* (Dicko, Diemert et al. 2007), and when the study was repeated in children from the same study site, the rise in antibodies (pre- to post-vaccination) was considerably lower than what had been observed in the adults (Dicko, Sagara et al. 2008).

In one study, antibodies against the 3D7 and FVO alleles of AMA1 were raised in mice following immunization with either or both of the alleles, and then tested in invasion-inhibition assays using a panel of five *P. falciparum* strains, bearing homologous and heterologous alleles. This not only showed that invasion-inhibition was more efficient with parasites bearing homologous, compared to heterologous AMA1 alleles, but also that in the heterologous assay, the magnitude of invasion-inhibition correlated negatively with genetic
distance (number of amino acid differences between the immunizing AMA1 allele and that tested in the heterologous invasion-inhibition assay) (Kennedy, Wang et al. 2002). This does not seem to be the case for antibodies detected by ELISA. In both acute and convalescence samples, similarity between the AMA1 allele in the parasite causing the clinical episode, and the sequences of three alternative allelic versions of AMA1, did not correlate with antibody reactivity. However, the analysis of allele-specific antibodies to AMA1 is not straight-forward. To detect antibodies to allele-specific epitopes in AMA1 induced by infecting parasite isolates more definitively, one would have to clone and express AMA1 antigens from each isolate. Even if this were achieved, it is unclear how many competition ELISAs (and with which AMA1 antigens) would be required to dissect out antibodies to the allele-specific epitopes. Notably, antibodies to all three AMA1 antigens were highly correlated, consistent with high sequence conservation observed in the greater part of the gene. Additionally, it is well-established that protective anti-AMA1 antibodies are conformation-dependent, and therefore, direct site by site amino acid sequence comparisons are probably not ideal for this type of analysis.
8 STRINGENT PREDICTION AND TESTING OF SITES UNDER IMMUNE-MEDIATED BALANCING SELECTION IN APICAL MEROZOITE ANTIGEN 1 (AMA-1)

8.1 INTRODUCTION

Complete sequencing of the *Plasmodium falciparum* genome revealed the presence of at least 5500 genes, many of which could be potential targets for protective immunity. However, identifying which of these genes are important in this regard is daunting, and molecular population genetic tools provide one way of homing in on potential candidates, by detecting the presence of natural selection using the variants of a nucleotide sequence in a population. These variants, or polymorphisms, are likely to be maintained, because they confer a survival advantage. In the case of pathogenic organisms, the presence of variants within a gene suggests that the gene codes for a protein that is either a target of protective immunity, or, plays a role in evading immune responses. Proof of principle for a malaria antigen was first provided by work on merozoite surface protein-1 (MSP-1). Population genetic analyses on allele-frequency distributions were applied to identify the region of MSP-1 that appeared to be under the strongest selection to maintain alleles in the population, predicting that this would be an important target for protective immunity. Predictions were supported by a longitudinal study showing that antibodies to this region of MSP-1 (block 2) were associated with protection from clinical episodes of malaria (Conway, Cavanagh et al. 2000).
For AMA1, it is clear from large population studies, that the locus is under strong balancing selection, which is most evident within sub-domains I and III (Polley and Conway 2001; Cortes, Mellombo et al. 2003; Polley, Chokejindachai et al. 2003). In this work, I have extended the previous findings, by finely mapping the precise sites within domains I and III of AMA1 that are under the strongest selection, in a sample of AMA1 alleles drawn from a Kenyan population. In a set of preliminary experiments conducted within the same population, I have demonstrated that these specific sites contain T-cell epitopes that are likely to be important in mediating protection.

8.2 Specific Aims

1. To determine whether there is evidence of balancing selection maintaining polymorphisms within a population sample of AMA1 alleles from Kenya

2. To use the Kenyan data in conjunction with similar population data from Nigeria and Thailand, in an attempt to map the precise sites under the strongest selection across three geographically distinct populations

3. In preliminary experiments, to test the hypothesis that this selection is immune-mediated by testing whether these sites contain important T- and/or B-cell epitopes that may be important components of naturally-acquired immunity to clinical malaria
8.3 METHODS

8.3.1 STUDY OUTLINE

The study was conducted in several discrete steps. First, *ama1* was sequenced from parasite isolates drawn from individuals recruited into the Chonyi cohort, from Kilifi, Kenya. These isolates were selected randomly from samples that contained parasites either at the time of the cross-sectional survey in October 2000, or when patients from the cohort subsequently presented to hospital with clinical malaria. Molecular population genetic analyses were then performed on AMA1 alleles from the Kenyan population, to identify the region(s) of the gene under the strongest selection. Once these regions were identified, the data were combined with similar population data from Nigeria (Polley and Conway 2001) and Thailand (Polley, Chokejindachai et al. 2003), to map the sites that were under the strongest selection across three geographically distinct populations. Antibody and cell-mediated immune responses to these sites were then tested in the Kenyan population. Antibody responses were tested using samples from the previously described Chonyi cohort. Cell-mediated responses were tested in a separate set of samples (Junju adults), drawn from adults living close to the Chonyi location, who had not been part of the original cohort. These adults were specifically recruited for this part of the study, as PBMC were not available from the Chonyi cohort to test for T-cell responses. The Chonyi cohort and Junju adults have already been described in detail (see Materials and Methods). I first present and discuss the results of the molecular population genetic analyses and then describe the immunological assay results.
8.3.2 LABORATORY METHODS

The PCR amplification and sequencing strategy for AMA1 has been detailed in the main 'Materials and Methods' section (Chapter 3).

8.4 RESULTS

8.4.1 POPULATION GENETIC ANALYSES

8.4.1.1 Sequence diversity

A total of 83 parasite isolates were available for sequencing. Of these, 34 (40%) had mixed infections and were excluded from further analyses. Forty nine (49) contiguous AMA1 alleles were thus obtained and included in the molecular population genetic analyses. Previously undescribed (when the sequencing was performed in 2005) polymorphisms were observed in ten of these samples, and sequencing was repeated from the initial PCR step to verify that these were not sequencing artefacts. The polymorphic nucleotides sequenced from 49 Kenyan alleles of the Plasmodium falciparum ectodomain of AMA-1 and their distribution within the three domains are shown in Figure 8.4.1. There were 66 polymorphic sites, 39 occurred in domain I, 10 in domain II, and 7 in domain III. There were 36 distinct haplotypes across the entire ectodomain, 27 of which were unique, the rest being shared by at least two individuals (6, 5, 2, 2, 2, 2). Within the sub-domains, the numbers of haplotypes observed were 31, 15, and 10, for domains I, II and III, respectively.
Figure 8.4.1 Polymorphic nucleotides in the *P. falciparum* ectodomain
Polymorphic nucleotides observed in the ectodomain of *P. falciparum* AMA1 sequenced from a population sample of 49 Kenyan alleles. Ten samples; C561, C111, C843, C597, C239, C443, C754, C411, C098, and C687 contained new polymorphisms.

**8.4.1.2 Linkage disequilibrium and recombination**

Linkage disequilibrium was calculated for all parsimony-informative polymorphic sites in the data set (i.e., sites that have a minimum of two nucleotides that are present at least twice, \( n = 51 \)). *Figure 8.4.2* and *Figure 8.4.3* show that for both indices, LD decreased rapidly with increasing nucleotide distance, indicating a high meiotic recombination rate. This was further confirmed by the tests for recombination, which are known to provide conservative estimates. A minimum number of 25 recombination events (\( R_M \)) were predicted to have occurred to give rise to the 36 haplotypes that were observed in the sample of 49 AMA1 alleles. The recombination parameter (\( C \)) was high at 0.0931 between adjacent sites, and 122 for the whole sequence.
Figure 8.4.2 LD estimated by D' and distance between polymorphic sites

LD as estimated by D' decreases with increasing distance between polymorphic nucleotides (measured in kilobases), indicating a high recombination rate. Dots represent the value of D' for pairs of polymorphic sites across the entire ectodomain. Red dots: p < 0.05 by Fisher's exact test. D', linkage disequilibrium. Sign, significant by Fisher's exact test.
Figure 8.4.3 LD estimated by $R^2$ and distance between polymorphic sites

LD as estimated by $R^2$ decreases with increasing distance between polymorphic nucleotides (measured in kilobases), indicating a high recombination rate. Dots represent the value of $R^2$ for pairs of polymorphic sites across the entire ectodomain. Red dots: $p < 0.05$ by Fisher's exact test.

8.4.1.3 Tests of neutrality

Tajima's $D$ was estimated for the entire ectodomain and is illustrated in the sliding window plot, Figure 8.4.4. It was also determined separately for each of the three domains, as were Fu and Li's $D$ and $F$ (Table 8.4.1). From both these tests, the most striking result is the strong signature of balancing selection detected in domain III from the Kenyan data. A strong signal of selection was also seen in domain I with positive values of Tajima's $D$, and Fu and Li's $D$ and $F$, but this did not reach statistical significance.
Figure 8.4.4 Sliding window plot of Tajima's $D$

Large positive values of $D$ indicate the presence of balancing selection acting to maintain alleles at intermediate frequencies in the population.
Table 8.4.1 Tajima’s D, and Fu & Li’s D and F for *ama1*

<table>
<thead>
<tr>
<th>Segregating sites</th>
<th>Tajima’s D</th>
<th>Fu &amp; Li’s D</th>
<th>Fu &amp; Li’s F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full ectodomain</td>
<td>66(74)</td>
<td>0.99286</td>
<td>0.39992</td>
</tr>
<tr>
<td>Domain I</td>
<td>39(45)</td>
<td>0.75067</td>
<td>0.32391</td>
</tr>
<tr>
<td>Domain II</td>
<td>10(10)</td>
<td>0.51416</td>
<td>0.01287</td>
</tr>
<tr>
<td>Domain III</td>
<td>7(7)</td>
<td>2.69580**</td>
<td>1.27840</td>
</tr>
</tbody>
</table>

Tajima’s D, and Fu & Li’s D and F (with an outgroup) for the entire *ama1* ectodomain, and separately for Domains I, II and III. **p < 0.01, *** p < 0.05

8.4.1.4 Coalescent simulations

To determine the effect that high levels of recombination would have on the estimates of departure from neutrality in comparison with those observed, 10,000 coalescent simulations for Tajima’s D were computed, Table 8.4.2. Observed estimates that did not fall within the confidence limits were considered statistically significant (p < 0.05). For domain III, allowing for the minimum number of recombination events ($R_m$), the observed value of Tajima’s D is far higher than the 95% upper limit of the expected. Similar results are obtained when free recombination (maximum theoretical value of the recombination parameter) is computed, Table 8.4.2.
Table 8.4.2 Coalescent simulations for Tajima's $D$ at varying levels of recombination

<table>
<thead>
<tr>
<th>Tajima's $D$</th>
<th>Ectodomain</th>
<th>Domain I</th>
<th>Domain II</th>
<th>Domain III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>0.992*</td>
<td>0.75067</td>
<td>0.51416</td>
<td>2.6958**</td>
</tr>
<tr>
<td>$C = 0$ (95% CI)</td>
<td>-1.60 to 1.80</td>
<td>-1.67 to 1.81</td>
<td>-1.69 to 1.89</td>
<td>-1.71 to 1.91*</td>
</tr>
<tr>
<td>$C = 25$ (95% CI)</td>
<td>-1.05 to 0.98*</td>
<td>-1.09 to 1.05</td>
<td>-1.38 to 1.49</td>
<td>-1.50 to 1.69*</td>
</tr>
<tr>
<td>Free recombination (95% CI)</td>
<td>-0.60 to 0.62*</td>
<td>-0.74 to 0.76</td>
<td>-1.28 to 1.32</td>
<td>-1.39 to 1.47*</td>
</tr>
</tbody>
</table>

Coalescent simulations for Tajima's $D$ allowing for variable rates of recombination. Ten thousand coalescent simulations were computed. Upper and lower limits (95% confidence interval) of the expected values are shown. For Domain III, the observed value of Tajima's $D$ considerably exceeds that expected under neutrality, even allowing for free recombination. **$p < 0.01$. 

8.4.1.5 Non-synonymous /synonymous (dN/dS) ratios

The dN/dS ratios were compared among fixed differences within and between $P.$ falciparum and $P.$ reichenowi. As shown in the Table 8.4.3, there is an excess of replacement polymorphisms within $P.$ falciparum, with 98% of polymorphisms within $P.$ falciparum being non-synonymous, compared with 65% of fixed differences between the two species, $p = 0.000015$, Fisher's exact (two tailed test).
Table 8.4.3 McDonald and Kreitman test between and within species

<table>
<thead>
<tr>
<th></th>
<th>Fixed differences between species</th>
<th>Polymorphisms within P. falciparum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonymous</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Non-synonymous</td>
<td>21</td>
<td>62</td>
</tr>
</tbody>
</table>

McDonald and Kreitman 2 x 2 table comparing the dN/dS ratio between and within species. 
Plasmodium reichenowi was used as the outgroup for P. falciparum. An excess of non-synonymous mutations was found within isolates of P. falciparum. Fisher's exact (two-tailed test) P = 0.000015.

8.4.1.6 Radical amino acid substitutions

The proportions of radical to conservative amino acid substitutions across the ectodomain were compared. As shown in Table 8.4.4, significantly more polymorphisms within P. falciparum (71%) resulted in radical amino acid changes, compared to fixed differences (38%) between P. falciparum and P. reichenowi, p = 0.02, Fisher's exact (two tailed) t test.
Table 8.4.4 Radical and conserved amino acid changes within and between species

<table>
<thead>
<tr>
<th></th>
<th>Fixed differences between species</th>
<th>Polymorphisms within P. falciparum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radical</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>Conservative</td>
<td>15</td>
<td>13</td>
</tr>
</tbody>
</table>

Radical (altered amino acid charge), versus conservative amino acid replacements were compared within and between species. *Plasmodium reichenowi* was used as the outgroup for *P. falciparum*. Significantly more substitutions within *P. falciparum* were radical, compared to fixed replacements between species, \( p = 0.02 \), Fisher's exact (two-tailed test).

### 8.4.1.7 Inter-population diversity

Wright's *F*<sub>ST</sub> index was calculated for pairwise comparisons between the three populations using a stringent criterion that only included polymorphic sites that had a minor allele frequency of >0.15 across three populations. Table 8.4.5 below shows the average *F*<sub>ST</sub> index across the entire ectodomain in pair-wise comparisons of the three populations. For all pair-wise comparisons, interpopulation divergence accounts for less than 5% of total nucleotide diversity (highest *F*<sub>ST</sub> = 0.041). Very low *F*<sub>ST</sub> values indicate that balancing selection is maintaining similar allele frequencies between populations. Figure 8.4.5 shows a site by site *F*<sub>ST</sub> analysis, and reveals that the *F*<sub>ST</sub> index was particularly low at specific sites compared to others, raising the possibility that these are functionally important sites.
Table 8.4.5 Pair-wise comparisons of Wrights $F_{ST}$ index across three geographically distinct populations

<table>
<thead>
<tr>
<th>Pair-wise comparisons</th>
<th>$F_{ST}$ (average)</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>Lower 99% CI</th>
<th>Upper 99% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya vs Nigeria</td>
<td>0.025</td>
<td>0.013</td>
<td>0.038</td>
<td>0.01</td>
<td>0.042</td>
</tr>
<tr>
<td>Thailand vs Kenya</td>
<td>0.041</td>
<td>0.02</td>
<td>0.067</td>
<td>0.014</td>
<td>0.077</td>
</tr>
<tr>
<td>Thailand vs Nigeria</td>
<td>0.036</td>
<td>0.015</td>
<td>0.06</td>
<td>0.009</td>
<td>0.068</td>
</tr>
<tr>
<td>Mean overall</td>
<td>0.034</td>
<td>0.021</td>
<td>0.049</td>
<td>0.017</td>
<td>0.054</td>
</tr>
</tbody>
</table>

The average $F_{ST}$ across the entire ectodomain in all comparisons was low, indicating that population divergence accounted for a small proportion (<5%) of the observed nucleotide diversity.
8.4.2 DISCUSSION: POPULATION GENETIC ANALYSES AMA1

This study provides further evidence that AMA1 is under strong balancing selection, as shown by the results of the tests of neutrality, the McDonald and Kreitman test, the preponderance of radical versus conserved amino acid changes, and the limited interpopulation diversity observed when three geographically distinct populations were analyzed. These findings are in agreement with those of other similar studies, where
alleles of AMA1 were sampled from large population samples in Nigeria (Polley and Conway 2001), Thailand (Polley, Chokejindachai et al. 2003), Papua New Guinea (Cortes, Mellombo et al. 2003) and India (Garg, Alam et al. 2007), though the latter two studies concentrated on polymorphisms within domain I.

Sequence diversity across the entire ectodomain was lower than that observed in a Nigerian population, but higher than that observed in Thailand (Polley and Conway 2001; Polley, Chokejindachai et al. 2003). In Kenya, 36 distinct haplotypes were observed among 49 isolates (73%), compared to 45/51 (88%) in Nigeria, and 27/50 (54%) in Thailand, and may reflect differences in malaria transmission intensity in the three areas. This idea is supported when the haplotypes within domain I are considered, with a higher proportion of haplotypes observed from populations in Africa (Kenya 63%, Nigeria 68% (Polley and Conway 2001)), compared to those from either India (36%) (Garg, Alam et al. 2007), Thailand (36%) (Polley, Chokejindachai et al. 2003), or Papua New Guinea (16%) (Cortes, Mellombo et al. 2003). As has been shown in other studies on AMA1 (Polley and Conway 2001; Polley, Chokejindachai et al. 2003), and on MSP-1 (Conway, Roper et al. 1999), linkage disequilibrium decreased with increasing distance between polymorphic nucleotides, reflecting the high recombination rate within natural populations of *P. falciparum*.

The results of both Tajima’s (Tajima’s D) and Fu and Li’s (Fu and Li’s F) tests, and the coalescent simulations based on these tests, indicate a strong signature of balancing selection within domain III in the Kenyan population studied here, as has been previously reported in the studies from Nigeria (Polley and Conway 2001) and Thailand (Polley,
Chokejindachai et al. 2003). This is despite the fact that in the Kenyan samples, domain III had considerably fewer polymorphisms (7), than either domain II (10) or domain I (39), which would be expected to limit the power of the analyses. These findings are further strengthened by the test of between population diversity (Wrights $F_{ST}$). A site-by-site analysis of $F_{ST}$ using data from all three populations indicated specific sites at which the $F_{ST}$ was particularly low (<0.1), highlighting these sites as potential targets of protective immunity. However, in spite of all this evidence in support of domain III as being under strong balancing selection, to-date, the evidence that it is an important antibody target is conflicting, and it remains possible that the observed variation is in T-cell epitopes.

Two studies have reported that domain III is an important target of invasion inhibitory antibodies. Nair and colleagues affinity purified human antibodies from Papua New Guinea on refolded recombinant domain III ($P. falciparum$ 3D7 strain) and found that these antibodies inhibited invasion in an allele-specific manner, being more effective against 3D7 as compared to HB3 parasites (Nair, Hinds et al. 2002). However they do not indicate what proportion of sera had high titre antibodies to domain III, or, had invasion-inhibitory capacity (Nair, Hinds et al. 2002). In a separate study, Mueller and colleagues elicited antibodies that were capable of inhibiting parasite growth by immunizing mice with a long synthetic peptide from domain III (loop 1) in a virosomal formulation (Mueller, Renard et al. 2003). Although loop I of domain III is semi-conserved, and therefore an attractive vaccine candidate, they did not report whether the observed invasion-inhibition appeared to be allele-specific (Mueller, Renard et al. 2003).
Other data on the importance of antibodies to domain III of AMA1 have been less convincing. In the first report of AMA1 sub-domain constructs, polyclonal antibodies were raised in rabbits against the entire ectodomain, and then tested for binding to single, or double sub-domains (Lalitha, Ware et al. 2004). In contrast to sub-domains I and II, sub-domain III was poorly recognized by these antibodies. However, although domain III specific antibodies (singly and in combination with either domain I or II), did not have a major contribution to growth-inhibition, optimal inhibition was achieved with antibodies raised to the entire ectodomain, as compared to the combination of domains I and II (Lalitha, Ware et al. 2004). These findings suggested that domain III, in the context of immunization with the whole protein, nevertheless had a subtle influence on the generation of functional antibodies. In the first immuno-epidemiological study using sub-domain AMA1 constructs in humans, antibodies to domain III were rare with a prevalence of between 0.7% and 1.3% in the two populations studied (Polley, Mwangi et al. 2004). However, in the same study, antibody reactivity to the full ectodomain was higher than that to the combination of domains I and II, again indicating that some antibody epitopes were present in domain III, and that these were perhaps not optimally captured in the domain III construct (Polley, Mwangi et al. 2004). Moreover, although antibodies to the domain III were rarely detected, antibody reactivity to the combination of domains II and III was in some cases higher than that to domain II on its own, suggesting a contribution from epitopes within domain III (Polley, Mwangi et al. 2004). In a separate study in Papua New Guinea, antibodies appeared to be more commonly directed against epitopes in domain I, than to those in either domain II, or III (Cortes, Mellombo et al. 2005). In this study by Cortes et al., difficulties in expressing a properly refolded domain I construct precluded a
more direct comparison of antibodies to the three separate domains (Cortes, Mellombo et al. 2005). An important consideration is the fact that protective antibodies to AMA1 have been shown to be conformation-dependent (Crewther, Matthew et al. 1996; Anders, Crewther et al. 1998; Hodder, Crewther et al. 2001), and as such important antibody epitopes may require the contribution of all three domains. Nevertheless, taken together, the evidence that protective immunity is mediated by antibodies to domain III of AMA1 is not particularly strong.

The strong signature of selection observed in domain III could be a result of protective T-cell driven immunity. To-date, only two studies have examined T-cell responses to AMA1 in malaria endemic communities (Lal, Hughes et al. 1996; Udhayakumar, Kariuki et al. 2001). Lal and colleagues used a computer algorithm to predict putative T-cell epitopes which spanned the entire ectodomain, and tested whether these peptides were able to induce T-cell proliferation in clinically immune adults in western Kenya, and identified at least 9 T-cell epitopes (Lal, Hughes et al. 1996). Subsequently, a longitudinal study conducted in the same area of Kenya, using the same peptides, found overall that T-cell responses (measured by lymphoproliferation assays) were short-lived, not being detectable after a 3-month interval, and that lympho-proliferative responses to one peptide (PL191, which lies within domain I) out of eight tested, was associated with a lower risk of parasitaemia at subsequent follow up (Udhayakumar, Kariuki et al. 2001).

In the second part of this study I therefore performed preliminary work to test the hypothesis that this strong signature of balancing selection detected in domain III in the Kenyan population was driven by T-cell dependent immune responses rather than humoral
immunity. I first began by doing competition assays with sub-domain AMA1 constructs in an attempt to dissect out any previously undetected antibodies to domain III. I then designed synthetic overlapping peptides spanning domain III, and tested these in *ex-vivo* T-cell ELISPOT assays for IFN-γ and IL-2.

## 8.5 Antibody Responses to Domain III

### 8.5.1 Objectives

To determine whether antibodies to domain III of AMA1 contributed significantly to the relatively high antibody reactivity to the combination of domains II and III, previously observed in the Chonyi cohort.

### 8.5.2 Methods

Competition ELISAs were used to dissect out antibody reactivity to domain III of AMA1 in specific sera. The presence of antibody reactivity to a panel of 8 AMA1 antigens had been previously established within the Chonyi cohort (Polley, Mwangi et al. 2004). This panel included a range of sub-domain constructs, including three containing separately, domains II-III (pf9mH, DII-III), domain II (pf8mH, DII), and domain III (pf10mH, DIII)(Polley, Mwangi et al. 2004). The design of the experiment was limited by the availability of the recombinant sub-domain constructs. Sera that had detectable antibody to either DII-III or DIII were identified and 3 sets of competition ELISAs were performed as follows to detect antibody reactivity attributable to epitopes within domain III:

i. Samples that were positive for both DII-DIII and DIII (n = 6)
a. ELISA plates were coated with DII-III and increasing amounts of DIII were 
used in competition. Any reactivity detected in this fashion would be 
primarily due to epitopes within domain II as opposed to those in domain III. 
Lack of reactivity would indicate that antibodies were directed solely against 
epitopes in domain III, with no contribution from domain II.

ii. Samples where the measured reactivity to DII-III was higher than that against DII by 
>0.2 OD units (n =11)

a. ELISA plates were coated with DII-III and increasing amounts of DII were 
used in competition. Any reactivity detected in this fashion would be due to 
epitopes within domain III as opposed to domain II.

iii. Samples where the reactivity for DII-III was high (this selection was limited by 
antigen amounts to sera with OD >1.7, n= 25)

a. ELISA plates were coated with DII-III (50ng/100ul) and sera pre-incubated 
with an excess of DIII (1000ng/100ul) as the competing antigen. Any 
decrease in OD would be due to epitopes within domain III.

8.6 Cellular responses to domain III

8.6.1 Objectives

8.6.1.1 Preliminary objectives

To determine whether any T-cell epitopes could be identified within domain III of AMA1 in 
semi-immune adults in Kenya
8.6.2 METHODS

8.6.2.1 Peptide design

Overlapping peptides spanning polymorphic and conserved regions of domain III were designed as described below. The predicted amino acid sequence of domain III of AMA1 from one Kenyan isolate is shown in

**Figure 8.6.1.** A schematic of the amino acid sequence illustrates the overlapping peptide (17-20 mer) strategy. **Figure 8.6.2.** Shows the between population divergence (Wright's fixation index, $F_{ST}$) at the corresponding loci. The frequencies of the potential peptide haplotypes at each polymorphic site of domain III among the Kenyan isolates is shown **Table 8.6.1.** At each polymorphic locus, the two most common haplotypes in the Kenyan population were selected for the design of synthetic peptides.

![Sequence](image)

**Figure 8.6.1 Domain III sequence from one Kenyan isolate**

Polymorphic sites occurring at a frequency of >0.15 in three populations (Kenya, Nigeria and Thailand) are highlighted in bold. There are 66 amino acids in total. Domain III is defined by cysteines as described in Hodder *et al.* 1996 (Hodder, Crewther *et al.* 1996).

300
Figure 8.6.2 Schematic showing polymorphic sites and overlapping peptide design

Polymorphic sites are indicated in black. Letters below the figure identify the location of potential peptides. * Peptide E contains a polymorphic site close to the end of domain III and another that lies just outside the domain.

Figure 8.6.3 Between population divergence at polymorphic loci within domain III

The analysis was performed using the Wright's fixation index of inter-population variance in allele frequencies ($F_{ST}$). Populations from Nigeria, Thailand and Kenya were compared.

493a and 512b lie just outside domain III). The $F_{ST}$ value is <0.1 for the majority of the polymorphic sites.

Table 8.6.1 The frequencies of potential peptide haplotypes at each polymorphic site

<table>
<thead>
<tr>
<th>Potential peptides</th>
<th>Haplotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location A</strong> (20 mer)</td>
<td></td>
</tr>
<tr>
<td>CSLYKDEIKKEIERESKRIK</td>
<td>30/49 (61%)</td>
</tr>
<tr>
<td>CSLYKNEIMKEIERESKRIK</td>
<td>7/49 (14%)</td>
</tr>
<tr>
<td>CSLYKDEIMKEIERESKRIK</td>
<td>12/49 (24%)</td>
</tr>
<tr>
<td><strong>Location B</strong> (20mer)</td>
<td></td>
</tr>
<tr>
<td>ESKRIKLNDNDDEGNKKIIA</td>
<td>Conserved</td>
</tr>
<tr>
<td><strong>Location C</strong> (20 mer)</td>
<td></td>
</tr>
<tr>
<td>KIIAPRIFISDDIDSLKCPC</td>
<td>27/49 (55%)</td>
</tr>
<tr>
<td>KIIAPRIFISDDKDSKLKCPC</td>
<td>22/49 (45%)</td>
</tr>
<tr>
<td><strong>Location D</strong> (17 mer)</td>
<td></td>
</tr>
<tr>
<td>DSLKCPDPEIMVSNSTC</td>
<td>28/49 (57%)</td>
</tr>
<tr>
<td>DSLKCPCAPEIVSNSTC</td>
<td>10/49 (20%)</td>
</tr>
<tr>
<td>DSLKCPCDPEIVSNSTC</td>
<td>9/49 (18%)</td>
</tr>
<tr>
<td>DSLKCPCAPEIMVSNSTC</td>
<td>2/49 (4%)</td>
</tr>
<tr>
<td><strong>Location E</strong> (20mer)</td>
<td></td>
</tr>
<tr>
<td>NSTCRFFVCKCVERRAEVT</td>
<td>20/49 (41%)</td>
</tr>
<tr>
<td>NSTCNFFVCKCVERKAEVTS</td>
<td>16/49 (33%)</td>
</tr>
<tr>
<td>NSTCRFFVCKCVERKAEVTS</td>
<td>10/49 (20%)</td>
</tr>
<tr>
<td>NSTCHFFVCKCVERRAEVT</td>
<td>3/49 (6%)</td>
</tr>
<tr>
<td>NSTCNFFVCKCVERRAEVT</td>
<td>1/49 (2%)</td>
</tr>
</tbody>
</table>

The frequencies of the potential peptide haplotypes at each polymorphic site of domain III found in the Kenyan isolates. The two peptide haplotypes which occurred at the highest frequencies at each location were selected for testing in cellular assays.
8.6.2.2 Peptide synthesis

Peptides were synthesized using Fmoc chemistry and a solid support resin (Sigma Genosys, Haverhill, UK). Peptide composition was verified by the MALDI-TOF (matrix-assisted laser desorption/ionization – time of flight) method. Peptide purity was checked by HPLC (high performance liquid chromatography) and was confirmed to be > 95% for all the peptides. The peptides situated at location E proved difficult to synthesize and thus were not available for the study. As such, a total of 7 AMA1 peptides were tested in the preliminary assays, and included a peptide spanning a conserved region of domain III, and 3 pairs of peptides that spanned polymorphic sites within the domain. The T cell assays and the cohort (Junju adults) in which they were performed have been described (Section 3.2.1.3). Antibody responses to full-length ectodomain constructs of AMA1 (3D7, HB3 and FVO) were also tested in standard indirect ELISA assays in the same individuals.

8.6.3 RESULTS

8.6.3.1 Antibodies to domain III

The individuals recruited into the Chonyi cohort have already been described (Section 3.2.1.1). A total of 42 specific samples were selected for this part of the study. The mean age of these individuals was 16.7 years, range (4 – 58 years), and 26/42 (62%) were parasitaemic at the time the serum sample was collected.
8.6.3.1.1 Antibody reactivity to both DII-III and DIII

In all sera that had reactivity to both DII-III and DIII, reactivity to DII-III could not be competed out with an excess of DIII, indicating that the detected reactivity was mainly due to epitopes in domain II as opposed to domain III (Figure 8.6.4).

Figure 8.6.4 Competition ELISA in sera with reactivity to both DII-III and DIII

In all cases competition with increasing concentrations of DIII did not compete out reactivity to DII-III indicating that the antibodies were mainly directed to epitopes within DII.
8.6.3.1.2 Antibody reactivity to DII-III greater than that to DII

In sera that had higher OD values to DII-III than to DII alone, two patterns of reactivity were seen in the competition ELISA assays. In 4/11 sera reactivity to DII-III was competed out with increasing concentrations of DII indicating that the reactivity was due to epitopes in domain II (Figure 8.6.5, C763, C067, C189 and C244). In the remaining 7/11 sera competition with increasing amount of DII did not compete out the reactivity to DII-III, thus demonstrating the presence of reactivity to epitopes within domain III (Figure 8.6.5, C149, C027, C141, C450, C338, C282 and C689).
Figure 8.6.5 Competition ELISA in sera with reactivity to DII-III that was higher than that to DII
8.6.3.1.3 High antibody reactivity to DII-III

For the last set of sera that had high antibody reactivities to DIII, pre-incubation of test sera with high amounts of DIII did not decrease the reactivity detected to DII-III as shown in the left panel of Figure 8.6.6, in contrast to homologous competition as shown in the right panel of the same figure. This indicates that antibodies in these sera were directed at epitopes within domain II.

![Figure 8.6.6](image)

**Figure 8.6.6 Competition ELISA in sera with high antibody reactivity to domain III**

Antibody reactivity to DII-III is compared to that obtained following competition with excess amounts of DIII (heterologous competition, left panel) and DII-III (homologous competition, right panel).
8.6.4 T-CELL RESPONSES TO DOMAIN III

8.6.4.1 Study population

The baseline characteristics of the Junju adults have been described (Section 3.3.3).

Previous studies have shown that PBMCs taken from individuals never exposed to malaria are able to respond to some malaria peptides (Zevering, Amante et al. 1992; Good 1994), (Ndungu, Sanni et al. 2006). Therefore, to gauge the responses of individuals with minimal exposure to malaria, PBMCs were taken from non-immune volunteers visiting Kilifi, Kenya (n=6). At the time of sampling, these visitors had been in Kilifi for between 3 days and 6 weeks. All but one of these visitors had previously travelled to malaria endemic countries, always on prophylaxis, and never had a confirmed episode of malaria.

8.6.4.2 IFN-γ T-cell responses to domain III peptides

The IFN-γ T-cell responses to all seven AMA1 peptides from domain III are shown in figure 9.9. A positive response was defined as at least one spot per 300,000 PBMC, and was derived by subtracting spot numbers in wells containing only media from those containing peptides (McConkey, Reece et al. 2003; Keating, Bejon et al. 2005; Vuola, Keating et al. 2005; Webster, Dunachie et al. 2005). All samples showed strong IFN-γ T-cell responses to the positive control, SEB. Positive responses were detected for all seven domain III AMA1 peptides tested, with prevalence for each peptide ranging between 73.1% to 96.2%, amongst Junju adults (n=26). Interestingly, individuals with 'minimal' exposure to malaria (n=6), also made IFN-γ responses to the AMA1 peptides, with a prevalence of 50 – 100%.

However, it is clear from Figure 8.6.7 that the magnitude of responses was higher in the
Junju adults, compared to the visitors. Notably, the log transformed mean responses are comparable in both groups for some peptides (Table 8.6.2). This is because the number of non-responders (y axis =1, Figure 8.6.7) among the Junju adults is higher than that among the visitors. In 5 (3%) of 169 wells, for three different peptides, the IFN-γ T cell response was as strong as the positive control rendering enumeration of spots impossible. In these wells, an arbitrary value of 1000 spots per 300,000 PBMC was allocated. This value was chosen as it was greater than the highest enumerable spot count obtained for any peptide for either IFN-γ or IL-2 responses which were 355 and 455 respectively. For all the graphs, values of 0 were converted to 1 to enable visualization of the data on a logarithmic scale.

Table 8.6.2 IFN-γ T cell responses to peptides from domain III

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide abbrev.</th>
<th>Prevalence (%)</th>
<th>Mean response a</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSLYKD</td>
<td>A_DK</td>
<td>80.8</td>
<td>83.3</td>
<td>1.74</td>
</tr>
<tr>
<td>CSLYKE</td>
<td>B_NM</td>
<td>80.8</td>
<td>50</td>
<td>1.12</td>
</tr>
<tr>
<td>ESKRIKLND</td>
<td>C_Cons</td>
<td>96.2</td>
<td>100</td>
<td>1.25</td>
</tr>
<tr>
<td>KIIAPRIFISDD</td>
<td>D_1</td>
<td>73.1</td>
<td>66.7</td>
<td>0.89</td>
</tr>
<tr>
<td>KIIAPRIFISDD</td>
<td>E_K</td>
<td>88.5</td>
<td>66.7</td>
<td>1.16</td>
</tr>
<tr>
<td>DSLKCPC</td>
<td>F_DM</td>
<td>80.1</td>
<td>83.3</td>
<td>1.01</td>
</tr>
<tr>
<td>DSLKCPC</td>
<td>G_AI</td>
<td>80.7</td>
<td>83.3</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* Students t test used to compare the means of the log transformed IFN-γ T-cell responses between the two groups. * p < 0.05
Figure 8.6.7 IFN-γ T-cell responses to peptides from domain III of AMA1

Dotplot showing individual IFN-γ T-cell responses to peptides from domain III of AMA1. Actual values are shown on a logarithmic scale. Black and red dots indicate responses of Junju adults and non-immune volunteers with minimal exposure to malaria, respectively. Horizontal lines indicate medians amongst Junju adults.

8.6.4.3 Individual IFN-γ T-cell responses to domain III peptides

Figure 8.6.8 shows the profile of each individuals' IFN-γ T-cell responses to all seven peptides from domain III of AMA1. The majority of individuals made moderate to strong responses to one or more peptides, and weak or no responses to the others. Although not clearly visible on the graph, in two of the Junju adults, less than one IFN-γ producing T-cell/300,000 PBMC was detected, for any of the seven peptides.
Figure 8.6.8 Individual IFN-γ T-cell responses to domain III peptides from AMA1

Each colored line represents an individual. The key to the peptides is as follows: 1 - A_DK, 2 - B_NM, 3 - C_Cons, 4 - D_I, 5 - E_K, 6 - F_DK, and 7 - G_AI.
8.6.4.4 IL-2 T cell responses to domain III peptides

The IL-2 T-cell responses to all seven AMA1 peptides from domain III are shown in Figure 8.6.9. As for IFN-γ, a positive response was defined as at least one spot per 300,000 PBMC, and was derived by subtracting spot numbers in wells containing only media from those containing peptides (McConkey, Reece et al. 2003; Keating, Bejon et al. 2005; Vuola, Keating et al. 2005; Webster, Dunachie et al. 2005). A strong IL-2 T-cell response to the positive control SEB was observed in 25/26 samples from Junju adults, and the results of the sample where it was negative were excluded. As with the IFN-γ, positive IL-2 responses were detected for all seven domain III AMA1 peptides tested, with prevalence for each peptide ranging between 53.9% to 80.8%, amongst Junju adults (n =25). Individuals with ‘minimal’ exposure to malaria (n=6) also made IL-2 responses to the AMA1 peptides, with a prevalence of 16.7 – 66.7%. However, the magnitude of responses was consistently higher among the Junju adults, compared to the non-immune volunteers (Figure 8.6.9). Although the means of the log transformed responses appear comparable, as with the IFN-γ, the numbers of non-reponders in the Junju adults is greater than that among the visitors (Table 8.6.3). In three wells, the IFN-γ T cell response as strong in intensity to the positive control rendering enumeration of spots impossible. In these wells, as previously, an arbitrary value of 1000 spots per 300,000 PBMC was allocated.
Prevalence and magnitude of IL-2 T-cell responses to peptides from domain III of AMA1, in 25 adults from Junju and 6 non-immune volunteers with minimal exposure to malaria.

1 Wilcoxon rank sum test was used to compare the magnitude of responses in both groups.

### Table 8.6.3 IL-2 T-cell responses to peptides from domain III of AMA1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide abbrev.</th>
<th>Prevalence (%)</th>
<th>Magnitude</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Junju adults</td>
<td>Visitors</td>
<td>Junju adults</td>
</tr>
<tr>
<td>CSLYKDEIKKEIERESKRIK</td>
<td>A_DK</td>
<td>68</td>
<td>66.7</td>
<td>1.18</td>
</tr>
<tr>
<td>CSLYKNEIMKEIERESKRIK</td>
<td>B_NM</td>
<td>72</td>
<td>50</td>
<td>0.88</td>
</tr>
<tr>
<td>ESKRIKLNDNDEGNKKIA</td>
<td>C_Cons</td>
<td>80</td>
<td>66.7</td>
<td>1.01</td>
</tr>
<tr>
<td>KIAAPRIFISDDISLKCPC</td>
<td>D_I</td>
<td>64</td>
<td>33.3</td>
<td>0.48</td>
</tr>
<tr>
<td>KIAAPRIFISDDDSLKCPC</td>
<td>E_K</td>
<td>56</td>
<td>50</td>
<td>0.43</td>
</tr>
<tr>
<td>DSLKPCDPEMVSNSTC</td>
<td>F_DM</td>
<td>56</td>
<td>50</td>
<td>0.6</td>
</tr>
<tr>
<td>DSLKPCAPEIVSNSTC</td>
<td>G_AI</td>
<td>52</td>
<td>16.7</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Figure 8.6.9 IL-2 T-cell responses to peptides from domain III of AMA1
Dotplot showing individual IL-2 T-cell responses to peptides from domain III of AMA1. Actual values are shown on a logarithmic scale. Black and red dots indicate responses of Junju adults and medical students with minimal exposure to malaria, respectively. Horizontal lines indicate medians amongst Junju adults.

8.6.4.5 Individual IL-2 responses to domain III peptides

Figure 8.6.10 shows the profile of each individuals' IL-2 T-cell responses to all seven peptides from domain III of AMA1. As for IFN-γ, the majority of individuals made moderate to strong responses to one or more peptides, and weak or no responses to the others. In four of 25 Junju adults, less than one IL-2 producing T-cell was detected per 300,000 PBMC, against any of the seven peptides. For two of these four individuals, IFN-γ T cell responses were similarly not detected.
Figure 8.6.10 Individual IL-2 T-cell responses to domain III peptides from AMA1

Each colored line represents an individual. The key to the peptides is as follows: 1 - A_DK, 2 - B_NM, 3 - C.Cons, 4 - D_I, 5 - E_K, 6 - F_DK, and 7 - G_Al.

8.6.4.6 Correlation between responses to IFN-γ and IL-2

In general, no correlation was found between IFN-γ and IL-2 T-cell responses for all the peptides analyzed, Figure 8.6.11.
**Figure 8.6.11 Correlation between responses to IFN-γ and IL-2**

IFN-γ and IL-2 T-cell responses to peptides from domain III of AMA1 were not correlated. Graphs are plotted on a logarithmic scale.

8.6.4.7 T-cell responses to peptides spanning the same polymorphic sites

The magnitude of both IFN-γ and IL-2 T-cell responses to peptides spanning the same polymorphic sites was compared. **Figure 8.6.12** shows that the majority of individuals made responses of greater magnitude to one of two peptides from the same polymorphic site, suggesting that these polymorphisms affected T-cell epitopes that are commonly responded to.
Figure 8.6.12 T-cell responses to allelic peptides

IFN-γ (left panel) and IL-2 (right panel) T-cell responses compared for pairs of peptides spanning the same polymorphic sites. For the majority of comparisons, higher responses appear to be made to one peptide of the pair.
8.6.4.8 Correlation of T-cell responses to antibody responses

The antibody responses to full-ectodomain antigens of AMA1 were tested among all 26 Junju adults, as domain III specific reagents were not available. Given this limitation, the T-cell and antibody responses cannot be compared directly. Nevertheless, it was interesting to observe that the individuals making the highest IFN-γ and IL-2 T-cell responses to domain III also had high titres of antibodies to full-length AMA1 (data shown in Figure 8.6.13 for IFN-γ). Conversely, three individuals who were sero-negative for antibodies to full-length AMA1, had detectable IFN-γ and IL-2 T-cell responses to peptides within domain III (data not shown). None of the controls with minimal exposure to malaria had detectable antibodies to the full-length AMA1 although nearly half of them made low grade T-cell responses to both IFN-γ and IL-2.
Figure 8.6.13 Correlation of T-cell responses to antibody responses

Antibody responses to full-length ectodomain allelic versions of AMA1 (left panels) and corresponding IFN-γ T-cell responses to AMA1 peptides from domain III of AMA1. Individuals with the highest antibody levels tended to have the strongest T-cell responses. Note: the scale is different in each of the panels on the right.
In this descriptive study, little evidence of reactivity specific to domain III of AMA1 was detected by competition ELISA, except in a few sera. High titre antibodies to DII-III were mainly directed at epitopes within domain II, as opposed to those in domain III. These findings extend the previous observations of the low prevalence of antibodies to domain III of AMA1 in this population (Polley, Mwangi et al. 2004), and are in agreement with studies from other areas (Cortes, Mellombo et al. 2005). Epitopes that induce anti-AMA1 antibodies capable of inhibiting erythrocyte invasion of merozoites, or protect animal models from challenge with parasites bearing homologous AMA1 alleles, are known to be conformation sensitive (destroyed on reduction of disulphide bonds) (Crewther, Matthew et al. 1996; Anders, Crewther et al. 1998; Hodder, Crewther et al. 2001). It is possible that the recombinant domain III is not optimally folded, or that the important epitopes within this domain are conformationally dependent on domains I and II. Alternatively, cellular immune responses may be responsible for the selection observed in domain III.

In the preliminary experiments conducted here, all seven peptides were shown to contain T-cell epitopes. The precise number of actual epitopes is not possible to determine with certainty, due to the overlapping strategy employed in the peptide design. Furthermore, at three locations within domain III, three pairs of peptides that were identical save for the polymorphic loci were tested. Nonetheless, these are the first ex-vivo ELISPOT data on peptides from AMA1 to be reported, and suggest that cell-mediated responses may be an important component of naturally-acquired AMA1-mediated immunity.
Lympho-proliferative T-cell responses to peptides from AMA1 have been reported in immune adults from Western Kenya (Lal, Hughes et al. 1996; Udhayakumar, Kariuki et al. 2001). Although the methodology used to select, as well as test the AMA1 peptides in these studies differs from that used in my study, it is still worth noting that they detected lympho-proliferative T-cell responses to peptide PL172 (FPCSLYKDEIKKEIERESKR). This peptide is located within domain III, and save for 2 N- and 1 C-terminal amino acid(s), shares close to 100% sequence similarity with peptides A_DK and B_NM, of the present study. As such, for at least two of the seven peptides tested, there is independent evidence of the presence of T-cell epitopes. However, this needs to be interpreted with caution, as it is known from previous studies that the results of ex-vivo ELISPOT and lympho-proliferation assays do not correlate, and probably identify distinct sub-populations of T-cells (Hagiwara, Abbasi et al. 1995; Flanagan, Lee et al. 2001; Pinder, Reece et al. 2004). In any case, in the longitudinal study by Udhayakumar et al., only responses to PL191 (a peptide spanning a polymorphic epitope within domain I) were associated with a subsequently reduced risk of infection with *P. falciparum* (Udhayakumar, Kariuki et al. 2001).

In a recent phase 1 malaria vaccine trial in malaria naïve volunteers, immunization with a virosome-formulated synthetic peptide containing loop 1 of domain III (49 mer), failed to induce significant responses to IFN-Y responses as assayed by the ex-vivo ELISPOT, pre- and post-vaccination (Peduzzi, Westerfeld et al. 2008). However, in the same study, 50% of volunteers made significant lympho-proliferative T-cell responses. This 49 mer synthetic peptide spanned the region encoded for by peptides A_DK/B_NM, C_cons, and D_I/E_K in the present study, for which strong IFN-Y and IL-2 T-cells responses were observed. Apart
from the fact that this study focused on vaccine-induced immunity, and measured responses to a long synthetic peptide that may not have been optimally presented to T-cells, the threshold for positivity was high at 100 SFU/10^6 PBMC, with a minimum difference of 75 SFU/10^6 PBMC between stimulated and un-stimulated cells (Peduzzi 2008). They also report that the SFU of PBMCs incubated without stimulus ranged from 0 – 140 SFU/10^6 PBMC (Peduzzi, Westerfeld et al. 2008). This seems unusually high, as fewer than 1/10^3, or 0.001/10^6 PBMC have been shown to spontaneously secrete IFN-γ or IL-2 (Hagiwara, Abbasi et al. 1995), and suggests that the ELISPOT assay had not been sufficiently optimized. Nevertheless, the definition for positivity for ELISPOT assays, which markedly affects interpretation of data, as illustrated by the study of Peduzzi et al., needs development. For vaccine trials where ELISPOT assays have been used extensively, the issue of a threshold does not often arise, as SFUs are compared pre- and post- vaccination, the magnitude of change being the more important outcome. However, for naturally-acquired immunity to malaria for instance, a range of thresholds have been used by different investigators (Flanagan, Lee et al. 2001; Lee, Flanagan et al. 2001; Malhotra, Mungai et al. 2005), with no clear consensus emerging. For vaccine studies in malaria, the consensus, as has been applied in this preliminary work, appears to be the subtraction of the SFUs in the negative well from those in the peptide-containing wells (McConkey, Reece et al. 2003; Keating, Bejon et al. 2005; Vuola, Keating et al. 2005; Webster, Dunachie et al. 2005). Notably, these studies are all conducted by the same laboratory.

Individuals with minimal exposure to malaria also made ‘low-grade’ T-cell responses to the seven peptides from domain III of AMA1. This is not surprising as previous studies have
documented that PBMCs taken from individuals never exposed to malaria are able to respond to some malaria peptides (Zevering, Amante et al. 1992; Good 1994), (Ndungu, Sanni et al. 2006). Interestingly, in the T-cell lymphoproliferation assays with AMA1 peptides, the stimulation index (measure of T-cell lymphoproliferation) using PBMC from individuals never exposed to malaria was always below the positivity threshold (Lal, Hughes et al. 1996). The lack of truly non-malaria exposed donors in this study precludes the drawing of firm conclusions, based on this data.

For some individuals, the magnitude of IFN-γ and IL-2 responses was nearly as high as that detected to the positive control antigen, SEB. Interestingly, the four individuals that made this type of IFN-γ response, were distinct from those making similarly strong IL-2 responses, and overall, there was a poor correlation between IFN-γ and IL-2 T-cell responses. This is in keeping with reports that the number of unstimulated T-cells secreting either of these two cytokines are not correlated (Hagiwara, Abbasi et al. 1995). However, both naturally-acquired and vaccine-induced ex-vivo IFN-γ and IL-2 T-cell responses to the pre-erythrocytic malaria antigens, CS and ME-TRAP, were highly correlated (Bejon, Keating et al. 2006). In general, although direct comparisons cannot be made, for some individuals, the magnitude of T-cell responses observed with these AMA1 peptides is considerably higher than that reported for peptides from MSP-1 (Lee, Flanagan et al. 2001; Malhotra, Mungai et al. 2005) and EBA-175 (Malhotra, Mungai et al. 2005). In this preliminary work, the data is deliberately presented as SFU/300,000 PBMC to enable the raw data to be viewed, without the enhancement introduced by presenting data as SFU/million PBMC.
In conclusion, although this study is small, it provides new albeit preliminary data on T-cell responses to short synthetic peptides from domain III of AMA1, and provides proof of principle, that there are T-cell epitopes in this region, as predicted by the population genetic analyses. The hypothesis that T-cell responses to these epitopes are important mediators of protection from malaria needs to be tested in longitudinal studies.
9 CONCLUDING REMARKS

The work presented in this thesis contributes significantly to the understanding of naturally acquired immunity to clinical malaria in several important ways. First is the finding that the amount of antibody, and not simple sero-positivity as has often been used in the past, is a better predictor of children who are protected from clinical episodes of malaria. Second was the finding that increasing breadth of specificity of high titre antibodies to a carefully selected panel of merozoite antigens correlated with increasing protective efficacy of the antibodies against clinical episodes of malaria. Thirdly, high titre antibodies to particular combinations of antigens, MSP-2, MSP-3 and AMA1, were more strongly predictive of protected children, than other combinations, in two separate groups of children. Fourthly, antibodies to allelic versions of the same antigen were equally predictive of the risk of clinical episodes, suggesting that in practice, the inability to include multiple allelic versions of highly polymorphic antigens into malaria vaccines may not be as limiting to vaccine efficacy as previously thought. This was supported by the findings that allele-specific antibodies did not appear to protect against clinical episodes associated with parasites bearing homologous alleles. All these findings have important implications for malaria vaccine development that's modeled on naturally acquired immunity.

The studies on AMA1 conducted here provide further evidence of the diversity at this locus, and confirm the presence of strong balancing selection acting to maintain these polymorphisms within natural populations of \textit{P. falciparum}. Preliminary experiments using peptides from Domain III of AMA1 suggest that T-cell mediated immune responses may contribute significantly to the signature of selection detected within this domain. This
work opens up an area of research into naturally-acquired T-cell mediated immune responses against AMA1 that has previously received little attention.
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