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MALARIA: MULTICLONAL INFECTIONS AND PROTECTIVE IMMUNITY

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Stockholm 2010

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ISBN 978-91-7409-874-7

ABSTRACT

The mortality and morbidity attributable to malaria remain high in Sub-Saharan Africa, especially among children less than five years of age. In areas of high transmission, immunity to clinical malaria is gradually acquired after repeated exposure to the polymorphic *Plasmodium falciparum* parasite. Increased knowledge of the interaction between the human host and the genetic diversity of *P. falciparum* infections is a prerequisite for understanding the mechanisms underlying acquisition of protective malaria immunity, an understanding important for the development of malaria control strategies e.g. vaccines.

This thesis has assessed how the genetic diversity of *P. falciparum* infections affects the risk of clinical malaria and how clearance of asymptomatic infections affects host protection. The thesis also includes establishment/development of a new technique to analyze the genetic diversity of parasite populations.

P. falciparum infections were genotyped based on sequence and size polymorphisms of the genes encoding the parasite antigens merozoite surface protein 1 and 2 (*msp 1* and *2*). A nested PCR method widely used to characterize parasite populations was adapted to fluorescent PCR and capillary electrophoresis in a DNA sequencer. The improved sensitivity and specificity of allelic discrimination forwards this new method as an important tool in molecular epidemiology studies and antimalarial drug trials.

Factors associated with the genetic diversity of *P. falciparum* infections were investigated in different transmission settings in Tanzania, Ghana and Kenya. The number of concurrent clones increased with age in all studies. Individual exposure, analyzed by antibody levels to the circumsporozoite protein, increased with age but was not associated with the number of clones in a high transmission setting in Tanzania.

The number of *P. falciparum* clones was correlated to the individual's subsequent risk of clinical malaria. In Tanzania, the lowest risk was found in asymptomatic children infected with 2-3 clones. In Ghana, intermittent preventive treatment administered during 6 months of the peak malaria season reduced the infection diversity. Although temporary, this reduction affected susceptibility to malaria during the following high transmission season. Infections composed of ≥ 2 clones again predicted a lower risk of febrile malaria, however only in children given placebo. These findings suggest that persistence of antigenically diverse *P. falciparum* infections is important for protective immunity and that clearance of multiclinal infections might contribute to the rebound in clinical disease observed after IPT was stopped in some studies. In an area of lower transmission in Kenya, children with ≥ 2 clones had a marked decreased risk of febrile malaria only when the parasites had been cleared with a course of an antimalarial drug. In Kenya, the number of clones was associated with level of exposure. When excluding children who remained uninfected after treatment and thus considered less exposed, the protection associated with multiclinal infections were even more evident and associated with blood stage immunity.

A reduced risk of malaria in asymptomatic individuals with persistent multiclinal *P. falciparum* infections suggests that controlled maintenance of diverse infections is important for clinical protection in continuously exposed individuals. The findings need to be considered in the design and evaluation of new malaria control strategies such as vaccines and interventions aiming to clear asymptomatic infections.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. **Multiclonal asymptomatic *Plasmodium falciparum* infections predict a reduced risk of malaria disease in a Tanzanian population**
Sándor Bereczky, Anne Liljander, Ingegerd Rooth, Lea Faraja, Fredrik Granath, Scott M. Montgomery, Anna Färnert.
Microbes and Infection 2007, 9:103-110
- II. **Optimization and validation of multi-coloured capillary electrophoresis for genotyping of *Plasmodium falciparum* merozoite surface proteins (*msp1* and *2*)**
Anne Liljander, Lisa Wiklund, Nicole Falk, Margaret Kweku, Andreas Mårtensson, Ingrid Felger, Anna Färnert.
Malaria Journal 2009, 8:78
- III. **The effect of Intermittent Preventive Treatment on the genetic diversity of *Plasmodium falciparum* infections and malaria morbidity in Ghanaian children**
Anne Liljander, Daniel Chandramohan, Margaret Kweku, Daniel Olsson, Scott M. Montgomery, Brian Greenwood, Anna Färnert.
Submitted
- IV. **Clearance of asymptomatic multiclonal *Plasmodium falciparum* infections; effect on subsequent risk of clinical malaria in Kenyan children**
Anne Liljander, Philip Bejon, Jediah Mwacharo, Oscar Kai, Edna Ogada, Norbert Peshu, Kevin Marsh, Anna Färnert.
Manuscript

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
AQ	Amodiaquine
AS	Artesunate
bp	Base pair
CI	Confidence interval
CSP	Circumsporozoite protein
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
Hb	Haemoglobin
HR	Hazard ratio
Ig	Immunoglobulin
IL	Interleukin
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITN	Insecticide-treated net
MSP	Merozoite surface protein
PCR	Polymerase chain reaction
RBC	Red blood cell
Rfu	Relative fluorescent units
SP	Sulphadoxine-pyrimethamine
WHO	World Health Organization
VSA	Variant surface antigen

DEFINITIONS

Definitions of terms used throughout this thesis:

Allele: one of several alternative forms of a gene that occupy the same locus.

Allelic type: alleles of a gene that can be grouped based on similar characteristics e.g. sequence similarity of the allelic types of *msp1* and *msp2*, also referred to as allelic families.

Clone: a set of genetically distinct blood-stage parasites derived from one parasite by asexual reproduction.

Genotype: combinations of alleles that determines a particular genetic characteristic.

Infection diversity: the number of clones detected within one sample. This number represents the minimum number of circulating clones; also referred to as genetic diversity of infection or multiplicity of infection.

Strain: the term strain has been widely used within the field of malaria research to describe distinct parasite populations that are distinguishable based on a variety of features e.g. biological or epidemiological, thus the term is difficult to define (McKenzie *et al.* 2008). In this thesis the term strain-specific is used in the context of malaria immunity; implying immune responses specific to one parasite strain that do not protect against a heterologous strain.

1 INTRODUCTION

1.1 THE MALARIA PARASITE AND THE MOSQUITO VECTOR

Malaria is caused by unicellular, protozoan parasites belonging to the genus *Plasmodium*. Over 100 distinct *Plasmodium* species have been identified that are capable of infecting mammals, birds and reptiles. Until recently four *Plasmodium* species were considered infectious to humans; *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*. However, *Plasmodium knowlesi*, the natural host of which are the long- and the pig-tailed macaque monkeys, has now been suggested as the fifth human malaria parasite (White 2008). *P. falciparum* is the main cause of malaria mortality and morbidity and is the focus of this thesis.

Human malaria is transmitted exclusively by female mosquitoes of the genus *Anopheles*. In sub-Saharan Africa, the predominant vectors belong to the *Anopheles gambiae* complex that includes some the most efficient transmitters of human malaria, *A. gambiae* and *A. arabiensis*.

1.2 GLOBAL BURDEN OF MALARIA

In 2008 it was estimated that malaria transmission occurred in 108 countries (Figure 1) (WHO 2009b). With the 243 million clinical cases and the roughly 900 000 deaths per year, malaria is one of the most important infectious diseases and one of the leading causes of death. The disease burden is highest in Africa (85% of cases) where a vast majority of the fatal cases occur in children under the age of five years. In part as a result of the scaling up of effective interventions i.e. insecticide-treated bednets (ITNs), indoor residual spraying (IRS) and treatment with artemisinin-based combination therapy (ACT) a remarkable reduction (>50%) in malaria cases has been reported from several countries in and outside Africa (WHO 2009b). However, countries with the highest incidence rate reported the smallest decrease in number of clinical cases. The burden of disease extends well beyond mortality and morbidity. Malaria transmission and poverty share geographical distribution, and malaria is considered to have a profound effect on the economic growth in endemic countries (Sachs *et al.* 2002).

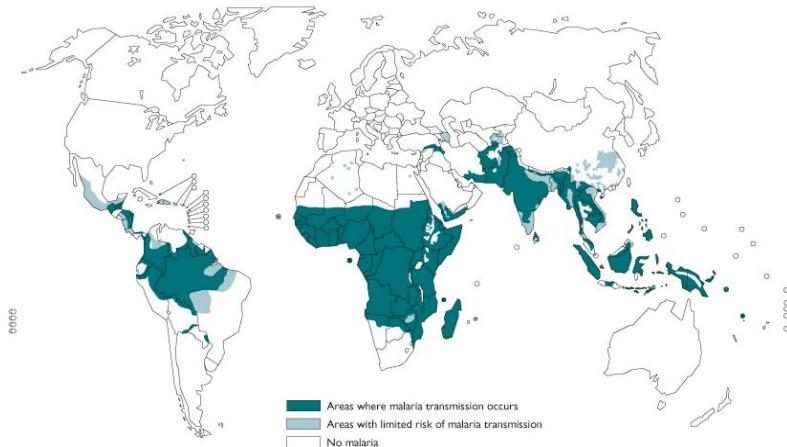


Figure 1 Malaria transmission 2008. Printed with permission from WHO.
[\(\[http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_RiskAreas_2007.png\]\(http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_RiskAreas_2007.png\)\)](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_RiskAreas_2007.png)

1.3 THE LIFE CYCLE OF *P. FALCIPARUM*

The life cycle of the malaria parasite is very complex and comprises morphologically and antigenically distinct stages both in the *Anopheles* mosquito and in the human host. The life cycle is depicted in Figure 2. Briefly, when an infected female *Anopheles* mosquito penetrates the human skin for a blood meal, sporozoites are injected along with the anticoagulant saliva. The sporozoites readily migrate through the bloodstream to the liver where they invade the hepatocytes. Within the hepatocytes, the sporozoite can either undergo initial growth followed by asexual replication (liver schizogony) into a mature liver schizont containing up to 30 000 merozoites, or as for *P. vivax* and *P. ovale*, the sporozoite can enter a dormant stage (hypnozoite) that can cause clinical relapses weeks, months or years after the primary infection. The liver schizogony takes 5-16 days depending on species (5-7 days for *P. falciparum*) and is asymptomatic. As the mature liver schizonts rupture merozoites are released. Most are ingested by liver macrophages i.e. Kupffer cells, however, the merozoites that do escape rapidly invade the erythrocytes (red blood cells). Once inside the erythrocyte the merozoite re-differentiate to an immature trophozoite (ring form), then to a mature trophozoite, followed by asexual replication to a mature schizont containing 10 to 20 merozoites. The merozoites are released upon erythrocyte rupture and rapidly infect new erythrocytes. The duration of the erythrocytic cycle differs between species; 24 h for *P. knowlesi*, 48 h for *P. falciparum*, *P. vivax* and *P. ovale*; while 72 h for *P. malariae*.

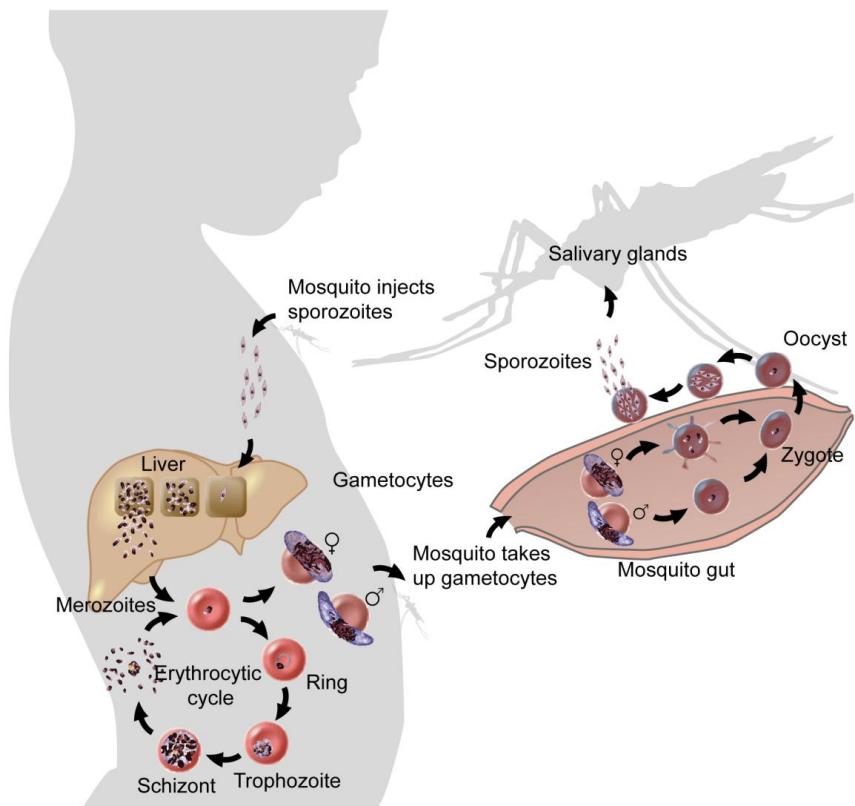


Figure 2 Life cycle of *P. falciparum*.

Printed with kind permission from Dr. Christin Sisowath. Illustration by Leopold Roos.

A characteristic of *P. falciparum* is sequestration i.e. binding of infected erythrocytes to endothelium in the deep vascular system during the second half of the erythrocytic cycle. Therefore only ring forms and early trophozoites are detectable in peripheral blood.

As the erythrocytes rupture, parasite debris are released. This induces host responses e.g. fever and cytokines and the symptomatic phase of the infection starts. The clinical manifestations vary from asymptomatic infections to severe life-threatening conditions.

Some merozoites do not undergo further asexual replication; instead they develop into male and female gametocytes. Erythrocytes containing gametocytes do not rupture; instead they circulate, waiting to be extracted from the human host by a blood feeding mosquito. Within the mosquito's gut the gametocytes, triggered by the presence of specific mosquito factors and the drop in temperature, form male and female gametes.

A male and a female gamete fuse, forming a diploid zygote that undergoes meiosis and recombination. The resulting ookinete penetrates the mosquito's mid-gut wall, transforms into an oocyst that, through repeated mitotic divisions, produces a large number of haploid sporozoites. These will migrate and invade the salivary glands from which they can be injected into the human host as the mosquito takes a blood meal, thus starting the life cycle of the parasite again. The process takes 10-18 days (at 28°C) depending on *Plasmodium* species. The mosquito remains infectious for 1-2 months.

1.4 THE CLINICAL DISEASE

Of the five *Plasmodium* species that can cause disease in humans, most cases of severe malaria are attributable to *P. falciparum*. The other species cause rather mild infections with low parasite loads. Nonetheless, exceptions have been reported, in particular for *P. vivax* (Poespoprodjo *et al.* 2009) and *P. knowlesi* (Cox-Singh *et al.* 2008).

1.4.1 Clinical presentation of malaria

Malaria infections are asymptomatic during the liver stage and clinical symptoms do not develop until rupture of the infected erythrocytes. Early symptoms are often rather non-specific such as fever, headache, weakness, muscle/joint and abdominal pains, diarrhea and vomiting. Furthermore, children often present with cough, difficulty in breathing as well as enlarged spleen and signs of anaemia.

The mechanisms associated with *P. falciparum* pathogenesis are still largely unknown; however the parasite's ability to sequester in the deep vascular system along with the high multiplication rate are thought to be key features. The outcome and severity of disease depends on age, genetic disposition, immune status and general health of the person.

Besides infants and children, pregnant women represent another risk group for malaria disease. Despite repeated infections during childhood and adolescence resulting in acquired malaria immunity, women become susceptible to disease during pregnancy. Pregnancy-associated malaria is characterized by placental malaria i.e. sequestration of parasites in the placenta. The infection might be asymptomatic; however, adverse consequences of maternal infection include maternal anaemia and low birth weight of the infant (Shulman *et al.* 1996; Aribodor *et al.* 2009).

Malaria symptoms can be categorized as uncomplicated i.e. symptoms as described above, without signs of severe malaria. Provided that efficient treatment is given, the case fatality rate is low for uncomplicated falciparum malaria (1/1000).

However without prompt treatment the disease can progress to life-threatening severe malaria within hours. The clinical manifestations include cerebral malaria (unrousable coma), severe anaemia (haemoglobin concentration (Hb) \leq 5.0g/dl), respiratory distress, acidosis and hypoglycemia (WHO 2000). Case fatality with treatment is 10-20 %, without treatment severe malaria is almost always fatal (WHO 2010). Mortality is particularly high in children who have impaired consciousness or respiratory distress (Marsh *et al.* 1995).

Severe anaemia is common among infants in high transmission areas while cerebral malaria increase in incidence among older children and adults as the transmission intensity decreases (Snow *et al.* 1997; Reyburn *et al.* 2005; Okiro *et al.* 2009).

Many children that survive cerebral malaria suffer from transient or permanent neurological sequelae e.g. cognitive impairment, ataxia, hemiparesis and cortical blindness (Newton *et al.* 1998).

Susceptibility and disease progression might be influenced by nutritional status and other infecting pathogens. Malnutrition and deficiencies in micronutrients e.g. zinc, iron and vitamin A has been associated with an increased risk of malaria morbidity and mortality (Caulfield *et al.* 2004; Berkley *et al.* 2009). Concurrent infection with HIV and/or bacteria e.g. nontyphoidal salmonellae was associated with increased case fatality rate among children with severe malaria (Berkley *et al.* 2009).

1.4.2 Malaria diagnosis and treatment

Malaria is a curable disease provided that prompt diagnosis and effective treatment is available. Fever or history of fever within the past 24h and/or pronounced anaemia is often the basis for a clinical diagnosis in remote areas. However, due to the overlapping clinical presentation of malaria with other diseases, e.g. influenza and pneumonia (O'Dempsey *et al.* 1993; English *et al.* 1996), a confirmed malaria diagnosis is desirable to reduce unnecessary treatment with antimalarials.

Light microscopy of stained thick and thin blood smears remains the conventional method for malaria diagnosis. The technique is relatively cheap and is fairly sensitive with detection down to 50-100 parasites / μ l blood under field conditions (Wongsrichanalai *et al.* 2007). Moreover, slide examination allows for species identification and quantification of the parasite load. However, the method requires skilled personnel with sufficient time for reading each slide, functional microscopes and electricity.

Rapid diagnostic test (RDT) for malaria is a more simple method that do not require skilled personnel for interpretation or electricity. The test detects malaria antigens e.g. histidine-rich protein 2 (HRP-2) for *P. falciparum* in small blood volumes (5-15 µl) in 5-20 min. Available RDTs can detect *P. falciparum* alone or can distinguish between *P. falciparum* and other human malaria species, although with varying sensitivity (Wongsrichanalai *et al.* 2007). They can however not quantify the parasite load and *P. falciparum* might be hard to detect at low densities. Although more expensive than microscopy, RDTs might be valuable in the diagnosis of febrile illnesses in remote areas where microscopy is not available.

The recommended first line treatment of uncomplicated malaria is a combination of antimalarials i.e. artemisinin-based combination therapy (ACT) given for a minimum of three days (WHO 2010). Severe malaria is treated with intravenous quinine or with certain artemisinin derivatives e.g. artemether and artesunate.

1.5 MALARIA TRANSMISSION AND EPIDEMIOLOGY

Malaria transmission is restricted to geographical areas where *Anopheles* mosquitoes thrive and where the climate and temperature is favorable for the parasite i.e. mainly in sub-tropical and tropical regions. Indigenous malaria can be either endemic or epidemic. Endemic transmission is characterized by consistent transmission over a long period of time. The transmission can be either stable, characterized by continuous transmission (constant over many years) with or without seasonal fluctuations (rainy/dry seasons); or unstable with considerable fluctuations. The level of transmission is reflected by the entomological inoculation rate (EIR) i.e. number of infective mosquito bites received per person per year. Stable transmission is associated with an EIR >10 per year while in areas with unstable transmission the EIRs are between <1 and <5 infective bites per year (WHO 2010). Malaria epidemics may occur in areas with low and unstable transmission and are characterized by a sudden increase in the number of clinical cases.

The level of transmission intensity i.e. endemicity was previously classified according to the proportion of children with enlarged spleen in a community (spleen rate). The classification has been revised to parasite rate i.e. prevalence of peripheral blood-stage infection among children 2-9 years old (Metselaar 1959). Spleen and parasite rates provide literally the same definitions of malaria endemicity i.e. holoendemicity (>75%), hyperendemicity (50-75%), mesoendemicity (11-50%) and hypoendemicity (<10%). However, the measures only provide a rough estimation of the transmission setting since seasonal changes is not captured at a single cross-sectional survey.

Malaria transmission can occur without a mosquito vector through blood transfusions and contaminated needles/syringes as well as from mother to fetus during pregnancy.

1.6 MALARIA CONTROL IN AFRICA

The malaria parasite has persisted through decades of global eradication efforts, development of efficient drugs and over 30 years of vaccine research. In 1955 the Global Malaria Eradication Program was launched by WHO. Although successful in some countries e.g. USA and parts of Europe, transmission could not be interrupted in many high endemic countries and the program was abandoned in 1972. Malaria resurged in many areas alongside the emergence of parasite resistance to chloroquine and sulphadoxine-pyrimethamine (SP) and insect resistance to DDT. Subsequently the efforts were reoriented from eradication to malaria control. WHO defines malaria control as; reducing the burden of disease to a level at which it is no longer a public health problem (WHO 2008a). The main tools for malaria control are; effective antimalarial drugs, including artemisinin-based combination therapy (ACT), insecticide treated nets (ITN), indoor residual spraying (IRS) and intermittent preventive treatment (IPT).

1.6.1 Artemisinin-based combination therapy (ACT)

Early diagnosis and prompt treatment are cornerstones in malaria control. A new concept of antimalarial treatment has been adopted i.e. artemisinin-based combination therapy (ACT) to increase the rate of clinical and parasitological cure and to decrease the emergence of parasite resistance to antimalarials. The concept comprises administration of two antimalarial drugs with different modes of action; an artemisinin derivative with rapid reduction of the parasite biomass and gametocyte carriage, combined with a long acting drug. ACT is considered the best available treatment for uncomplicated malaria and is recommended as first line treatment in malaria endemic areas (WHO 2010). Currently WHO recommends five different ACTs; artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-SP and dihydroartemisinin-piperaquine. Although accepted as treatment policy, the ACT coverage remains low in most African countries (WHO 2009b).

1.6.2 Insecticide-treated nets (ITNs)

The use of insecticide-treated nets and curtains are effective strategies for malaria control. ITNs are largely efficacious in reducing malaria associated mortality (with 25% and 33% respectively) and morbidity (with ~50% and 44% respectively) among children (D'Alessandro *et al.* 1995; Nevill *et al.* 1996). Moreover, ITNs have also reduced the all-cause mortality among children in high endemic areas by 18% (Lengeler 2004). Besides the protection granted the users, high coverage of ITNs also

provide benefits for the non-users by killing and/or diverting mosquitoes away from houses with treated nets.

Recently, long-lasting ITNs (LLIN) have been introduced that maintain protective levels of insecticides for more than three years. The coverage of ITNs and LLINs has increased in Africa the past years however the percentage of children under five years sleeping under a treated net is still low (24%) (WHO 2009b).

1.6.3 Indoor residual spraying (IRS)

Indoor spraying with long-lasting insecticides on the roof and walls kills mosquitoes that rest on treated surfaces. IRS has had a great impact on parasite prevalence and malaria morbidity (Mabaso *et al.* 2004) and the use of IRS has increased drastically over the past years, however the number of people protected by IRS is still low (WHO 2009b).

1.6.4 Malaria vaccines

The development of a malaria vaccine has been difficult. Nonetheless, great investments and research developments have resulted in a large number of potential vaccine candidates that are now in preclinical development or in clinical trials.

The primary objective of a pre-erythrocytic vaccine is to prevent blood-stage infection and thus protect against any clinical malaria. Trials are ongoing evaluating synthetic sub-unit vaccines based on the TRAP and the CS proteins of the sporozoite (Targett *et al.* 2008) with the latter being the major constituent of the RTS, S vaccine. Immunization with RTS,S resulted in almost a 50% protection against severe malaria and 30% protection against clinical malaria in children (Alonso *et al.* 2005). Recent studies have confirmed the efficacy of RTS,S in infants and children (Abdulla *et al.* 2008; Bejon *et al.* 2008).

A blood-stage vaccine will not prevent infection but might protect against clinical symptoms. The extensive polymorphism in many of the *P. falciparum* blood-stage proteins has complicated the task of developing a blood-stage vaccine. There are however certain promising vaccine candidates including MSP3 (Druilhe *et al.* 2005) and MSP1/MSP2 and RESA (Genton *et al.* 2002) and more recently the recombinant AMA-1 (Spring *et al.* 2009). Immunity induced by polymorphic vaccine antigens is largely allele specific and the allelic types of the antigens included in a vaccine are likely to affect the outcome. In a trial of the vaccine Combination B (Genton *et al.* 2002) comprising the 3D7 allele of MSP2 there was an increased incidence of malaria morbidity attributable to the FC27 allele of MSP2 among vaccine recipients suggesting that vaccination induced selection of parasites expressing the alternative

allele. Thus, vaccine formulations should include components covering all important allelic types and/or conserved antigens.

1.6.5 Intermittent Preventive Treatment (IPT)

Intermittent preventive treatment (IPT) is a new strategy for malaria control. IPT involves administration of curative doses of antimalarials at specific time points to vulnerable populations (pregnant women, infants and children) in endemic areas, regardless of whether a subject is known to be infected. The concept of IPT is a development from the previously used long term chemoprophylaxis. Sustained chemoprophylaxis reduced malaria related mortality and morbidity, however, it never became a recommended strategy due to logistic problems, concerns of impaired development of malaria immunity and fear of emergence of drug resistant parasites. The advantage of intermittent treatment over sustained chemoprophylaxis is reduced drug exposure.

1.6.5.1 IPT in pregnancy (IPT_p)

IPT_p involves full therapeutic doses of SP given 2 to 3 times from the 2nd trimester concurrent with visits to the antenatal clinic. The administration of SP during pregnancy is efficient in reducing the risk of placental parasitemias, maternal anaemia and in preventing low birth weight and neonatal death (Menendez *et al.*; Shulman *et al.* 1999; Gies *et al.* 2009). The strategy was recommended by the WHO in 1998 and is now a policy in several African countries. Nonetheless, the coverage is still low with only 20% of the pregnant women receiving two IPT doses (WHO 2009b).

1.6.5.2 IPT in infants (IPT_i)

Several studies of IPT with SP given to infants alongside the extended program on immunization (EPI) at the age of 2, 3 and 9 months have shown a reduction in the incidence of clinical malaria and anaemia by 20-30% (Aponte *et al.* 2009). IPT_i is now recommended for implementation in areas with high burden of malaria and low SP resistance (WHO 2009a). Although the first IPT_i study showed a prolonged protective efficacy extending beyond the pharmacological effect of the drug (Schellenberg *et al.* 2001; Schellenberg *et al.* 2005), subsequent IPT_i trials with SP have not reported any sustained protection (Chandramohan *et al.* 2005; Macete *et al.* 2006; Kobbe *et al.* 2007; Mockenhaupt *et al.* 2007). With the widespread and increasing resistance to SP, other long- and short-acting antimalarial drugs are now being investigated for IPT_i (Cairns *et al.*; Gosling *et al.* 2009). IPT_i is likely to be most effective in areas with continuous high transmission where the highest burden of disease falls on the infants.

1.6.5.3 IPT in children (IPTc) and school-aged children (IPTsc)

In many areas of sub-Saharan Africa, especially in those with seasonal transmission, the main burden of malaria is in children less than five years of age. Children in school-age have developed partial immunity to malaria and infections are often not fatal. Nonetheless, they are still at risk of clinical disease, asymptomatic parasitemias and anaemia. Clinical malaria has been associated with inferior school performance (Fernando *et al.* 2003).

One approach for children in these areas is administration of IPT during the most intense transmission season. In two trials of seasonal IPTc with SP and/or AS, the incidence of clinical malaria was reduced by 42.5% and 86% respectively (Cisse *et al.* 2006; Dicko *et al.* 2008). Moreover, in Ghana, monthly administration of artesunate plus amodiaquine (AS+AQ) reduced clinical malaria by 69% whereas bimonthly doses of either AS+AQ or SP resulted in a decrease of 17% and 24% respectively (Kweku *et al.* 2008).

In children aged 6-13 years, seasonal IPTsc with SP/AS or AS+AQ substantially reduced the incidence of clinical malaria, asymptomatic parasitemias and anaemia (Barger *et al.* 2009).

1.6.5.4 IPT and impaired immunity to malaria?

The mode of action of IPT is both by clearing existing parasitemias and through post-treatment prophylaxis protecting from new infections. Administration of antimalarial drugs to asymptomatic individuals could potentially lead to a delayed acquisition of immunity to malaria.

Rebound in malaria morbidity has been reported following sustained chemoprophylaxis in some studies (Greenwood *et al.* 1995; Menendez *et al.* 1997). Furthermore, some IPTi/c studies have reported an increased incidence of clinical malaria or anaemia when the intervention was stopped (Chandramohan *et al.* 2005; Cisse *et al.* 2006; Mockenhaupt *et al.* 2007; Kweku *et al.* 2008).

Infants in Ghana assigned to IPTi during their first year of life had an increased incidence of high-density clinical malaria (parasite density more than 5000 per μl) during their second year of life (Chandramohan *et al.* 2005). In another trial in Ghana, the risk of severe malarial anaemia was higher in children who had received IPTi compared to those who received placebo (Mockenhaupt *et al.* 2007). Nonetheless, in spite of these reports of rebound in the year after IPTi was stopped, a recent meta-analysis of all the published trials of IPTi did not find any evidence to support the idea that IPTi leads to a rebound in malaria morbidity (Aponte *et al.* 2009).

In Senegal, there was a tendency for children older than two years at the time of IPT to have an increased risk of clinical malaria during follow-up as compared to younger children (Cisse *et al.* 2006). Ghanaian children receiving monthly IPTc with AS+AQ also experienced more clinical episodes during follow-up (Kweku *et al.* 2008). These results suggest that immune impairment by IPT may occur in infants and children with different levels of acquired immunity.

1.7 IMMUNITY TO MALARIA

Immunity to malaria develops after repeated infections by *P. falciparum* parasites. The acquisition of immunity is largely dependent on the level of malaria transmission. In areas of high transmission, immunity develops in an age-dependent manner where children under five years of age are at highest risk of disease, and clinical manifestations among adults are rare; while in areas of low/unstable transmission, immunity is not acquired and therefore all age groups are at risk.

1.7.1 Innate immunity

Macrophages and dendritic cells (DCs) expressing Toll-like receptors (TLRs) are believed to be important in early immune responses to malaria. TLR9 has recently been identified as receptor for *P. falciparum* derived antigens e.g. hemozoin (Coban *et al.* 2007). Binding of hemozoin to the receptor activates macrophages and DCs and induce release of pro-inflammatory cytokines e.g. TNF as well as anti-inflammatory cytokines e.g. IL-10. Moreover infected erythrocytes are recognized by host receptors such as CD36 on DCs and macrophages resulting in phagocytosis of the infected cells. Evidence is also emerging of the importance of NK cells and the production of IFN- γ in early responses to malaria (Korbel *et al.* 2004).

Certain host genetic factors have been associated with resistance to malaria and protection from severe disease e.g. sickle-cell trait, beta- and alpha-thalassaemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Williams 2006).

1.7.2 Acquired immunity

Under intense transmission children first develop an anti-disease immunity that protects against severe clinical manifestations. Anti-parasite immunity, protecting against high parasite burdens is acquired more slowly. Sterilizing immunity is never fully achieved and asymptomatic infections are common in children and adults in endemic areas. This state of equilibrium between the immune response and the nearly constant low-level parasitemia has been termed premunition (Sargent 1935) and implies that immunity to malaria is mediated by the presence of parasites rather than by previous exposure. The

immunity to malaria is lost if the exposure is interrupted for longer time periods (Colbourne 1955).

During the first months of life, infants are protected from high parasitemias, fever and severe clinical manifestations and the infections that do occur are often asymptomatic and self clearing (Franks *et al.* 2001). This protection has been associated with fetal haemoglobin (Pasvol *et al.* 1976) and passive transfer of maternal IgG over the placenta (Riley *et al.* 2001). The duration of this passive immunity seems to be related to transmission intensity, decreasing faster in areas of high transmission. Between 4 to 6 months of age the infant becomes susceptible to severe disease with severe anaemia as the most common manifestation. Immunity to severe non-cerebral malaria has been suggested to be acquired after 1 to 2 infections (Gupta *et al.* 1999). By school age children have acquired considerable immunity to malaria as reflected by a decrease in number of clinical episodes and lower parasite loads (Marsh 1992). Adults rarely develop symptomatic disease and are often infected without having any symptoms. However, during pregnancy, in particular during the first and second pregnancies, women are again susceptible to malaria. Maternal susceptibility is thought to be related to immune suppression during gestation and accumulation of parasitized erythrocytes in the placenta (Menendez 2006).

1.7.3 Pre-erythrocytic immunity

Vaccination with live attenuated sporozoites induces strong and sterilizing immunity in humans involving both cellular and humoral responses (Herrington *et al.* 1991; Egan *et al.* 1993). Antibodies to pre-erythrocytic stages can protect either through opsonization leading to sporozoite clearance before reaching the liver, or by interfering with the hepatocyte invasion process. Antibodies have been found in humans that recognize several surface proteins of the sporozoite e.g. CSP, LSA1 and TRAP (Marsh *et al.* 2006). The most successful vaccine in trial, RTS,S, containing parts of the circumsporozoite (CS) protein, elicited high IgG concentrations in protected vaccine recipients (Moorthy *et al.* 2009). Moreover, the number of CS-specific CD4+ T-cells secreting IFN- γ or a combination of IFN- γ , TNF, IL-2 and CD40 ligand was greater among the protected vaccine recipients. Similar results were obtained after sporozoite challenges in humans (Roestenberg *et al.* 2009).

1.7.4 Blood-stage immunity

Antibodies are important in reducing parasite densities during blood-stage infection (Cohen *et al.* 1961). Possible functions of the antibodies include opsonization of free merozoites or infected red blood cells (RBCs) to promote phagocytosis by macrophages, prevent processing of proteins important for invasion (e.g. MSP1) or

blocking erythrocyte binding sites. Humoral responses to blood-stage infection appear to be acquired in an age-dependent manner with highest levels achieved by late childhood or early adolescence (Riley *et al.* 1992; Polley *et al.* 2006; Osier *et al.* 2007). Moreover, IgG antibodies against many of the blood-stage proteins of *P. falciparum* e.g. MSP1, MSP2, MSP3, AMA1, and the VSAs (variant surface antigens) have been found to be associated with protection against clinical disease (Riley *et al.* 1992; Conway *et al.* 2000; Dodoo *et al.* 2001; Kinyanjui *et al.* 2004; Polley *et al.* 2004; Polley *et al.* 2006; Osier *et al.* 2007). Furthermore, high antibody levels to a combination of different antigens appear more protective against clinical malaria than antibodies to a single antigen (Osier *et al.* 2008). Nonetheless, antibody responses are rather short-lived (Kinyanjui *et al.* 2007) and may be lost without persisting infection (Akpojeneta *et al.* 2008). In Kenyan children, IgG1 and IgG3 antibody levels against MSP1-19, MSP2, EBA-175 and AMA-1 decreased swiftly within six weeks after a clinical episode (Kinyanjui *et al.* 2007). Likewise, without infection, antibody responses (to the same antigens as above) were lost within four months after sampling (Akpojeneta *et al.* 2008).

Although antibodies of different isotypes have been found, IgG appear to be the most important with the IgG1 and IgG3 sub-classes prevailing (Taylor *et al.* 1995; Jouin *et al.* 2001; Ndungu *et al.* 2002). High levels of IgE have been reported during severe clinical malaria (Perlmann *et al.* 1994; Perlmann *et al.* 2000) and have also been associated with protection against clinical disease (Bereczky *et al.* 2004).

Studies of induced blood-stage infection in humans showed proliferative T-cell responses (CD4+ and CD8+) and production of IFN- γ and nitric oxide synthase activity in mononuclear cells in protected individuals (Pombo *et al.* 2002).

1.7.5 Strain-specific and cross-reactive immunity

Immunity to malaria develops both in a strain-specific and cross-reactive manner. Early evidence of strain-specificity came from studies of induced malaria for treatment of neurosyphilis (Jeffery 1966). Altered disease progression (lower peak parasite densities and rapid termination of clinical symptoms) was seen after repeated inoculations with homologues *P. falciparum* strains (Jeffery 1966). Protection against homologous strains has been shown in other human studies (Wilson *et al.* 1976) and in animal models (Jones *et al.* 2000).

Immune responses to the VSAs also develop largely in a specific manner i.e. variant-specific. Among semi-immune children, clinical episodes were primarily caused by parasites expressing VSA variants not recognized by the pre-existing VSA-specific antibodies in the individual children studied (Bull *et al.* 1998). Such clinical episodes

were followed by an increase in antibodies specific to the VSAs expressed by the parasite causing the episodes (Ofori *et al.* 2002; Kinyanjui *et al.* 2003).

Besides the strain-specific components, cross-reactive responses to heterologous strains have been documented. *Aotus* monkeys could control the parasiteamia induced by a heterologous challenge with *P. falciparum* (Jones *et al.* 2000). Modification of disease progression during second heterologous *P. falciparum* infections in humans have been reported (Jeffery 1966). Moreover, a number of studies have described cross-reactive antibody recognition (Ofori *et al.* 2002; Felger *et al.* 2003; Franks *et al.* 2003).

1.8 GENETIC DIVERSITY OF *P. FALCIPARUM*

The *P. falciparum* genome is 23 megabases long, consists of 14 chromosomes and encodes for approximately 5300 genes (Gardner *et al.* 2002). A large number of genes exhibit extensive polymorphism. In particular, the loci encoding proteins displayed on the surface of the sporozoite (e.g. CSP) and the merozoite (e.g. MSP1, MSP2, AMA1) and thus accessible to the host immune components, are highly polymorphic (Escalante *et al.* 1998). In these genes, conserved and semi-conserved regions are interspersed with variable regions containing repetitive units that differ in sequence, length and copy number. The diversity is preserved through a high number of non-synonymous nucleotide substitutions (Escalante *et al.* 1998) as well as duplications and/or deletions of repetitive units (Felger *et al.* 1997; Rich *et al.* 2000).

Sequential expression of alternate forms of an antigen is an additional mechanism for genetic variation in *P. falciparum*. Gene switching is associated with alternating expression of the genes (*var*) encoding the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). The *var* genes form a multi-gene family, comprising approximately 60 genes dispersed over several chromosomes (Gardner *et al.* 2002). During early stages of the parasite's intra-erythrocytic development, multiple *var* genes may be transcribed, however during late stages, one transcript dominates and only a single variant of PfEMP1 is expressed on the surface of the infected erythrocyte (Chen *et al.* 1998b). The switching in *var* gene expression results in the transcription of a new dominant *var* gene and the expression of a different PfEMP1 variant. PfEMP1 is known to mediate cytoadhesion of infected RBCs to endothelial cells (Smith *et al.* 1995) and binding to uninfected erythrocytes i.e. rosetting (Chen *et al.* 1998a), mechanisms believed to be associated with immune evasion and pathogenesis.

Other characterized *P. falciparum* proteins that exhibit great diversity are the RIFINs and STEVORs which are encoded by one of the around 200 *rif* genes (Kyes *et al.* 1999) and 30-40 *stevor* genes (Blythe *et al.* 2004) respectively. Moreover, genetic

polymorphisms in a number of genes (e.g. *pfnmdr1*, *pfCRT* and *dhfr*) has been associated with parasite resistance to antimalarials (e.g. chloroquine, amodiaquine and SP) and treatment failure (Picot *et al.* 2009).

1.8.1 Genotyping of *P. falciparum*

The introduction of PCR based genotyping techniques in malaria research has substantially improved the understanding of the parasite biology and epidemiology. A number of highly polymorphic genetic markers of *P. falciparum* have been characterized and can be used to distinguish individual parasite populations. The most widely used markers for genotyping of *P. falciparum* are the genes encoding the MSP1, MSP2 and GLURP i.e. *merozoite surface protein 1* (*msp1*) and 2 (*msp2*) and *glutamate-rich protein* (*glurp*). These genes are suitable to characterize parasite populations since as they are unlinked single copy genes and remain stable throughout the erythrocytic life cycle (as opposed to e.g. *var* genes). In epidemiological studies, genotyping is used to investigate the infection diversity i.e. number of infecting parasite clones, in relation to factors such as transmission intensity and host immunity. In antimalarial drug trials, genotyping is recommended to define treatment outcome by differentiating recrudescence parasites from new infections (WHO 2008b). To distinguish recrudescence from a new infection, genotyping is often performed stepwise adding several consecutive markers (*msp1*, *msp2* and *glurp*) (Mugitu *et al.* 2006). In epidemiological studies assessing infection diversity, a single marker is often sufficient and *msp2* has been shown to be the most informative marker (Farnert *et al.* 2001).

In this thesis the *msp1* and *msp2* markers were optimized for a new genotyping methodology. Antigens coded by these genes are described in more detail below. The *msp2* marker was used as the main marker to characterize parasite populations throughout the different studies.

1.8.1.1 Merozoite surface protein 1 (*msp1*)

Merozoite surface protein 1 (MSP1) (previously referred to as p190 and p195) is a 195 kDa polypeptide anchored to the plasma membrane of the merozoite (Holder 1988). MSP1 is the most abundant protein on the surface of the merozoite and is thought to be involved in RBC invasion. The protein has been extensively studied and is considered a major vaccine candidate. During maturation the protein undergoes two distinct proteolytic processing events; initial processing as the merozoite is released from the rupturing schizont followed by a second processing as the merozoite invades the RBC. The MSP1 complex is shed during invasion, except for the 19kDa C-terminal fragment (MSP1₁₉) that remains attached (Holder 2009).

The gene encoding MSP1 is located on chromosome 9 and divided into 17 blocks (1-17) based on level of sequence variance i.e. conserved, semi-conserved and variable regions (Tanabe *et al.* 1987). The sequence displays a dimorphic pattern defining two distinct allelic types, the MAD20- and the K1-type (Tanabe *et al.* 1987). Block 2 however represents an exception to the dimorphism as a third allelic type (RO33) has been defined. Block 2 of the MAD20- and the K1-type contain tri and hexapeptide repeat units (9 and 18bp) that differ in sequence and copy number while block 2 of the RO33-type lacks repeats (Tanabe *et al.* 1987; Miller *et al.* 1993; Ferreira *et al.* 2003). Flanking the repetitive regions in block 2 are non-repetitive sequences that are highly conserved within the allelic type but differ between types. Recently, a novel allelic type, the MR-type was described as a recombinant with the 5' end being a MAD20-type while the 3' end is an RO33-type (Takala *et al.* 2002).

Antibodies to MSP1 have been shown to inhibit parasite invasion *in vitro* through agglutination of free merozoites, preventing MSP1 processing, and by inhibiting interactions with host receptors (Holder 2009). Antibodies to the conserved as well as the repetitive regions (block 2) of the protein have been identified (Riley *et al.* 1992; Da Silveira *et al.* 1999; Conway *et al.* 2000; Jouin *et al.* 2001) and associated with protection from clinical disease (Riley *et al.* 1992; Conway *et al.* 2000).

1.8.1.2 Merozoite surface protein 2 (*msp2*)

The merozoite surface protein 2 (MSP2), previously referred to as merozoite surface antigen 2 (MSA 2), is a ~30kD glycoprotein anchored in the plasma membrane of the merozoite (Smythe *et al.* 1988; Snewin *et al.* 1991). MSP2 is thought to be involved in RBC invasion and has been well characterized as a potential vaccine candidate. The *msp2* gene, located on chromosome 2, contains a single open reading frame with conserved, semi-conserved and variable sequences. According to sequence type the gene has been divided into five blocks (1-5) (Snewin *et al.* 1991). The N and C-terminal sequences (block 1 and 5) are highly conserved while block 2 and 4 are semi-conserved. Block 3 contains variable non-repetitive sequences flanking repetitive units that differ in length and copy number (Smythe *et al.* 1990). The non-repetitive sequences define the two allelic types, the FC27- and the IC- (elsewhere also referred to as 3D7) allelic types (Smythe *et al.* 1991). Block 3 of the FC27-type alleles contains varying number of structurally conserved 96 bp (1-4 copies) and 36 bp (0-5 copies) repeat units (Smythe *et al.* 1988; Smythe *et al.* 1991; Felger *et al.* 1994; Ferreira *et al.* 2007). Additionally, a 9 bp repeat unit has also been described to occur in 2-23 copies in the FC27 family (Irion *et al.* 1997). In contrast, the repeat units of the IC-type are less conserved; and highly variable in length (6-30 bp), copy number (up to 45) and in sequence (Smythe *et al.* 1990; Felger *et al.* 1997; Putaporntip *et al.* 2008).

Recombination during meiosis between alleles of the different types has been reported (Marshall *et al.* 1991; Snewin *et al.* 1991). The 5'end of the *msp2* hybrids is an IC-type while the 3'end is an FC27-type. Hybrids have been found in only 3.1% of the sequenced *msp2* alleles (Ferreira *et al.* 2007).

Antibodies to MSP2 inhibit merozoite invasion *in vitro* (Epping *et al.* 1988; Clark *et al.* 1989) and have been associated with protection from clinical malaria (Polley *et al.* 2006). While a relatively small proportion of the antibodies to MSP2 react with the N and C terminal conserved epitopes, a majority recognize the allelic type specific and repetitive regions (Thomas *et al.* 1990; Taylor *et al.* 1995). Antibody cross-reactivity within the allelic types has also been reported (Felger *et al.* 2003; Franks *et al.* 2003).

1.8.1.3 Methods for genotyping *msp1* and *msp2*

The most widely used techniques for genotyping of *P. falciparum* are based on two-step PCR amplification (nested PCR). The entire gene segment of interest is amplified in a primary amplification e.g. block 2 of *msp1* and block 3 of *msp2*, followed by a nested amplification targeting the allelic type specific regions (Contamin *et al.* 1995; Zwetyenga *et al.* 1998; Felger *et al.* 1999a; Snounou *et al.* 1999). Nested PCR is used to increase the specificity and sensitivity of the DNA amplification and is therefore suitable for detection of parasite genotypes present in low concentrations in a sample. The nested PCR products are usually distinguished from each other based on fragment size after separation by gel electrophoresis and visualization under UV-light after ethidium bromide staining. Fragment sizes are estimated compared to a DNA size standard by the naked eye or with digital software. Interpretation of agarose gels and comparisons between separate runs might however be difficult since the exact base pair (bp) size and variations between fragments are often hard to detect. Moreover, samples with high parasite densities often generate non-specific bands and smears detectable after gel separation (illustrated in Figure 3).

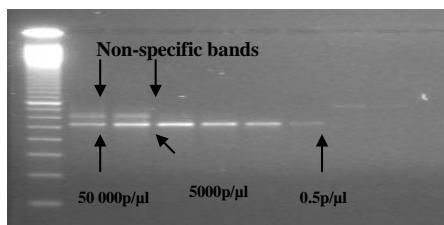


Figure 3 Genotyping of *msp2* of the F32 laboratory line in different concentrations (parasites/ μ l) exemplifies non-specific bands that often appear in high density samples following electrophoresis on agarose gel

A new technique for fragment analysis is capillary electrophoresis (CE) performed in an automated DNA sequencer. Fragments amplified with fluorescently-labeled primers are separated by electrophoresis in fine capillaries and detected by laser. Distinct allelic types are distinguished using primers labeled with different fluorescent dyes, which are detected as different colors upon laser excitation. The relative bp sizes of the fragments are estimated in relation to migration time of an internal fluorescent size standard using specific software e.g. Gene Mapper. CE has a resolution of one bp and the results are highly reproducible. CE has been applied to *P. falciparum* *msp2* genotyping in both an allele type specific (Falk *et al.* 2006) and non-specific manner (Jafari *et al.* 2004). CE can be used for quantification provided that only a single round of PCR amplification is used (Jafari *et al.* 2004). CE has been used for genotyping of *P. falciparum* microsatellites (Anderson *et al.* 1999; Nyachieo *et al.* 2005; Greenhouse *et al.* 2006).

1.8.1.4 Other genetic markers and techniques for genotyping of *P. falciparum*

The gene encoding the glutamate rich protein (*glurp*) has been used for genotyping of *P. falciparum* (Zwetyenga *et al.* 1998; Farnert *et al.* 2001). The gene contain two repeat regions (RI and RII), in which the RII region is most diverse, and therefore often the target for genotyping (Borre *et al.* 1991). Other genetic markers that have been used for *P. falciparum* genotyping, although less frequently include circumsporozoite protein gene (*csp*), erythrocyte binding antigen 175 gene (*eba-175*) (Brown *et al.* 1992; Ohrt *et al.* 1997) and a variety of microsatellites (Anderson *et al.* 1999; Greenhouse *et al.* 2006).

Another technique for genotyping is restriction fragment length polymorphism (RFLP) analysis where PCR products are digested with restriction enzymes e.g. *Hinf I* and *Dde I* followed by fragment separation on polyacrylamide gels (Felger *et al.* 1993). The protocol has been developed for *msp2* and the restriction fragment patterns provide high resolution of individual clones and mixed infections.

Using heteroduplex tracking assay (HTA) both sequence and size polymorphisms as well as quantitative data for *msp1* can be obtained (Ngrenngarmlert *et al.* 2005; Kwiek *et al.* 2007). Radiolabeled probes are annealed to PCR fragments and will migrate at different speed through a polyacrylamide gel depending on the complementarity of the probe to the fragment.

1.9 MOLECULAR EPIDEMIOLOGY OF *P. FALCIPARUM* INFECTIONS

The importance of the genetic diversity of *P. falciparum* infections has become increasingly recognized along with the development of molecular techniques that offer the possibility to enumerate and genotype multiple infecting parasite clones. Genetic characterization of parasites enables studies regarding host-parasite interactions and infection dynamics. Moreover, the genetic diversity of *P. falciparum* infections has received profound interest in the search for putative vaccine candidates. Several studies have also highlighted the significant epidemiological importance of multiclinal infections in the context of malaria morbidity and the development of protective immunity to malaria.

Multiclinal infections are the result of either an inoculation from a mosquito carrying several genetically different sporozoites, or as a result of superinfection i.e. additional infections. Several interacting factors affect the number of infecting parasite clones harbored by an individual.

1.9.1 Infection diversity, age and malaria transmission intensity

The association between the number of concurrent clones and age is largely dependent on the transmission intensity in a particular area. In low/moderate transmission settings, infection diversity is often low in asymptomatic individuals and there is no age-dependence in number of infecting clones (Babiker *et al.* 1997; Zwetyenga *et al.* 1998; Konate *et al.* 1999; Vafa *et al.* 2008). In high transmission areas, infections are often composed of multiple distinct parasite clones, and the accumulation is age-dependent (Ntoumi *et al.* 1995; Konate *et al.* 1999; Smith *et al.* 1999a; Bendixen *et al.* 2001). Asymptomatic infections are accumulated already during infancy but these infections tend to be less diverse than in older children (Felger *et al.* 1999b; Owusu-Agyei *et al.* 2002). The infection diversity peaks at the age of 3 to 14 years (Konate *et al.* 1999; Smith *et al.* 1999a; Owusu-Agyei *et al.* 2002) and the diversity decreases with increasing age thereafter. This peak coincides with the development of an anti-parasitic immunity, consistent with the notion that cumulative exposure to numerous antigenically different parasite clones is a prerequisite for efficient malaria immunity.

Although infection diversity is associated with transmission intensity, the correlation is far from linear as illustrated in Tanzania where a 50-fold increase in EIR did not significantly increase the infection diversity (Bendixen *et al.* 2001).

1.9.2 Infection dynamics

The turnover in *P. falciparum* populations over time within a single asymptomatic human host is high in endemic areas. In Senegal, in an area of intense transmission,

individual genotypes persisted for 2 to 3 weeks; however some genotypes were only detectable for a few days (Daubersies *et al.* 1996). A daily periodicity in genotype detection was described in asymptomatic children in Tanzania (Farnert *et al.* 1997). The longevity of individual infecting parasite clones appears to be affected by host age since infants often clear infections faster than older children (Smith *et al.* 1999c; Franks *et al.* 2001) with the average duration of an asymptomatic infection of < 4 weeks in infants (Franks *et al.* 2001). In contrast, individual *P. falciparum* genotypes can persist for over 2 months in children, while infection duration decreases during adolescence (Bruce *et al.* 2000).

The transmission pattern may also affect the turnover rate. In areas with seasonal transmission, individual genotypes can persist as asymptomatic infections for several months during the dry season (Babiker *et al.* 1998; Roper *et al.* 1998).

1.9.3 Infection diversity and risk of subsequent clinical malaria

With the increasing understanding of the dynamics of *P. falciparum* infections, subsequent studies have emphasized on the possible role of genetic diversity on different infection outcomes. For instance, certain allelic types of *msp1* and *msp2* have been associated with malaria morbidity (Engelbrecht *et al.* 1995; Beck *et al.* 1999; Ofosu-Okyere *et al.* 2001).

Different levels of diversity have been reported in children with febrile malaria compared to asymptomatic infections. In a study in a highly endemic area in Tanzania, infants experiencing a febrile episode had significantly higher parasite loads and were infected with a higher number of clones compared to their asymptomatic counterparts (Felger *et al.* 1999b). In contrast, multiclonal infections were significantly less frequent during episodes of clinical malaria in older children (Engelbrecht *et al.* 1995; Contamin *et al.* 1996).

Genotyping of parasites during asymptomatic infections over the dry season and then following acute infections during the transmission season revealed that clinical malaria was often caused by novel parasite clones (Babiker *et al.* 1998; Roper *et al.* 1998). The same pattern was reported in areas of intense transmission where parasite genotypes causing febrile episodes were genetically distinct from the genotypes carried asymptotically prior to the clinical episode (Contamin *et al.* 1996; Kun *et al.* 2002).

Whether the number of clones predicts the subsequent risk of clinical malaria has been investigated in a number of studies in different settings, with contradictory results. Some studies have concluded that an increasing number of infecting parasite clones increase the risk of clinical malaria (Branch *et al.* 2001; Ofosu-Okyere *et al.* 2001;

Mayor *et al.* 2003). Nonetheless, the opposite has been reported from other settings. In a highly endemic area in Papua New Guinea, multiclinal infections were associated with a significantly decreased risk of subsequent clinical episodes in children less than 18 years (al-Yaman *et al.* 1997). A similar finding was reported from Tanzania where children who were persistently parasitized were less likely to develop clinical malaria compared to parasite negative children and there was a tendency for children with multiclinal infections to be at a lower risk of clinical disease during the subsequent follow-up period (Farnert *et al.* 1999). Similar results were reported in another setting in Tanzania, although age-dependent, with parasites being protective only in children > 3 years (Henning *et al.* 2004). Moreover, in an area of lower endemicity in São Tomé an increased number of clones were protective against febrile malaria over all ages (Muller *et al.* 2001).

Further insight on the significance of multiclinal infection on the subsequent risk of disease comes from studies into various malaria control interventions such as chemoprophylaxis, use of ITNs and malaria vaccines trials. Sustained chemoprophylaxis with Deltaprim™ in children significantly reduced the infection diversity; and the reduction was implicated in the rebound in clinical malaria that was observed after the prophylaxis was stopped (Beck *et al.* 1999). Moreover, vaccination with the malaria vaccine SPf66 also reduced the number of infecting parasite clones and infection diversity was associated with protection against clinical episodes only in the placebo group (Beck *et al.* 1997). A reduction in infection diversity has also been reported among adults vaccinated with RTS,S compared to the control group (Waitumbi *et al.* 2009). However, the use of ITNs did not affect the infection diversity (Fraser-Hurt *et al.* 1999; Smith *et al.* 1999b).

In some settings, multiclinal infections might represent a marker of exposure, thus better immunity and the parasites might confer protection against clinical malaria through cross-reactive immune responses against superinfections i.e. premunition (Smith *et al.* 1999d). However, in other settings, the diversity appears to be a risk factor for disease. Further understanding regarding the interaction between the host and the genetic diversity of *P. falciparum* infections is needed to elucidate the mechanism behind the acquisition of protective malaria immunity.

2 AIM OF THIS THESIS

The overall aim of this thesis was to contribute to the understanding of the genetic diversity of *P. falciparum* infections in relation to the risk of disease and the acquisition of protective immunity.

Specific aims:

The specific aims of the presented papers were as follows;

- I. To investigate the diversity of *P. falciparum* infections in relation to individual exposure and immunity
- II. To improve the methodology for genotyping of *P. falciparum*
- III. To study the effect of intermittent preventive treatment on *P. falciparum* diversity and immunity
- IV. To study the effect of single clearance of asymptomatic multiclonal infections on risk of subsequent clinical malaria

3 MATERIAL AND METHODS

3.1 STUDY POPULATIONS

This section describes the geographical areas, study populations and clinical trials included in this thesis. All study sites are located in Sub-Saharan Africa and represents areas with different malaria transmission (Figure 4).

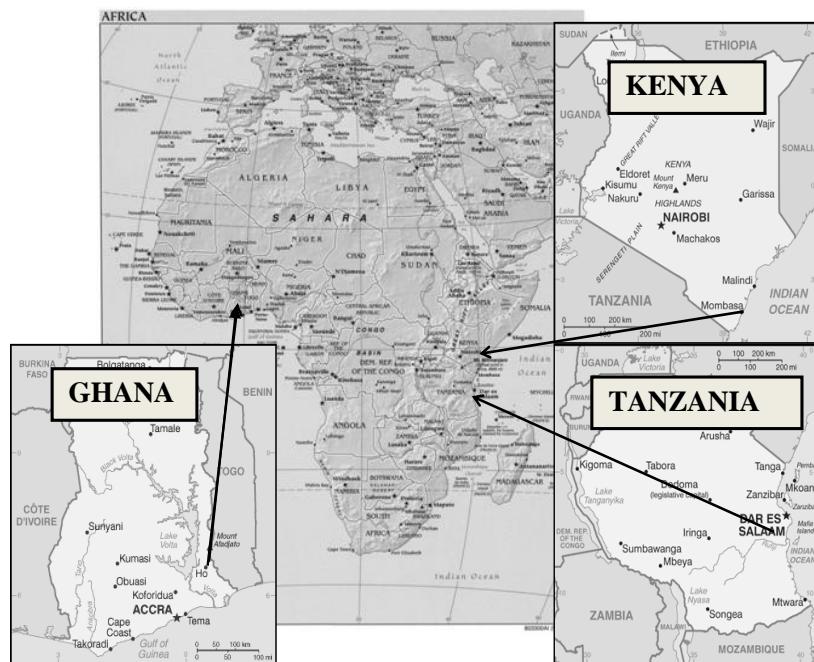


Figure 4 Study sites; Tanzania-high transmission, Ghana-seasonal high transmission and Kenya-moderate transmission

Illustration "Courtesy of the University of Texas Libraries, the University of Texas at Austin"

3.1.1 Tanzania, high transmission (study I)

Nyamisati village is situated in Rufiji River Delta, Rufiji District, coastal Tanzania. Malaria transmission is perennial with some increase following the two rainy seasons in April to June (long rains) and November to December (short rains). Previous assessment of the parasite prevalence in children 2 to 9 years put the figure at >75%, suggesting a holoendemic setting (Rooth 1992). A research team, also providing health care, lived in the village between 1985 and 2003. During 1993 to 1999 the population of about 1000 individuals was continuously monitored with regards to malaria by assessments of all fever cases, microscopy for malaria diagnosis, provision

of free antimalarial treatment and registration of all malaria episodes and other clinical data. At the time of the study the use of bednets was limited. In March to April 1999, before the rainy season, 890 villagers aged 1 to 84 years participated in a malariometric cross-sectional survey including collection of venous blood, assessment of health status and haemoglobin levels. In total 873 individuals had complete data sets and available blood/plasma samples and were included in study I.

3.1.2 Ghana, high seasonal transmission (study III)

The study site is located in Hohoe District, Volta region in Ghana. Malaria is endemic in the area with peak transmission following the two rainy seasons in April to July (long rains) and September to November (short rains) with an estimated transmission intensity of ~65 infective bites per person per year (Kweku *et al.* 2008). During 2005 to 2006, a randomized, placebo-controlled IPTc trial was conducted in the area (Kweku *et al.* 2008). In brief, 2451 children aged 3 to 59 months were enrolled and allocated to either of four treatments; SP bimonthly (every second month), AS +AQ bimonthly, AS +AQ monthly, or a placebo monthly, given over a six month period spanning the high transmission season. A drug-resembling placebo was given on alternate months in the bimonthly groups. During the six months intervention period, children were visited weekly at their homes for health assessment. Three cross-sectional surveys were performed during the 12 months follow-up (after stopped IPT) in addition to passive surveillance for clinical malaria and anaemia at the study hospital. Malaria morbidity and anaemia was reduced during the intervention period; AS+AQ monthly reduced by 69% and 45% respectively, SP bimonthly by 24% and 30% while AS+AQ bimonthly by 17% and 32% compared to placebo. No protection was seen beyond the pharmacological effects of the drugs. Children less than one year when receiving IPT with monthly AS+AQ had a higher incidence of clinical malaria during follow-up compared to those who received placebo. Rebound in clinical malaria was not observed in older children (Kweku *et al.* 2008).

Blood samples collected on filter paper from the three post-intervention surveys were included in study III. In total 2227 of the participating children, provided comprehensive data required for this study.

3.1.3 Kenya, moderate transmission (study IV)

The study site, Junju sublocation, is located in Kilifi District, coastal Kenya.

Peak malaria transmission is associated with two rainy seasons in May to July (long rains) and in November (short rains), but low level transmission occurs all year around. The five study villages can be divided into “high” (n=2) and “low” (n=3) transmission villages based on previous surveillance of re-infection rates (Bejon *et al.* 2009). In 2005-2006 a randomized controlled trial of a candidate malaria vaccine was conducted

in the area (Bejon *et al.* 2006). Briefly, 405 children aged 1-6 years were enrolled and immunized at three occasions four weeks apart. The vaccine regimen, FFM ME-TRAP, consisted of sequential immunizations with two attenuated poxvirus vectors (FP9 and modified vaccinia virus Ankara) both carrying the pre-erythrocytic antigen construct multiple epitope-thrombospondin-related adhesion protein (ME-TRAP). Children were visited weekly at home for a health assessment during the 18 months follow-up. Scheduled venous blood samples were collected at an initial screening in February 2005 and at post-vaccination in May 2005. All children were then treated with directly observed dihydroartemisinin monotherapy for seven days to clear asymptomatic parasitemias, beginning on the day of the blood sample taken in May. Parasite clearance was confirmed by blood films one week after ended treatment. Additional blood samples were taken at cross-sectional surveys in August 2005 and January 2006.

The vaccine did not elicit any protective responses to malaria and did not affect the incidence of parasitemia during follow-up (Bejon *et al.* 2006; Bejon *et al.* 2007).

Blood samples collected at the four cross-sectional surveys were included in study IV.

3.1.4 Samples used for method evaluation in study II

Evaluation of the fluorescent PCR genotyping method included laboratory cultured parasites as well as a set of randomly selected samples ($n=240$) from the IPTc trial in Ghana (study III). In addition, finger prick blood samples from 57 children participating in an efficacy trial of AS+AQ versus artemether-lumefantrine in Zanzibar, Tanzania (Mårtensson *et al.*, *in press*) were included. These children were below 5 years of age, and experienced an acute episode of uncomplicated *P. falciparum* malaria. Blood samples were taken before the initiation of treatment and at recurrent parasitemia between days 21–42 of follow up ($n=114$).

3.2 ETHICAL CONSIDERATIONS

Ethical approval has been obtained from the respective endemic countries where the studies have been performed and from the Regional Ethical Review Board in Stockholm, Sweden. Informed consents were obtained from all participants or their parents/guardians.

3.3 GENOTYPING OF *P. FALCIPARUM*

The number of concurrently infecting *P. falciparum* clones was investigated in all studies in this thesis by genotyping the *merozoite surface protein 1* and/or 2 gene (*msp1* and 2). The genotyping method is presented in detail including the optimization of the assay done in study II.

3.3.1 DNA extraction

3.3.1.1 From whole blood collected in EDTA

DNA was extracted from venous whole blood collected in EDTA in studies I and IV. In study I, DNA extraction was performed using phenol-chloroform and ethanol precipitation (Snounou *et al.* 1993). Briefly, packed erythrocytes were lysed with saponin and after centrifugation the pellet containing the parasite DNA was resuspended in lysis buffer and incubated in proteinase E. DNA was extracted by phenol and phenol-chloroform followed by ethanol precipitation with sodium acetate. The extracted DNA was resuspended in TE buffer. In study IV, DNA was extracted in 96-well format in an ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems) or PUREGENETM DNA Isolation Kit (Gentra systems).

3.3.1.2 From whole blood collected on filter paper

In study III, DNA was extracted from whole blood collected on filter paper (Grade 541, Whatman) using ABI Prism 6100 Nucleic Acid PrepStation. Whole blood spots were cut into small pieces and soaked in water before DNA extraction.

*3.3.1.3 From laboratory cultured lines of *P. falciparum**

DNA from laboratory cultured lines i.e. K1, F32 and 7G8 of *P. falciparum* were used as positive controls in all PCR amplifications. Parasite DNA was extracted from red blood cell cultures using E.N.Z.A Blood DNA Kit (Omega Bio-Tek, Inc).

3.3.2 PCR method

Genotyping of *P. falciparum* was performed using a two step (nested) PCR reaction targeting *msp1* or *msp2* (Snounou *et al.* 1999) with some modifications. In the primary reaction the primers span the entire genetic segments, block 2 for *msp1* and block 3 for *msp2*. In the nested reaction, separate primer pairs target the respective allelic types of *msp1* (K1, MAD20, and RO33) and *msp2* (FC27 and IC). The 1st reaction was identical in the original and fluorescent assays, whereas the second reaction was modified in the fluorescent assay.

3.3.2.1 Original PCR method non-fluorescent

In the primary reaction the final concentration of the master mix consisted of 1× PCR buffer, 2 mM MgCl₂, 125 µM dNTP and 0.02 units/µl of AmpliTaq® DNA polymerase (Applied Biosystems), and 250 nM each of the outer primer pairs *msp1* forward (F)/reverse (R) and *msp2* F/R.

The cycle conditions were as follows:

step 1- initial denaturation for 5 min at 95°C

step 2- annealing for 2 min at 58°C
step 3- extension for 2 min at 72°C
step 4- denaturation for 1 min at 94°C, steps 2-4 was repeated 24 times.
step 5- final annealing for 2 min at 58°C
step 6- final extension for 5 min at 72°C

In the separate nested non-fluorescent reactions the final concentration of the master mix consisted of 1×PCR buffer, 1 mM MgCl₂, 125 µM dNTP and 0.02 units/µl of AmpliTaq® DNA polymerase, and 250 nM of the respective *msp1* allelic type-specific primers and 125 nM of the respective *msp2* type primers. One µl product from the primary reaction was used as a template in the respective nested reactions.

The cycle conditions were as follows:

step 1- initial denaturation for 5 min at 95°C
step 2- annealing for 2 min /1 min at 61° C /58°C
step 3- extension for 2 min /1 min at 72°C
step 4- denaturation for 1min /30 sec at 94°C, steps 2-4 was repeated 29 times.
step 5- final annealing for 2 min/1 min at 61°C /58°C
step 6- final extension for 5 min at 72°C

Temperatures and times e.g. 2 min/1 min, 1 min/30 sec are different settings for amplification of *msp1* / *msp2* respectively.

All PCR amplifications were performed on 96-well plates with a total reaction volume of 20 µl per well.

3.3.2.2 Fluorescent PCR method

The PCR protocol for the capillary electrophoresis (CE) method was based on the *msp1* and *msp2* genotyping assay as described above. The primary PCR reaction was identical in reagent concentrations and cycle conditions to the original non-fluorescent PCR method. In the nested fluorescent reaction, the allelic type-specific primers were modified as follows: (i) the forward primers were tailed with a 7-bp tail (Applied Biosystems) at the 5'-end. The tail sequence is added in order to promote the non-template adenosine (A) addition by the *Taq* DNA polymerase at the 3' end of the PCR products (Brownstein *et al.* 1996), (ii) the reverse primers were labeled with different fluorophores at the 5'-end: *msp1* K1 with NED™ (yellow), MAD 20 with PET® (red), and RO33 with VIC® (green); *msp2* FC27 with 6-FAM™ (blue) and IC with VIC® (green). The addition of the tail, promoting the additional A, counteracts amplification of fragments differing with a single nucleotide i.e. fragments ± A. Due

to the addition of the tail on the forward primers, all GeneMapper®-estimated bp sizes presented include an extra 8 bp segment (7-bp tail +A).

After evaluating different modifications of the original nested reaction (performed within study II) the final protocol included the following adjustments: (i) the concentration of all *msp1* and the *msp2* FC27 allelic type-specific primer pairs was decreased to 125 nM each (F/R); (ii) in the *msp2* IC reaction, the primer concentrations were increased to 300 nM each (F/R) and the AmpliTaq® DNA polymerase to 0.05 units/µl; (iii) the number of cycles was reduced to 23 in all nested fluorescent PCR amplifications. All other concentrations and conditions were identical to the original non-fluorescent method (described above).

When the nested reaction was multiplexed (evaluated in study II), the allelic type-specific primers were mixed accordingly; *msp1* K1+MAD20+RO33 and *msp2* FC27+IC. In the hybrid assay (evaluated in study II), the *msp2* type-specific primers were mixed as follows; FC27 F-tail/IC R-VIC and FC27 R-6-FAM/IC F-tail, in two separate nested reactions. The amount of AmpliTaq® DNA polymerase was increased to 0.05 units/µl, in the multiplexed and in the hybrid assays.

3.3.2.3 Fragment analysis by gel electrophoresis

Amplified products from the non-fluorescent nested reaction were separated using electrophoresis on a 2% high resolution agarose gel (MetaPhor, BMA Rockland [study I] and Agarose 3:1 HRB™, Amresco Inc [study II]) in 1× TBE buffer. Following staining with ethidium bromide, the fragments were visualized with UV light in a Universal hood II (Bio-Rad Laboratories). Fragment size was estimated in relation to a 100 base-pair DNA ladder (Amersham Pharmacia Biotech [study I] and Invitrogen Corporation [study II]) both by the naked eye and with Quality One® analysis software version 4.4.1 (Bio-Rad Laboratories). The total number of alleles of the respective allelic types corresponds to the number of parasite clones in that particular sample. This method was used in study I and for comparison to capillary electrophoresis in study II.

3.3.2.4 Fragment analysis by capillary electrophoresis

Separation of fluorescent fragment was performed by capillary electrophoresis on DNA sequencers. In study II and III analysis was performed on a 3730 DNA sequencer (Applied Biosystems) equipped with 48 capillaries (36 cm), using POP-7™ polymer; and in study IV on a 3130xl DNA sequencer (Applied Biosystems) with 16 capillaries (50 cm) (performed at the KEMRI laboratory, Kilifi, Kenya).

From the nested reaction, 1-2 µl product (diluted 1:10 or 1:20 in water) was added to 9 µl Hi-Di formamide and 0.5 µl size standard (GST™-LIZ® 1200, Applied Biosystems) per well on 96-well plates. The fluorescent size standard contains 68 single-stranded DNA fragments ranging in size from 20 bp to 1200 bp. Due to competition for separation between the smaller VIC-labeled *msp1* RO33 fragments and the larger VIC-labeled *msp2* IC fragments, the *msp1* and *msp2* markers were run separately during CE.

The separation was run at 8.0 kV for 4000 sec in the 3730 DNA sequencer while at 8.5kV for 6700 sec in the 3130xl DNA sequencer. The results were interpreted using GeneMapper® Software version 4.0 (Applied Biosystems). To assist in the interpretation a fluorescent cut off was set to 300 relative fluorescent units (rfu) for the 3730 system and 150 rfu for the 3130xl system.

3.4 DETECTION OF ANTI-*P. FALCIPARUM* ANTIBODIES BY ELISA

Plasma levels of antibodies against the circumsporozoite protein (CSP) were measured using enzyme-linked immunosorbent assay (ELISA) (Hogh *et al.* 1991) in study I. 96-well microtiter plates (Costar Corporation) were coated with 50 µl of the synthetic peptide (NANP)₆ (10 µg/ml) conjugated to BSA and incubated at 4°C over night followed by saturation with 0.5% BSA at 37°C for 3h. Plasma samples diluted 1:1000 were added to the wells and incubated at 37°C for 1h, washed four times, and then incubated with the secondary antibody, goat antihuman IgG conjugated to alkaline phosphatase (1:1000) again at 37°C for 1 h. After additional washing the plates were developed by adding p-nitrophenylphosphate (Sigma Aldrich) for 1 h at room temperature. Optical densities (OD) were determined at 405 nm in a Multiscan EX reader (Labsystems). Antibody levels were determined against a standard curve and expressed as mg/ml. Sera from African donors with high antibody levels and sera from unexposed Swedish donors were used as positive and negative controls, respectively.

3.5 STATISTICAL APPROCHES

Data analysis was performed using Statistica and SAS (v 8.0) software in study I, R (v 2.9.0) and SPSS (v 17) in study II and III and STATA (v10) in study IV.

In study II Kruskal-Wallis test was used to analyze differences in number of detected fragments between the two methods.

In study I, III and IV similar approaches for analysis were used. The number of clones was categorized to avoid assumptions about linearity. Factors potentially

associated with number of clones were investigated i.e. age, sex, parasite density, previous clinical episode with antimalarial treatment, haemoglobin levels, CSP-antibody levels [study I], village of residence (high/low transmission) [study IV], ITN (bednet use) [study III and IV] and transmission season (dry/rainy) [study IV]. Factors found to be significantly associated with number of clones were included in the risk analyses.

To strictly ensure that only asymptomatic individuals are included in the risk assessments, individuals with clinical malaria at the survey or within the period 28 days (4 weeks) before to 7 days after the survey were excluded in all studies.

Prospective risk of clinical malaria during follow-up was assessed as time to first event by Cox regression in relation to the respective clone categories in asymptomatic children. Hazard ratios (HRs) were adjusted for factors significantly associated with number of clones. In study IV data from survey 1, 3 and 4 i.e. without treatment were pooled for analysis while data from survey 2 i.e. with treatment was analyzed separately.

In study IV a separate analysis taking exposure into account was performed using survey 2 as baseline and the outcome during the three months follow-up after parasite clearance was classified into three categories; clinical malaria during follow-up, asymptomatic parasitemia at the cross-sectional survey three months later (survey 3), or remaining uninfected i.e. having no clinical episode during follow-up nor any detectable parasites at the following cross-sectional survey. Factors associated with the different outcomes were investigated by logistic regression. When the outcome was re-infection (i.e. clinical malaria or asymptomatic parasitemia), the analysis was performed excluding children who remained uninfected as these children were considered less exposed.

4 RESULTS

4.1 STUDY I: MULTICLONAL ASYMPTOMATIC *PLASMODIUM FALCIPARUM* INFECTIONS PREDICT A REDUCED RISK OF MALARIA DISEASE IN A TANZANIAN POPULATION

*Aim: To investigate the diversity of *P. falciparum* infections in relation to individual exposure and immunity*

In March to April in 1999, before the rainy period, 890 villagers aged 1 to 84 years living in Nyamisati village, costal Tanzania, participated in a cross-sectional survey including collection of venous blood and assessment of clinical status. In total 873 individuals had complete data sets and available DNA/plasma samples. Only strictly asymptomatic individuals were included in the analysis. Infection diversity was assessed by genotyping of *msp2* and individual exposure was determined by CSP antibody levels. The number of clones at survey was correlated to exposure and risk of subsequent clinical malaria during 40 weeks follow up.

The key findings in study I are:

- ❖ Multiclonal infections (composed of ≥ 2 *msp2* alleles) were detected in 70% of the PCR positive samples. The number of clones increased with age with a peak at 6 to 10 years of age and then decreased in adults.
- ❖ Levels of antibodies against CSP were stable in children up to 10 years and increased in teenagers and adults. Parasite positive children (1-10 years) had higher antibody levels than their parasite negative counterparts. Anti-CSP antibody levels were not associated with the number of clones or with the subsequent risk of clinical malaria.
- ❖ Multiclonal infections were more common in children who had not been treated with SP for a clinical episode in the 40 weeks prior to survey.
- ❖ Haemoglobin levels decreased with increasing parasite densities but were not associated with number of clones.
- ❖ The subsequent risk of malaria decreased with age, and was higher in subjects with a history of malaria.

- ❖ Being parasite negative or infected with ≥ 2 clones was, compared to infections with 1 clone, was associated with reduced risk of subsequent clinical malaria. Following adjustments for age, sex and history of clinical malaria 40 weeks before, the risks were HR 0.28 (95%CI, 0.10-0.78) for infections with 2-3 clones 0.42 (95% CI, 0.15-1.17) for ≥ 4 clones and 0.53 (95% CI, 0.29-0.96) in parasite negative children.

4.2 STUDY II: OPTIMIZATION AND VALIDATION OF MULTI-COLOURED CAPILLARY ELECTROPHORESIS FOR GENOTYPING OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEINS (*msp1* AND *msp2*)

*Aim: To improve the methodology for genotyping of *P. falciparum**

One of the most widely used nested assays for *msp1* and *msp2* genotyping of *P. falciparum* populations (Snounou *et al.* 1999) was adapted to capillary electrophoresis (CE). The fluorescent PCR method was optimized and several modifications of the original nested protocol were evaluated. The final fluorescent PCR protocol is presented in the methodology section 3.3.2.2. The method was compared to standard PCR followed by gel electrophoresis. Determination of sensitivity, specificity, and reproducibility was performed on step-wise diluted series and mixtures of DNA from the laboratory lines F32, K1, and 7G8. Moreover, two sets of field samples were used to evaluate the technique in molecular epidemiology studies (samples from study III) and anti-malarial drug trials (samples from the study described in section 3.1.4). A multiplexed approach and an assay for detecting hybrids were evaluated.

The key findings in study II are:

- ❖ Optimizing required i) the introduction of a fluorescent cut-off to distinguishing true allele peaks from fluorescent background and non-specific low background artifacts, ii) reduced primer concentration to lower the fluorescent background, iii) dilution of products before CE to avoid artifacts e.g. “companion peaks” and iv) reduced cycle number in the nested reaction to reduce peak height and the appearance of stutter peaks.
- ❖ The CE and gel-electrophoresis based methods had the same detection sensitivity (5–10 parasites/ μ l) for the *msp1* and *msp2* markers. In samples with mixed genotypes the low concentration genotype (10 parasites/ μ l) was more often detected by CE than by gel electrophoresis.

- ❖ In high density laboratory samples (> 5000 parasites/ μl), the gel method often generated non-specific bands and smears, whereas the CE-based method was more specific and generated only one single peak on the electropherograms irrespective of parasite density (see Figure 2 in paper II).
- ❖ The CE method had a size resolution of 1 bp, and when PCR runs were repeated the size variation was $<0.5\text{--}1$ bp, thus demonstrating high reproducibility and size precision. When the same products were separated on gel they differed with 2-16 bp.
- ❖ A higher number of *msp1* and *msp2* fragments were detected by CE in the 240 blood samples from asymptomatic children in Ghana and the number of distinct alleles in the population could easily be determined.
- ❖ The methods performed equally well in distinguishing recrudescence parasites from new infecting after step-wise genotyping of *msp1* followed by *msp2* blood samples from 57 children collected prior to antimalarial treatment and at the day of recurring parasites.

In summary, the CE-based genotyping assay allows for improved size resolution and highly reproducibility in typing of *P. falciparum* *msp1* and *msp2* alleles compared to the original gel electrophoresis-based assay. The method required extensive optimization to overcome problem with background “noise” and stutter peaks. The CE-method represents a clear improvement compared to the gel-based separation since inherent issues including non-specific fragments and smears at high DNA concentrations have been resolved. Additionally, the sample processing has been simplified as all steps in the CE-method are done in 96-well format. The risk of cross-contamination is also reduced since e.g. paired samples in drug trials no longer have to be run next to each other on the gel. The improved sensitivity and specificity of allelic discrimination makes CE an important tool in molecular epidemiology studies as well as antimalarial drug trials. Moreover, a more detailed and reliable discrimination of individual alleles will also contribute to an increased knowledge of the frequency and dynamics of the different allelic types of *msp1* and *msp2* in different epidemiological settings.

4.3 STUDY III: THE EFFECT OF INTERMITTENT PREVENTIVE TREATMENT ON THE GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM* INFECTIONS AND MALARIA MORBIDITY IN GHANAIAN CHILDREN

Aim: *To study the effect of intermittent preventive treatment on *P. falciparum* diversity and immunity*

A randomized, placebo-controlled IPTc trial was conducted in Hohoe district, Ghana during 2005 to 2006 (Kweku *et al.* 2008). In total 2227 of the 2451 participating children (aged 3 to 59 months), provided comprehensive data required and were therefore included in study III. Enrolled children were allocated to either treatment regimen; SP bimonthly (every second month), AS +AQ bimonthly, AS +AQ monthly, or a placebo monthly, given over a six month period of intense malaria transmission. Blood samples from the three cross-sectional surveys after stopped IPT were genotyped for *msp2*. The number of infecting clones at the first survey after stopped IPT was associated with risk of subsequent clinical malaria during the 12-months follow-up.

The key findings in study III are:

- ❖ At the first survey one month after IPT was stopped, the prevalence of *P. falciparum* infection by microscopy was lower (5.2%) in children who had received monthly AS+AQ treatment compared to children given placebo, bimonthly SP or AS+AQ (17.5-20.5%).
- ❖ Six months post-intervention, the prevalence was relatively higher in children who had received IPT compared to children receiving placebo. Twelve months post-intervention, parasite prevalence was similar in all groups.
- ❖ Multiclonal infections (≥ 2 clones), were detected in 60.6-70.1% of the PCR positive samples from asymptomatic children at the first survey. No child in the AS+AQ monthly group was infected with >2 clones. Six and 12 months after IPT, the number of clones was equally high in all groups.
- ❖ Children with parasites at the first surveys after stopped IPT were more likely to be anaemic than children without parasites (OR 2.65, 95% CI 1.91-3.68). Multiclonal infections did not increase the risk of anaemia during follow-up (OR 1.67, 95% CI 0.87-3.36) compared to single clone infection.

- ❖ IPT was associated with an increase in the risk of clinical malaria during the 12-month follow-up compared to placebo; HR (95% CI) 1.55 (1.05-2.27) for AS+AQ monthly, 1.36 (0.94-2.10) for SP bimonthly and 1.20 (0.78-1.83) for AS+AQ bimonthly.
- ❖ Children who remained asymptomatic throughout the follow-up had higher number of clones at the first post-intervention survey, especially in the placebo group ($p=0.003$ MW).
- ❖ Within the AS+AQ monthly group, only children who were parasite negative just after intervention developed clinical malaria during follow up.
- ❖ In an analysis including all asymptomatic children, baseline infections composed of more than 2 clones were associated the decreased risk of disease, with an adjusted (age and treatment group) HR of 0.43 (0.19-0.99).
- ❖ The association with protection was significant in the placebo group where parasite negative children had a decreased risk of disease compared to those with single clone infections. HR for the age adjusted analysis was 0.07 (95% CI 0.0078-0.56) for infections with ≥ 2 clones while 0.30 (95% CI 0.12-0.73) for parasite negative children compared to children infected with 1 parasite clone. A similar, non-significant trend was seen in the AS+AQ bimonthly group, whereas no such association was found in the SP group.
- ❖ An interaction between infection diversity and treatment showed that clones were important only when IPT had not been given; and the protective effect of having ≥ 2 clones was ten-fold higher in children with placebo compared to children who had received bimonthly IPT (HR 10.83, 95% CI 1.02-114.91).

4.4 STUDY IV: CLERANCE OF ASYMPTOMATIC MULTICLONAL *PLASMODIUM FALCIPARUM* INFECTIONS; EFFECT ON SUBSEQUENT RISK OF CLINICAL MALARIA IN KENYAN CHILDREN

Aim: To study the effect of single clearance of asymptomatic multiclonal infections on risk of subsequent clinical malaria

The study included samples collected during a randomized controlled trial of a candidate malaria vaccine in Kilifi, Kenya (Bejon *et al.* 2006). In total 405 children

aged 1-6 years living in Junju sublocation were included. Initial blood samples were collected before and after vaccination. All children were treated with directly observed dihydroartemisinin monotherapy for seven days to clear asymptomatic parasitemias after the second survey. Additional blood samples were taken at cross-sectional surveys three and nine months after treatment. Blood samples collected at the four cross-sectional surveys were genotypes for *msp2* and number of clones was analyzed in relation to risk of subsequent clinical malaria. Data from the follow-up periods without treatment i.e. survey 1, 3 and 4 were pooled for analysis while data from the survey followed by treatment i.e. survey 2 was analyzed separately. Vaccination had no effect on number of clones measured in this study ($P=0.9$). The vaccine groups, i.e. malaria/control were therefore pooled for further analysis.

The key findings in study IV are:

- ❖ Multiclonal infections were detected in 75% and 76% of the PCR positive samples at the two first surveys while in 59.3% and 59.1% in the two last surveys after treatment.
- ❖ There was a high intra-individual consistency in the number of clones between the surveys without treatment while the number of clones correlated to a lesser extent between the survey before and after treatment i.e. between survey 2 and 3.
- ❖ At all surveys, the number of clones was associated with age (IRR= 1.17, 95% CI 1.11-1.23 for each year of age), village of residence (IRR=1.14, 95%CI 1.01-1.41 in the high transmission villages compared to the low transmission) and Hb levels (IRR=0.9, 0.87-0.94 per g/dl increase) however not with ITN use (IRR=0.88, 95%CI 0.73-1.04).
- ❖ Children who were parasite negative at the cross-sectional surveys had a lower risk of subsequent malaria both in the follow-up periods without and with treatment; HR 0.47 (95% CI 0.22-0.98) and HR 0.52 (95% CI 0.27-0.99), respectively.
- ❖ The number of clones was not associated with risk of subsequent malaria at the surveys not followed by treatment (compared to one clone HR=1.15 95% CI 0.60-2.19).
- ❖ Children infected with ≥ 2 clones had a clearly reduced risk in the period after treatment; HR 0.46 (95% CI 0.23-0.91).

- ❖ The interaction term between the number of clones and the effect of treatment (without and with treatment) was HR=3.54 (95%CI 1.4-9.1) for the effect of ≥ 2 clones and treatment. This confirms that the number of clones acts significantly differently depending on whether not treatment was given after the survey.
- ❖ In the separate analysis of lack of exposure vs. immunity, ≥ 2 clones was associated with an increased risk of re-infection (OR=1.97 95% CI 0.99-3.93). In those re-infected, being parasite negative or infected with ≥ 2 clones at survey 2 was associated with a reduced risk of clinical malaria compared with asymptomatic malaria (OR=0.19 95 CI 0.05-0.73 and OR=0.06 95% CI 0.02-0.25 respectively).

5 DISCUSSION

The importance of the genetic diversity of *P. falciparum* infections for immunity to malaria is important to establish. Previous studies have showed that a high number of clones predicted an increased risk of disease in some settings (Branch *et al.* 2001; Ofosu-Okyere *et al.* 2001; Mayor *et al.* 2003) while the opposite has been seen in other areas (al-Yaman *et al.* 1997; Farnert *et al.* 1999; Muller *et al.* 2001).

The studies presented here included assessments of different host factors that might affect the number of clones e.g. age, parasite density, clinical status, time to previous antimalarial treatment and individual exposure. The emphasis of these studies has been asymptomatic infections to determine how the host natural status reflects immunity. We investigated how the number of clones correlates to the subsequent risk of disease and how the risk is affected by clearing asymptomatic infections with effective antimalarial drugs both as intermittent treatment (IPT) during peak transmission season or as a single treatment course.

Age and individual exposure affect the number of clones in the individual. Our studies confirm previous findings of peak diversity in school aged childhood in areas with high to moderate transmission (Smith *et al.* 1999a; Bendixen *et al.* 2001; Owusu-Agyei *et al.* 2002). The age-dependent increase in number of clones suggests a cumulative exposure to diverse infections. The level of previous exposure was investigated in Tanzania (study I), and the levels of anti-CSP antibodies, the best available serological marker of previous exposure (Druihle *et al.* 1986; Webster *et al.* 1992), indeed increased with age. Nonetheless, no correlation between number of clones and anti-CSP antibody levels was found. This is in concordance with a previous study (Engelbrecht *et al.* 2000) and suggests that the number of clones an individual harbors is influenced by other intrinsic factors and not merely a marker of previous exposure.

Compared to single clone infections children infected with multiple clones had a decreased risk of subsequent clinical malaria. In Tanzania, the lowest risk was found in asymptomatic children infected with 2-3 parasite clones. More clones (≥ 4) were not associated with a higher protection. In Ghana infections composed of ≥ 2 clones predicted a lower risk of febrile malaria, however only in children who had not been given seasonal IPT. Interestingly, in Kenya the protection associated with infection diversity was only evident after treatment.

Immunity to malaria develops as a result of repeated infections with a variety of antigenically different parasite clones. Without exposure the immunity wanes

(Colbourne 1955), thus suggesting that continuous exposure and persistent infections are prerequisites for a sustained immunity. The importance of persisting infections was demonstrated in the IPTc study Ghana (Study III). Seasonal IPT cleared infections temporary; however one month after ended IPT, children that received bimonthly SP or AS+AQ were infected with similar number of clones as untreated children i.e. placebo group. Thus, multiclonal infections accumulate fast in this setting with high seasonal transmission. Multiclonal infections predicted a lower risk of malaria however only among untreated children, representing the natural condition in this setting. Suggestively, multiclonal infections in this group reflect persistent infections boosting the immunity rather than recent inoculations. The importance of persistent infections was also seen in our study in Kenya where children with multiclonal infections, that subsequently were protected against clinical malaria once the infections were cleared could control novel infections as the transmission season started, suggestively due to previous exposure that boosted the immunity.

Children who were parasite negative were also at lower risk of subsequent clinical malaria than children infected with a single parasite clone. Parasite negativity might reflect lack of exposure, a conceivable explanation in low endemic areas. To distinguish the effect of protective immunity from lack of exposure, Kenyan children that remained uninfected during the three months follow-up after treatment were considered unexposed and were excluded in a separate analysis. Nonetheless, the association between parasite negativity and protection remained significant suggesting a population with efficient immunity. Considering the non-sterilizing nature of the malaria immunity it is likely that some of these individuals had low-level infections not detectable by our PCR method. Moreover, parasite negativity was also associated with reduced risk in high transmission areas in both Tanzania and Ghana where individuals are expected to be repeatedly infected, thus the absence of detectable parasites rather reflects an efficient anti-parasitic immunity than lack of exposure.

The mechanisms by which multiclonal infections act appear rather complex. Infections composed of several distinct clones challenge the host's immune system with a greater antigenic diversity. In high endemic areas diverse infections might be controlled by cross-reactive immune responses primed by previous infections, whereas in areas where individuals are less exposed, multiclonal infections might be more difficult to control compared to single clone infections.

Considering the short half-life of malaria specific antibody responses (Kinyanjui *et al.* 2007) we have hypothesized that parasites *per se* are important to stimulate protective immune responses. Indeed, antibody responses are more long-lived in the presence of persistent infections (Akpojeneta *et al.* 2008). Detectable parasitemias also elicit

higher antibody levels (Bull *et al.* 2002) and in some studies antibodies have been protective against clinical malaria only in children with asymptomatic parasitemias (Polley *et al.* 2004; Osier *et al.* 2007). Clearing asymptomatic infections, although temporarily, might thus affect these antibody/ immune responses. An increase in malaria morbidity was reported following sustained chemoprophylaxis (Greenwood *et al.* 1995; Menendez *et al.* 1997), suggesting an impaired development of a protective immunity to malaria. A decrease in infection diversity among infants receiving chemoprophylaxis was proposed as an underlying mechanism for the rebound in one study (Beck *et al.* 1999). Increased incidence of clinical malaria and anaemia has been reported after intermittent preventive treatment (Chandramohan *et al.* 2005; Mockenhaupt *et al.* 2007; Kweku *et al.* 2008). In Ghana, children that were between 3 and 11 months of age when they received IPT with monthly AS+AQ were of highest risk for clinical malaria during follow-up (Kweku *et al.* 2008). The increased risk of disease was associated with a decreased number of clones. This suggests that exposure during the first year of life is crucial for development of protective immunity to malaria.

In the IPTc study, repeated dosage with long half-life drugs (e.g. AQ or SP) with prophylactic effect affected natural exposure. In Kenya a short acting drug, with no or negligible prophylactic effect, was used and thus allowed for assessment of clearance of parasites without affecting exposure. Without clearance the number of clones was not associated with disease risk. However, with clearance, the number of clones harbored at the survey prior to treatment predicted the risk of subsequent clinical malaria. Compared to children infected with a single parasite clone, children infected with more than 2 clones prior to treatment had a reduced risk of developing febrile malaria during follow-up. Why multiclonal infections only protected once cleared remains unclear. Suggestively, infections might be somewhat immunosuppressive at earlier stage of immune acquisition and thus better when cleared; however previous encountering of multiclonal infections has induced broader immunological memory protecting against novel infections.

Nonetheless, it is evident that the effect on immunity attributable to the number of clones differs, even in areas with differences in transmission. In three closely located areas in Kenya, the infection diversity correlated differently with malaria morbidity. In the area with low transmission the number of clones did not predict the risk of disease while under moderate transmission conditions multiple clones were associated with an increased risk. (Farnert *et al.* 2009). In contrast, in our study in an area with more moderate transmission clones did not predict disease risk unless they were cleared after which they did protect against clinical malaria. Moreover, infection diversity was associated with protection in high transmission settings in Tanzania and Ghana,

suggesting a transmission dependent component in the immunological balance and tolerance to multiclonal infections.

In summary, our studies have confirmed the importance of asymptomatic multiclonal *P. falciparum* infections for protective malaria immunity. Moreover, we have shown that clearing infections with effective antimalarial treatment, intermittent or single course, affects the infection diversity during follow-up and the subsequent risk of clinical malaria. We can moreover conclude that there are intriguing differences in how multiclonal infections predict the risk of malaria in different settings, which most probably reflect different levels of exposure and acquired immunity as well as need to tackle subsequent infection pressure and antigenic diversity. Understanding of how immunity to multiclonal *P. falciparum* infections, develops and how it is affected by different interventions is a prerequisite for the development and evaluation of future strategies for malaria control.

6 CONCLUSIONS

- ❖ *P. falciparum* infections composed of several distinct clones are commonly detected in asymptotically infected individuals living in endemic areas.
- ❖ The number of clones in an individual increase with age and transmission intensity.
- ❖ Exposure to malaria, assessed by anti-CSP antibody levels, does not alone affect the number of clones.
- ❖ Asymptomatic multiclinal infections are associated with protection from subsequent clinical malaria in areas of high transmission.
- ❖ In an area of moderate transmission multiclinal infections were only protective once they were cleared.
- ❖ Clearance of asymptomatic infections with effective antimalarials used intermittent or as a single course affect the infection diversity and risk of disease.
- ❖ A reduction in number of clones may explain the rebound in malaria morbidity seen after stopped IPT.
- ❖ Persistent multiclinal infections are important for protective immunity in high transmission areas.
- ❖ Multiclinal infections predict the risk of malaria differently in different exposure settings, which might reflect different levels of acquired immunity.
- ❖ Fluorescent PCR and capillary electrophoresis represent an improvement of the original method with gel based fragment separation.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Malaria är en av vår tids mest allvarliga infektionssjukdomar. Sjukdomen orsakas av en parasit tillhörande släktet *Plasmodium* och sprids mellan människor av blodsugande *Anopheles*-myggor. Det finns fem olika *Plasmodium*-arter som kan infektera människor, varav arten *Plasmodium falciparum* orsakar de svåraste infektionerna med högst dödlighet

Malaria sprids i 108 länder i sub-tropiska och tropiska områden. Bekämpning genom ökad användning av nya kombinationsbehandlingar med effektiva antimalariamediciner, myggnät och inomhussprayning av insektsmedel, ligger delvis bakom att förekomsten av malaria har minskat i flera länder i Afrika och Asien. Trots detta uppskattades 2009 fortfarande ca 243 miljoner fall av malaria, varav ca 900 000 dödsfall. Sjukdomsbördan är störst i Afrika, söder om Sahara, där en majoritet av de som avlides är barn under fem års ålder.

Till följd av upprepade infektioner, utvecklar människor som lever i malariadrabbade områden immunitet mot malaria. Immunförsvaret blir dock aldrig så effektivt att alla parasiter elimineras och individer som lever i malariaområden är därför ofta infekterade utan att utveckla symptom.

P. falciparum parasiten har en mycket stort genetisk mångfald och infektioner består ofta av flera stammar samtidigt. Studier har visat att asymptomatiska infektioner med flera genetiskt olika parasitstammar, s.k. kloner, är särskilt vanliga hos barn mellan 3 och 14 års ålder, vilket sammanfaller med utvecklingen av immuniteten mot malaria.

Det övergripande målet med denna avhandling var att öka förståelsen av *P. falciparum* infektioners genetiska mångfald och hur den påverkar individens sjuklighet och immunitet mot malaria. Studier från Tanzania, Ghana och Kenya ingår i avhandlingen (Studie I, III och IV).

I samtliga studier har vi använt en molekylärbiologisk metod, polymerase chain reaction (PCR), för att amplifiera parasit-specifika gener från blodprover. De olika parasitstammarna särskiljs utifrån storleken och typen på parasitens DNA-fragment. Vidareutveckling av denna metod, med användning av DNA sekvenserare, utgjorde en delstudien i avhandlingen (Studie II) och resulterade i avsevärt större precision för att definiera olika parasitkloner.

I den första studien undersökte vi vilka faktorer som påverkar antalet parasitkloner/stammar som infekterar en individ. Studien inkluderade 873 personer mellan 1-84 års ålder (i Nyamisati, en fiskeby) i Tanzania. Antal stammar ökade med åldern under barndomen och reflekterade inte endast tidigare exponering för malaria. Det visade sig att barn som var friska bärare av 2-3 olika stammar hade en lägre risk att utveckla klinisk malaria än barn infekterade med endast en stam.

I studie III undersökte vi konsekvenserna av att behandla bort asymtomatiska infektioner i en klinisk prövning av ny kontrollstrategi (intermittent preventiv behandling) där upprepade doser av malarialäkemedel gavs för att förebygga infektioner. 2451 barn (3 till 59 månader gamla) i Ghana behandlades med effektiva malarialäkemedel alternativt placebo varje eller varannan månad i sex månader då malariatransmissionen var som högst. Vi studerade hur barnens skydd mot malaria påverkades till följd av att deras asymtomatiska infektioner elimineras. Vi kom fram till att behandling av asymtomatiska infektioner ökade risken för att drabbas av klinisk malaria efter att behandlingen upphört. Barn som ej behandlats (de som fått placebo) och var infekterade med flera parasitkloner hade lägre risk att bli sjuka i malaria under uppföljningstiden jämfört med barn infekterade med en parasitstam.

Konsekvenserna av att behandla asymtomatiska infektioner undersöktes vidare i studie IV, i vilken barn i åldrarna 1 till 6 år i Kenya fick effektiv behandling vid endast ett tillfälle. Vi kom fram till att denna behandling ändrade individers risk för att bli sjuka i malaria och de barn som tidigare varit infekterade med flera parasitstammar hade en lägre risk att utveckla klinisk malaria under uppföljning.

Sammantaget visar dessa studier att asymtomatiska malariainfektioner är viktiga för bibehållandet av en skyddande immunitet hos individer som lever i malariaområden. Våra resultat bidrar till förståelsen av hur immunförsvaret mot malaria byggs upp och upprätthålls, och är av värde för vidare utveckling och utvärdering av nya bekämpningsmetoder mot malaria såsom vaccin.

8 ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all of you who saw me through, thank you!
Special thanks to...

Anna Färnert, my supervisor, for your guidance, trust and support, your never-ending enthusiasm for science and “clones” and for your sense of details and perfection. It has been a pleasure working with you, thank you for believing in me. **Jan Andersson**, my co-supervisor for support and encouragement. **Kristina Broliden**, for welcoming me to your group. It has been great to be a part of your team. **Marita Troye-Blomberg**, for introducing me to the fascinating world of malaria research. **Benedict Chambers**, my mentor, for getting me back on track when I really needed it.

To all my colleagues and friends in the CMM building; The Färnert-group, **Klara Lundblom, Josea Rono, Saduddin Dashti and Khayrun Nahar**: You have been fantastic team-mates. Good luck to all of you in conquering the malaria research world! **Anna Lindblom, Pauline Levinsson and Nina Wolmer Solberg**: for all “stänkare” and countless laughs. I will always have a bottle of wine at home with your names on it. **Mia Ehnlund**, for endless enthusiasm and for being the “rock” in the lab. **Michelle Wong**, for always being so positive and happy, even when cutting about a million filter papers with me. All the great scientists in the Broliden group; **Pernilla Petersson, Klara Hasselrot, Taha Hirbod, Igge Gustafsson, Tove Kaldensjö, Birgit Sköldenberg, Christian Smedman, Thomas Tolvenstam and Oscar Norbeck**, for good company and many laughs, it has been a pleasure working with you all! **Biborka Bereczky-Veress**, for your positive attitude and all the good advices you have given me. I am keeping my fingers crossed for you. Good luck! **Lars Öhrnalm**, for being the best colleague and friend one can wish for. Thank you for all your help, your encouraging words and for believing in me/Häxan Surtant. **Selim Sengül** for teaching me everything I know about the “3730”, for always being there when I needed help and for being a true friend.

To all my colleagues and collaborators at Karolinska Institutet and Stockholm University; **Daniel Olsson**, for patiently teaching me everything I know about statistics, it can’t have been an easy task! Thank you! **Andreas Mårtensson**, for good collaboration in paper II. **Scott Montgomery**, for all your good ideas. My former colleagues at the Malaria Research Unit, **Sándor Bereczky, Lisa Wiklund, Berit Schmidt, Christin Sisowath, Sabina Dahlström, Isabel Veiga, Pedro Ferreira, Johan Ursing, José Pedro Gil, Akira Kaneko, Gabrielle Holmgren, Achuyt Bhattacharai and Anders Björkman**, for making “M9” a very nice place to work. The

malariaologists at SU, **Salah Farouk** and **Manijeh Vafa** for great friendship.
Margareta Hagstedt for teaching my ELISA.

To all my colleagues and collaborators around the world; The great team at KEMRI, Kilifi, Kenya, **Kevin Marsh** and **Norbert Peshu** for welcoming me to KEMRI, a place of scientific excellence; I have really enjoyed every stay. **Philip Bejon, Brett Lowe, Moses Mosobo, Oscar Kai, Alex Macharia, George Githinji,** you are all a constant source of inspiration to me! To all my co-authors at the London Schools of Hygiene and Tropical Medicine, United Kingdom, **Daniel Chandramohan, Brian Greenwood** and **Margaret Kweku**, it was nice working with you. To my co-authors at the Swiss Tropical Institute, **Ingrid Felger** and **Nicole Falk**, you have taught me a lot about capillary electrophoresis, thank you. **Ingegerd Rooth**, for your devoted work in Nyamisati that made study I possible.

To all my fantastic friends here and far away; The “wazungus” in Kilifi, Kenya: **Elise Schieck**, for sharing my love for Kenya, **Agnes Prins**, for being there for me in good times and bad times. I hope to see you in Nairobi soon! **Liz Stevenson**, for great friendship! You are a kick-ass immunologist! **Benjamin”Dr Phil” Edvinsson**, for all coffees and great conversations we have had through the years. The constituents of “Nuppalisterna”, **Caroline O, Caroline K** and **Nina**. My oldest and dearest friends. From parties at Sörgårdsvägen to “köttfrossa” på Grill. You mean the world to me. And just to clarify things- I was not bluffing! **Irena**, for always being available for a sushi and a horror movie. **Elin**, for great friendship and many nice dinners.

Anish, for support and encouragement, for giving me something to look forward to and for patiently waiting for my return. Thank you for being who you are!

Till min lilla ”klick”, familjen Kärki-Österlund-Hansson-Jansson-Ljungkvist-Liljander; tack för att Ni alltid finns vid min sida. Min underbara **mammis** och mina fantastiska systrar **Pia** och **Eva**, ni betyder allt för mig. Utan er hade det här ej varit möjligt.

Pappis, jag saknar dig!/Himlahumlan

This PhD project and included studies received financial support from: Swedish International Development Cooperation Agency (SIDA-SAREC), Anna Whitlocks Foundation, Erik and Edith Fernströms Foundation, Sigurd and Elsa Goljes Foundation, KI Travel funds and the Swedish Society for Tropical Medicine and International Health.

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