Genetic Diversity

of *Plasmodium falciparum* Infections

- Influence on Protective Malaria Immunity

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ABSTRACT

Detailed knowledge of the interaction between the human host and the antigenic and genetic diversity of *Plasmodium falciparum* infections is a prerequisite for understanding the mechanisms underlying acquisition of immunity to malaria. This thesis assessed the diversity of *P. falciparum* by genotyping the highly polymorphic vaccine candidate antigen *merozoite surface protein 2* (*msp2*) gene in different transmission areas. Factors influencing the number of genotypes and the relation between level of diversity and morbidity were investigated, as well as potential immunological markers of protection.

In a high endemic area in Tanzania, where a large part of the population harbours asymptomatic *P. falciparum* infections, the diversity was highest in children aged 6 to 10 years. Time to previous antimalarial treatment influenced the diversity of infections suggesting accumulation of genotypes with time. Individual exposure, assessed by anti-circumsporozoite protein antibody levels, did however not affect the number of concurrent genotypes. Asymptomatic multiclonal infections were associated with a reduced risk for subsequent febrile malaria without affecting hemoglobin levels. The number of *msp2* genotypes did not affect the crude anti-malarial IgG and IgE antibody levels. However, high anti-malarial IgE levels *per se* were associated with a reduced risk for subsequent malaria episode. In a mesoendemic area in Mali, ethnic difference in malaria susceptibility was not reflected in the diversity of *P. falciparum* but rather in different patterns of splenomegaly. In a low endemic area in Iran, the genetic diversity of *P. falciparum* in patients was higher than expected, suggesting a more intense transmission than previously reported. A new Tris-EDTA buffer-based DNA extraction method was developed and found to be useful for long term stored filter paper field samples.

The thesis contributes to a further understanding of the molecular epidemiology of the highly polymorphic *P. falciparum* parasite and highlights interesting interactions between the human host and multiclonal infections that may have to be considered in future strategies of malaria control.

**Key words:** malaria, *Plasmodium falciparum*, genetic diversity, *msp2*, IgE, subsequent risk, asymptomatic, DNA extraction, ethnic group, spleen enlargement, hemoglobin

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


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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AES</td>
<td>Average enlarged spleen</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CSP</td>
<td>Circumsporozoite protein</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological inoculation rate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>msp</td>
<td>Merozoite surface protein gene</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pRBC</td>
<td>Parasitized red blood cell</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SR</td>
<td>Spleen rate</td>
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<td>Th</td>
<td>T helper cells</td>
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**PAPERS**
INTRODUCTION

GENERAL BACKGROUND

Global situation of malaria

Malaria is the most important tropical parasitic disease and one of the major threats to human health in the world. Although malaria exists in about 100 countries, it is mainly confined to the poor tropical areas of Africa, Asia and Latin America, causing enormous toll in lives, in medical costs and in days of labour lost [Sachs and Malaney, 2002]. It has been estimated that some 300-400 million are infected with malaria and 1-2 million people die due to infection each year (WHO, 2004), but a more recent approach indicate that these numbers may be highly underestimated [Snow et al., 2005]. The great majority of clinical cases and malaria deaths occur in tropical Africa, mostly among children and pregnant women.

Epidemiology of malaria

The risk of malaria is highly dependent on interactions between the human host, parasite and mosquito vector. The causative agent of malaria is the single-celled protozoan parasites of the genus *Plasmodium*, transmitted by mosquitoes of the genus *Anopheles*. Four malaria species infect humans, *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*, the later being responsible for almost all cases of severe clinical malaria and death.

The epidemiology of malaria is highly dependent on the transmission pattern of the parasites. An area supporting active malaria transmission is termed endemic whereas sporadic outbreaks determine epidemic areas. In endemic areas transmission of infection may be fluctuating, leading to low- to high malaria incidence, or it may be stable, showing consistently high incidence over successive years. Malariologists have long graded endemic malaria according to risk of infection. Risk for malaria may be estimated by indirect measures, i.e. environmental (rainfall, altitude, temperature), entomological and clinical (spleen rate, serology) estimates, or by direct estimates as passive and active case detection [Baird et al., 2002]. An indirect marker, accepted by WHO (Kampala conference, 1950) as criteria for
classification of endemicity is the proportion of children and adults having enlarged spleens, known as spleen rate (SR).

Table 1. Criteria for classification of malaria endemicity

<table>
<thead>
<tr>
<th>Endemicity</th>
<th>Spleen rates (%)</th>
<th>Transmission</th>
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<tbody>
<tr>
<td></td>
<td>Children: 2-9 yr</td>
<td>Adults: &gt;16 yr</td>
</tr>
<tr>
<td>Hypoendemic</td>
<td>0 - 10</td>
<td>No measure</td>
</tr>
<tr>
<td>Mesoendemic</td>
<td>11 - 50</td>
<td>No measure</td>
</tr>
<tr>
<td>Hyperendemic</td>
<td>&gt; 50</td>
<td>≥ 25</td>
</tr>
<tr>
<td>Holoendemic</td>
<td>&gt; 75</td>
<td>&lt; 25</td>
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Life cycle of *P. falciparum*

The life cycle of malaria parasites is complex, including several stages both in the human host and in the mosquito vector (Figure 1).

The infection starts when an infected female mosquito, while taking a blood meal, injects saliva with malaria sporozoites into the human blood. The parasites will rapidly invade the liver cells (approximately after 30 minutes) and mature into liver schizonts during the next 5-15 days. Each hepatocytic schizont contains 10,000-30,000 merozoites, which are released into the blood stream, where they invade the red blood cells (RBC). In *P. vivax* and *P. ovale* infections parasites can stay dormant, as hypnozoites, in the liver for up to years before developing into blood stage infection and may also result in relapses long periods after initial clinical episodes. The erythrocytic stage of the life cycle is characterised by growth and asexual multiplication of the parasites. When the merozoites have entered into the RBC they grow into asexual trophozoites and form erythrocytic schizonts, each composed of 8-24 merozoites which are released at the burst of the erythrocytes. The duration of the erythrocytic cycle is 48 hours for the *P. vivax*, *P. ovale* and *P. falciparum* and 72 hours for *P. malariae*. 
A special characteristic for *P. falciparum* is a phenomenon known as sequestration. During the second half of the erythrocytic cycle the parasites “hides” into the deep vascular system by adhering to endothelial cells to escape clearance by the spleen.

Some of the merozoites that invade RBC will develop into gametocytes, i.e. male and female sexual forms. If ingested by a blood-feeding mosquito, the parasites will finally complete the life cycle in the mosquito gut, including fertilisation, production of oocysts, development and migration of the sporozoites to the salivary glands of the mosquito. The time of the sexual life cycle in the mosquito takes about 9-16 days, being largely dependent on temperature conditions.
Clinical manifestations of malaria

Malaria infection is asymptomatic during maturation in the liver. Clinical malaria disease will be apparent during the erythrocytic stage of infection and can present with a wide range of symptoms. The manifestations of non-severe malaria infection may be the classical flu-like symptoms such as fever, head- and body ache, but a wide spectrum of symptoms are possible. Severe disease and death can occur, especially in non-immune individuals. Sequestration in *P. falciparum* largely contributes to the pathogenesis of severe malaria. The main causes of death are: severe anemia, leading to profound hypoxia and congestive cardiac failure [Phillips and Pasvol, 1992], cerebral malaria leading to coma and other cerebral symptoms [Marsh and Snow, 1999] and respiratory distress syndrome [English et al., 1996]. In endemic areas the manifestation of severe disease will differ in function of malaria transmission [Snow et al., 1994; Modiano et al., 1998a], severe anemia being more common in high endemic areas, whereas cerebral malaria in areas of lower endemicity.

Malaria infection is associated with a reduction in hemoglobin (Hb) levels and is the main aetiological agent of anemia in young children and pregnant women living in highly endemic areas [Matteelli et al., 1994; Menendez et al., 1997]. Malaria-related anemia is determined by rupture of parasitized red blood cells (pRBC), phagocytosis of pRBC and unparasitized RBC, hypersplenism, hemolysis, dyserythropoiesis – i.e. both increased RBC destruction and decreased RBC production [Menendez et al., 2000].

Several factors influence the clinical outcome of malaria infections, mainly acquired and innate immunity [Marsh, 1992].

**MALARIA IMMUNITY**

*Plasmodium falciparum* infections in immunologically naive subjects of any age have high rates of morbidity and mortality. Protective immunity is gradually acquired to malaria in areas of high transmission. The mechanism of clinical immunity is however largely unknown and there is still no marker of individual protection to malaria.
Acquired immunity

Individuals living in endemic areas acquire immunity to clinical malaria disease after repeated exposure to the parasite but will still be infected and have detectable parasites in the blood, which reflects the non-sterile nature of antimalarial immunity [Bruce-Chwatt, 1963; Greenwood et al., 1991]. Moreover, immunity is regarded to be short-lived and will decrease when exposure to the parasites is interrupted [Colbourne, 1955]. Acquisition of immunity is also dependent of host age, as seen in adult immigrants to an area of endemic malaria who become less susceptible more rapidly than their younger counterparts [Baird et al., 1993; 1998].

In high endemic areas *P. falciparum* infections are more frequent and severe in young children than in adults. Protective immunity gradually develops with age and repeated infections. During the first months of life, children are protected by foetal Hb [Pasvol et al., 1977] and by the intrauterine transfer of protective antibodies from their immune mothers [Edozien et al., 1962]. After four to six months, infants lose this protection, and increasing exposure result in clinical malaria which may be severe, with high mortality risk. By school age, children have developed a considerable degree of immunity [Bruce-Chwatt, 1952; Lucas et al., 1969] manifested by fewer clinical episodes, lower parasitemias and enhanced parasite-specific immune responses [Marsh, 1992]. Adults rarely experience clinical episodes of malaria and have low grade infections. During pregnancy women are again highly susceptible to severe disease and death as well as foetal complications and low birth weight. This increased susceptibility is more pronounced during the first pregnancy and has been explained by sequestration of parasites in the placenta [Fried and Duffy, 1998]. In low endemic areas the whole population is at risk for malaria and clinical episodes have similar frequency throughout different age groups [Reyburn et al., 2005]. The pyrogenic threshold, i.e. the parasite density at which fever develops, decreases with age [Rogier et al., 1996] and differs also depending on transmission level [Mwangi et al., 2005].

Strain-specific immunity

The long period needed to build up an effective anti-malarial immunity has been explained by the poor immunogenicity of the parasite antigens but also by strain-specific acquisition of protection [Kitchen, 1949; Jeffery, 1966] Evidence for strain-specific immunity has been provided in studies of induced malaria in humans [Collins and Jeffery, 1999] as well as in
animal models [Jarra and Brown, 1989]. Several studies have indicated that the multiple allelic forms of antigenic genes differ in their ability to induce protective immunity in the host [Udhayakumar et al., 1994; Rich and Ayala, 2000; Weisman et al., 2001]. The concept of strain-specific immunity implies that immunity is mounted against one parasite variant at the time and that it requires exposure to the parasite variants circulating in an area [Day and Marsh, 1991]. Clinical malaria episodes may thus be caused by parasite variants which have not been previously encountered by the individual. Indeed, several studies have found new parasite genotypes within the host during a clinical malarial episode [Contamin et al., 1996; Roper et al., 1998; Kun et al., 2002] which suggests that lack of specific immune responses allows for rapid multiplication of this particular parasite leading to increased parasitemia and clinical symptoms.

**Humoral and cellular immunity**

Infection with *P. falciparum* induces both humoral and cell-mediated immune responses. Studies of passively transferred immunoglobulins from immune adults have shown that IgG antibodies are important in reducing parasite density during clinical malaria [Cohen et al., 1961; Sabchareon et al., 1991]. There is a significant production of parasite stage-specific antibodies that react with a large number of antigens and antibodies are specific for the different antigen variants. There is also accumulating evidence for a protective role of certain IgG subclasses, e.g. IgG1 [Ndungu et al., 2002], IgG2 [Aucan et al., 2000] and IgG3 [Taylor et al., 1998]. Malaria-specific antibodies may inhibit RBC invasion [Wahlin et al., 1984] and merozoite release from schizonts [Green et al., 1981]. Antibodies may also mediate parasite clearance as they inhibit sequestration [David et al., 1983] and rosetting, i.e. spontaneous binding of two or more uninfected erythrocytes to the pRBC [Carlson et al., 1990; Treutiger et al., 1992]. Other mechanisms of protection are those of antibody dependent phagocytosis involving FcR-bearing cells and antibody dependent cell mediated cytotoxicity (ADCC) [Boucharoun-Tayoun et al., 1990; Groux and Gysin, 1990].

The high IgE levels observed in acute severe malaria indicate a pathogenic role of these antibodies [Perlmann et al., 1994, 2000]. A protective role for malaria-specific IgE has also been proposed [Perlmann et al., 1997, 1999] but not previously demonstrated.
Malaria-induced cell-mediated immune responses may be protective against both extra- and intra-erythrocytic parasites. T-cells play a major role in the acquisition and maintenance of protective immunity to malaria. Studies indicate that CD4+ T-cells regulate immune responses to the asexual blood stages of the parasite by cytokine production and providing help for B cells to produce specific antibodies [Kabilan et al., 1987; Troye-Blomberg et al., 1994], whereas CD8+ T-cells has an important role in the pre-erythrocytic immunity [Malik et al., 1991] as well as protection against severe malaria [Hill et al., 1991]. There is also evidence that macrophages, dendritic cells and NK cells are important effectors of immunity against malaria [Orago and Facer, 1991; Gyan et al., 1994; Hansen et al., 2003; Pichyangkul et al., 2004].

**The role of the spleen**

Spleen enlargement is a well-established phenomenon in malaria endemic areas [Thomas et al., 1981; Bryceson et al., 1983; Greenwood et al., 1987] and spleen rate is a classical indicator of malaria endemicity as described above. Splenomegaly, although most common in chronic cases of malaria, may be detected a few days after an acute malaria attack in non-immune or in semi-immune individuals which then gradually decrease after recovery from disease [Bryceson et al., 1983]. The spleen has a crucial function for the control of malarial infection, both by blood filtration and as a lymphoid organ [Engwerda et al., 2005]. Decreased clearance of pRBC in patients with acute *falciparum* malaria, as well as RBC containing dead parasites after treatment, were observed in splenectomised humans [Looareesuwan et al., 1993; Chotivanich et al., 2002]. Moreover, in experimental model, patients with acute malaria having splenomegaly showed markedly accelerated clearance of labelled *P. falciparum* infected erythrocytes [Looareesuwan et al., 1987]. The importance of the spleen for erythropoiesis and hematopoiesis [Villéval et al., 1990a, 1990b], and generation of immunity against malaria has been confirmed in rodents [Kumar et al., 1989]. Splenomegaly in a murine malaria model has been associated with expansion of the splenic B-cell pool and enhanced parasite-specific antibody formation in response to *P. berghei* infection and showed that CD1-restricted NKT cells contributed to malarial splenomegaly [Hansen et al., 2003].
Immunogenetics of malaria including innate immunity

Infections with *Plasmodium* species have existed in humans throughout much of our evolutionary history and because the infections had significant effects on mortality before reproductive age, malaria has exerted a strong selective pressure on the human genome. The inherited disorders of hemoglobin, including certain Hb variants and the thalassemias, are the most frequent monogenic diseases in man [Nagel and Rooth, 1989; Weatherall, 2001]. Mutant variants of the β-globin gene result in certain hemoglobinopathies associated with susceptibility and pathogenesis of malaria, as HbS (sickle cell trait) [Allison, 1954; Pasvol et al., 1978], HbC [Agarwal et al., 2000; Fairhurst et al., 2005] or HbE [Hutagalung et al., 1999]. Thalassemia, defined by a defective synthesis of one of the globin chains, has been found to protect against malaria [Haldane, 1949; Willcox et al., 1983]. Other polymorphisms associated with protection against malaria infection are those regulating the glucose-6-phosphate dehydrogenase (G6PD) activity [Ruwende et al., 1995; Ruwende and Hill, 1998], those responsible for the red cell integrity, represented by erythrocytic band 3 protein causing Southern Asian ovalocytosis (SAO) [Kidson et al., 1981; Allen et al., 1999] or Duffy antigen expression on the surface of the RBC [Miller et al., 1976; Tournamille et al., 1995].

Several studies indicate that immunity against malaria, at least in part, is genetically regulated. Levels of antibody to a major malarial antigen Pf155/RESA in individuals living in a holoendemic area in Liberia were more concordant within monozygotic- than in dizygotic twins or in age- and sex-matched sibs living under similar environmental conditions [Sjöberg et al., 1992]. Moreover, comparative survey performed on three sympatric ethnic groups, Fulani, Mossi and Rimaibé living within the same geographical area and apparently exposed to the same malaria transmission in Burkina Faso, has shown lower susceptibility to malaria and higher levels of antibodies to various *P. falciparum* antigens in the Fulani [Modiano et al., 1995; 1996]. Interethnic differences in anti-malarial immune responses were further confirmed by more recent studies [Modiano et al., 1998; Louni et al., 2001; Farouk et al., 2005; Dolo et al., 2005].

The chromosome region 5q31-q33, containing genes coding for the interleukins IL-4, IL-9 and IL-13, was found to be critical in the control of malaria infection [Garcia et al., 1998; Rihet et al., 1998]. Furthermore, resistance or susceptibility to clinical malaria is associated with diverse immunogenetic polymorphisms, as HLA class I antigen (HLA-B53) and HLA
class II haplotypes (DQB1*0501, DRB1*1302) [Hill et al., 1991; May et al., 2001], intercellular adhesion molecule 1 (ICAM-1) [Fernandez-Reyes et al., 1997; Kun et al., 1999], complement component receptor 1 (CR1) [Xiang et al., 1999; Cockburn et al., 2004], CD36 [Aitman et al., 2000; Omi et al., 2003], mannose-binding protein (MBP) [Luty et al., 1998], inducible nitric oxide synthase 2 (iNOS-2) [Burgner et al., 1998; Kun et al., 2001] and tumor necrosis factor α (TNF-α) [McGuire et al., 1994; 1999; Knight et al., 1999].

GENETIC DIVERSITY OF P. FALCIPARUM

The P. falciparum genome, 23 Mb situated on 14 chromosomes, consists of about 5300 genes [Gardner et al., 2002]. A large number of genes encoding parasite antigens have been isolated and characterised. As expected for genes under strong diversifying selection for evasion of the immune system, antigenic genes of the parasite are characterised by high number of non-synonymous nucleotide substitutions [Escalante et al., 1998], as well as duplication and/or deletion of the repeated segments within the genes [Rich and Ayala, 2000]. A prominent feature of the P. falciparum surface proteins is the presence of the highly mutable repeating nucleotide sequences that encode iterative amino acid sequences [Anders et al., 1988; Arnot, 1991; Felger et al., 1997].

P. falciparum antigens are stage specific and many of them are characterised by a high variability. A major antigen, involved in the cytoadherence of the infected erythrocytes to endothelial cells [Smith et al., 1995] and binding of noninfected erythrocytes, i.e. rosetting [Chen et al., 1998a], is the P. falciparum erythrocyte membrane protein 1 (PfEMP1). This antigen is encoded by the var multigene family, comprising about 60 genes located on multiple chromosomes [Su et al., 1995; Gardner et al., 2002]. These genes, expressing variant forms of the protein, are switched on at different times leading to antigenic variation by translation and expression of only one PfEMP1 variant at a time on the erythrocyte surface [Chen et al., 1998b]. Several other antigens, specific for the different developmental stages of the parasite, have been identified: Pf45/48, circumsporozoite protein (CSP), thrombospondin-related adhesive protein (TRAP), liver-stage antigens 1-3 (LSA1-3), merozoite surface proteins 1-3 (MSP1-3), apical membrane antigen 1 (AMA1), erythrocyte binding antigen 175 (EBA175), ring-infected erythrocyte surface antigen (Pf155/RESA), R23 and Pf332 [reviewed by Bolad and Berzins, 2000].
Genotyping of *P. falciparum* infections

The extensive polymorphism of malaria antigens has been used to genetically characterise *P. falciparum* populations, and PCR based genotyping methods are now widely used in molecular epidemiological studies as well as in drug trials to differentiate between recrudescence and reinfection [Cattamanchi et al., 2003]. Among the polymorphic genes most commonly used for genotyping, i.e. *msp1*, *msp2*, *glurp*, the most informative single marker for epidemiology studies is *msp2* [Färnert et al., 2001] and was therefore chosen to characterise the genetic diversity of *P. falciparum* infections in this thesis.

**Merozoite surface protein 2 (msp2) gene**

MSP2, also known as merozoite surface antigen 2 (MSA2), is a glycoprotein expressed on the surface of merozoites, the stages of parasite that invade the RBCs. In areas of high malaria endemicity the age prevalence of antibodies against MSP2 has been established together with a reduced risk for clinical malaria [Taylor et al., 1998; Polley et al., 2005]. MSP2 has been widely studied as one of the major vaccine candidates [Sturchler et al., 1995; Polley et al., 2005]. Being one of the most polymorphic antigens of *P. falciparum*, the *msp2* gene has thus been shown to be useful marker to characterise *P. falciparum* populations.

The single-copy *msp2* gene is characterised by a conserved 5’ and 3’ region (blocks 1 and 5) [Smythe et al., 1990; 1991; Snewin et al., 1991], two non-repetitive variable regions (blocks 2 and 4) and a polymorphic central region (block 3) containing tandem repeats of varying sequence, copy number and length. The non-repetitive variable blocks may be grouped into two allelic families, FC27 and 3D7/IC (Figure 2).

The dimorphic family-specific domains are totally conserved over extended sequences but show in parts a remarkable length and sequence variation [Felger et al., 1999]. The central repetitive region differs considerably between and within allelic families giving rise to considerable length polymorphism, which is exploited in the genotyping analysis. The FC27-type central region consists of two relatively conserved repetitive sequences: one to four copies of a 32-mer motif followed by a conserved non-repetitive 7-mer fragment and none to five copies of a 12-mer motif. The 3D7/IC-type block 3 repeats are highly variable in length (six to 30 bp), sequence and number of copies [Felger et al., 1997]. MSP2 is indeed highly...
polymorphic with more than 150 different alleles described so far [Felger et al., 1999; Weisman et al., 2001].

**Figure 2.** Schematic representation of the *msp2* gene and the two allelic families of *msp2*, FC27 and 3D7/IC (adapted from Smythe et al., 1990)

Genotyping approaches are generally based on an initial PCR of the outer regions of the selected gene followed by different methods for determination of different allelic types. The methodology may include a second nested PCR with allelic type-specific amplification, hybridization reactions with Southern blotting or restriction fragment length polymorphism (RFLP) by restriction enzyme digestions [Dolan et al., 1993; Felger et al., 1993]. PCR products of different sizes are generally separated by gel electrophoresis or by a recently developed method of GeneScan [Falk, 2004]. The number of products of the two respective allelic types denotes the number of genotypes per infections [Snounou et al., 1999].

Other methods for typing *P. falciparum* are based on microsatellite (MS) analysis [Su et al., 1998], reverse transcriptase (RT) PCR [Menegon et al., 2000], amplified fragment length polymorphism (AFLP) [Rubio et al., 2001], sequence analysis [Zhu et al., 2002], fluorogenic PCR [Decuypere et al., 2003], minisatellite variant repeat (MVR) mapping [MacLeod, 2004] and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) [Marks et al., 2004]. These methods have however still limitations in determining multiple concurrent parasite genotypes in single samples in large epidemiological studies.
MOLECULAR EPIDEMIOLOGY OF *P. FALCIPARUM* INFECTIONS

An increasing number of studies have been reported on the epidemiology of genetic diversity of the parasite, mainly focusing on the polymorphism of *msp1* and *msp2* genes. Parasite populations have been studied in different geographical areas with different transmission, over time within an area as well as within the individual. Some studies have searched for allelic types as markers of parasite virulence and found that alleles of the FC27 family of *msp2* were associated with malaria morbidity [Felger et al., 1994; Engelbrecht et al., 1995; Beck et al., 1997; Genton et al., 2002], whereas other studies have found such association with other allelic types of *msp 1* or *2* [Robert et al., 1996; Al-Yaman et al., 1997a].

**Factors associated with diversity of infections**

The diversity of *P. falciparum* within a single host can reach up to about 10 different genotypes at a given time – but the mean number in a population is about 2-4 in most studies. The number of genotypes within an infection is a result of interaction between several factors: intensity of malaria transmission in the area, individual exposure to infective mosquitoes, natural and acquired immunity as well as chemoprophylaxis or recent treatment (Figure 3).

The parasite population determined in a single blood sample may however, underestimate the true number considering an extensive dynamics of parasite population within the host [Daubersies et al., 1996; Färnert et al., 1997; Bruce et al., 2000]. Although different parasites may be found over time, the number of genotypes at a given time is relatively constant [Färnert et al., 1999] suggesting that single samples are sufficient in epidemiological studies.

**Malaria transmission and individual exposure**

Transmission intensity can influence the genetic diversity of the parasite population. Sexual reproduction of the parasites in the mosquito allows for intragenic recombination and chromosome assortments, events which are more frequent in high transmission areas [Walliker, 1983]. Studies comparing different transmission intensities indeed established that *P. falciparum* populations, both in an area and in an individual, are more diverse in areas of high transmission compared to areas with low or seasonal transmission [Babiker et al., 1997; Konate et al., 1999]. Higher transmission levels are associated with a non-linear increase in
the number of parasite genotypes per host [Arnot, 1998; Babiker et al., 1999; Bendixen et al., 2001]. However, even in areas with the most intense transmission individuals can be infected with only a limited number of different genotypes [Felger et al., 1994; Engelbrecht et al., 2000].

**Figure 3.** Potential factors associated with the genetic diversity of *P. falciparum* infections

**Immunity and age**

Epidemiological studies have indicated that diversity of *P. falciparum* infections reflects the immune status of the hosts. In highly endemic areas the number of infecting parasite genotypes in asymptomatic infections decreases with age [Ntoumi et al., 1995]. The peak multiplicity of infection is however not found in young children and infants, but rather in children aged 3-7 years [Smith et al., 1999a], 5-9 years [Konate et al., 1999] or 8-10 years [Engelbrecht et al., 2000] in different settings. In areas with lower malaria transmission the same level of diversity is observed throughout different age groups [Zwetyenga et al., 1998; Konate et al., 1999].
The reduction in complexity of infection with age, observed in areas with intense malaria transmission, reflects gradual acquisition of antiparasitic immunity. Interestingly, the peak multiplicity is not found in the youngest ages, which suggests a complex pattern of immune acquisition to exposure to diverse infections. The low diversity of *P. falciparum* infections found in adults may also be due to the lower parasite density observed in this age group and thus lower sensitivity of detection [Smith et al., 1999a]. In this context the diversity of infections in adults may be higher than what can be shown. In low endemic areas a more constant level of diversity seen in all age groups rather reflects that the difference in immunity between children and adults in low transmission areas is not as apparent.

**Risk for subsequent clinical malaria**

Several studies have assessed how the number of genotypes in an individual correlates with clinical protection and risk for malaria episodes. The number of co-infecting parasite genotypes has been associated with reduced incidence of clinical malaria episodes [al-Yaman et al., 1997b; Beck et al., 1997; Färnert et al., 1999; Smith et al., 1999b; Henning et al., 2004]. Prospective studies conducted in areas of intense malaria transmission in Papua New Guinea [al-Yaman et al., 1997b] and Tanzania [Beck et al., 1997; Henning et al., 2004] revealed that the presence of multiple *P. falciparum* genotypes in asymptomatic individuals during cross-sectional surveys was associated with fewer disease episodes. Furthermore, longitudinal studies have established that the level of parasite diversity is consistent in the individual over time and that individuals with multiclonal infections were less likely to have clinical episodes of malaria [Färnert et al., 1999]. However, multiple *P. falciparum* infections have also been associated with higher morbidity in children less than three years in a high endemic area [Branch et al., 2001; Henning et al., 2004] and in individuals living in areas with low malaria transmission [Roper et al., 1998; Mayor et al., 2003]. In these populations multiclonal infections may rather be a marker of recent inoculation.

Persistent multiclonal infections may offer cross-protection against invading parasites through continuous stimulation of immune responses. Clinical studies [Powell et al., 1972; Smith et al., 1999b] as well as experimental model [Gatton and Cheng, 2004] have indeed indicated that persistent *P. falciparum* infections are important in acquisition of protective immunity in malaria endemic areas. It is therefore interesting to establish how malaria interventions affect diversity in relation to protection. Insecticide-treated bed nets (ITNs) are effective in reducing child morbidity and mortality in endemic area [D’Alessandro et al., 1995; Binka et al., 1996].
The use of ITNs in high endemic areas did not markedly affect the genetic diversity of *P. falciparum* infection [Fraser-Hurt et al., 1999; Smith et al., 1999c] nor the antibody response to blood stage antigens [Kariuki et al., 2003].

A potential negative effect of persistent asymptomatic multiclonal infections may be anemia, especially in infants and pregnant women. The low Hb levels in symptomatic and asymptomatic children with *P. falciparum* infections [McGregor et al., 1966; Greenwood, 1987b] may argue for the general use of chemoprophylaxis or treatment. The new concept of intermittent preventive treatment (IPT) has been introduced in pregnancy and is widely studied during infancy to reduce anemia [Greenwood, 2004]. There are also suggestions to introduce IPT in older children. Clearance of asymptomatic multiclonal low-grade infection may however alter maintenance of protective immunity. Further understanding of the importance of diversity of infections in acquisition and maintenance of protective malaria immunity is therefore needed for the development of effective control tools in endemic areas.
AIMS OF STUDY

- To describe the epidemiology of *P. falciparum* diversity in areas of different transmission (I, III, V) and in populations with different susceptibility to malaria (V)

- To explore factors that influence the number of parasite genotypes in an individual (I)

- To investigate the relationship between the diversity of *P. falciparum* infections and malaria morbidity including anemia (I, II)

- To explore potential markers of immunity related to the diversity of *P. falciparum* infections (II, V)

- To improve the DNA extraction methodology for PCR genotyping from field samples (IV)
MATERIAL AND METHODS

STUDY POPULATIONS

The studies were conducted in three areas with different malaria endemicity (Figure 4).

Figure 4. Study sites: Tanzania – highly endemic; Mali – mesoendemic; Iran – low endemic area
Tanzania, highly endemic area (I, II)

Nyamisati village is situated by the Rufiji River Delta, coastal Tanzania. A research team, which also provides health care, has lived in the village since 1985 and studied the epidemiology of malaria [Rooth and Björkman, 1992]. The area is holoendemic for malaria with perennial transmission which increase during the two rainy seasons (long rains April-June and short rains November-December). The population of about 1000 individuals has been continuously followed with regards to malaria, including examination of all fever cases, confirmation of malaria by microscopy, provision of free antimalarial therapy, and registration of all malaria episodes and other clinical data. In March-April 1999, preceding the long rain period, 890 villagers, 1-84 years of age, participated in a cross-sectional malariometric survey. All samples were obtained after informed consent of the participants and/or their guardians. The study was approved by the National Institute for Medical Research in Tanzania and by the Ethical Committee at the Karolinska Institutet (Dnr 00-084).

Mali, mesoendemic area (V)

Two ethnic groups, the Fulani and the Dogon, live in sympatry in rural villages located in a sahelian area of Mali, situated half of the distance between Mopti and the country border to Burkina Faso. The area is mesoendemic for malaria, with a dry season during October-May and a rainy season between June and September. Malaria transmission is seasonal, with most cases registered from July until December. Infections are mainly caused by *P. falciparum*. The Fulani are nomadic partly settled pastoralists, characterized by non-Negroid features of possible Caucasian origin, while the Dogon are Sudanese Negroid populations with a long tradition of sedentary farming. Several studies have demonstrated a lower susceptibility to malaria in the Fulani [Greenwood et al., 1987; Modiano et al., 1996; Dolo et al., 2005] why there are specific immunogenetic interests to study this population. A cross-sectional malariometric survey was performed in the two populations in September 2001, at the end of the rainy season. The study included 474 age-matched participants (237 Dogon and 237 Fulani). Informed consent was obtained from the participants and/or their guardians. The study was approved by the Ethical Committee of the Faculty of Medicine and Pharmacy of Mali and by the Ethical Committee at Karolinska Institutet (Dnr 03-536).
Iran, hypoendemic area (III)

Chabahar is located in the south-eastern part of Iran, an area with substantial human migrations originating mainly from Afghanistan and Pakistan. Malaria cases are reported during the whole year with two peaks, the first with predominantly *P. vivax* in April through September and the second peak with 45-50% *P. falciparum* infections after September. In April 2001 - March 2002 some 110 patients with microscopically confirmed *P. falciparum* were recruited at a malaria health center in Chabahar City Public Health Department in Sistan and Baluchistan province, Iran. The study was approved by the Review Board from Institute Pasteur, Tehran, Iran (Ethical Approval Reference N. 502).

**GENOTYPING OF *P. FALCIPARUM***

All studies in this thesis include determination of the diversity of *P. falciparum* infections, i.e. number of infecting clones, by genotyping the *merozoite surface protein 2*. The genotyping method is here described in detail as well as a new DNA extraction method developed within this thesis.

**DNA extraction**

Frozen blood specimens

Extraction of DNA from whole blood collected in EDTA tubes (paper I-III) was performed by phenol-chloroform extraction and ethanol precipitation as previously described [Snounou et al., 1993]. DNA was prepared from packed erythrocytes. Briefly, the cells were lysed with saponin and after centrifugation, the parasite pellet was resuspended in lysis buffert (40mM Tris pH 8.0, 80 mM EDTA, 2% SDS) and incubated in proteinase E. DNA was extracted with phenol followed by phenol-chloroform and precipitated in sodium acetate and ethanol. Finally, the DNA was resuspended in TE buffer (10mM Tris pH 8.0, 0.1 mM EDTA pH 8.0) so that 1 μl of DNA suspension, added in all PCR amplifications, corresponded to 5 μl of whole blood.

Filter paper specimens

In study V the material consisted of the archive blood spots on filter paper stored for up to three years. Initial extraction by several methods (H₂O boiling, methanol, Chelex®) generated
insufficient or negative PCR results. A new (TE buffer-based extraction) method was therefore developed (IV). Briefly, individual samples with a diameter of 4 mm were punched out from the dried blood spots, 65 µl TE buffer were added to each sample and were incubated in eppendorf tubes at 50°C for 15 min. The punches were pressed gently at the bottom of the tube several times, using a new pipette tip for each sample. The tubes were heated for 15 min at 97°C to elute the DNA. The method was then systematically compared to the established methods for extraction from filter paper, i.e. Chelex® (Bio-Rad Lab, CA) [Kain and Lanar, 1991; Plowe et al., 1995] and methanol [Long et al., 1995; Gil et al., 2003].

**PCR method**

Genotyping of *P. falciparum* infections was performed with a nested PCR-based method, targeting the *msp2* gene [Snounou et al., 1999]. This single-copy gene is highly polymorphic both in sequence and size, by a variable number of tandem repeats (see above), and enables establishment of the diversity of a *P. falciparum* population, i.e. number of infecting parasite populations or genotypes. The outer conserved region of the polymorphic repetitive block 3 of *msp2* is amplified in an initial reaction followed by two separate nested reactions with oligonucleotide primers specific for the two allelic types for *msp2* (FC27 and 3D7/IC) [Snounou et al., 1999]. Laboratory-cultured parasite lines were used as controls, the K1-line for the FC27 and the F32-line for the 3D7/IC allelic family of the *msp2*. Two positive controls of the two respective lines with different parasite concentrations (a low concentration corresponding to about 10 parasites/µl and a high concentration corresponding to about 5000 parasites/µl) as well as one negative control without DNA were included in each amplification set-up.

The total volume of the PCR reactions was 20 µl. The final concentration of the master-mix for the Nest1 amplification was: 1x PCR buffer, 2 mM MgCl₂, 125 µM dNTP, 250 nM of each oligonucleotide primer pair (F/R) and 0.02 units/µl of Taq polymerase. The Nest2 amplifications were carried out at a final concentration of: 1x PCR buffer, 1 mM MgCl₂, 125 µM dNTP, 125 nM of each oligonucleotide primer pair (F/R) and 0.02 units/µl of Taq polymerase, in double distilled and de-ionized water. An overlay of 40 µl mineral oil was added to each reaction tube in both amplifications. The quantity of the DNA template in the first reaction was 1 µl (corresponding to a blood volume of 5 µl). In case of the TE-buffer
based DNA extraction from blood spots on filter paper, 3 μl of DNA template was added to 18 μl total reaction volume (the quantity of water was reduced with 2 μl for each reaction). In both cases 1.2 μl of the Nest1 amplification product was used as template in the second reaction.

The amplification cycles were as follows:

**Nest1:** step 1 - initial denaturation at 95°C for 5 min,  
step 2 - annealing at 58°C for 2 min,  
step 3 - extension at 72°C for 2 min,  
step 4 - denaturation at 94°C for 1 min. Steps 2-4 were repeated 24 times.  
step 5 - final annealing at 58°C for 2 min,  
step 6 - final extension at 72°C for 5 min.

**Nest2:** step 1 - initial denaturation at 95°C for 5 min,  
step 2 - annealing at 58°C for 1 min,  
step 3 - extension at 72°C for 1 min,  
step 4 - denaturation at 94°C for 30 sec. Steps 2-4 were repeated 29 times.  
step 5 - final annealing at 58°C for 1 min,  
step 6 - final extension at 72°C for 5 min.

The temperature was reduced to 20°C when the reactions were completed.

The PCR products were separated by gel electrophoresis in TBE buffer on 2% MetaPhor® agarose gels (BMA Bioproducts, Rockland, ME, USA) and visualised by UV transillumination after staining with ethidium bromide. Length differences were determined using a 100-base pair ladder (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and a gel analysis program (GelDoc 2000, BioRad, CA, USA). The total number of product of the two respective allelic types denotes the number of *msp2* genotypes per infection (Figure 5).

**Figure 5.** *Msp2* genotyping with FC27 (a) and 3D7/IC (b) allelic family-specific amplifications (molecular weight marker: 100-base pair ladder)
ANTIBODY LEVELS

P. falciparum blood-stage extract preparation (II)

The *P. falciparum* laboratory strain F32 was maintained in continuous culture, at 5% hematocrit, and synchronized as previously described [Lambros and Vanderberg, 1979]. Parasites (schizont-infected RBC) were harvested at 10% parasitemia and washed twice in cold RPMI. After the second centrifugation the pellet were resuspended in cold RPMI (1:10), layered on top of 60% Percoll and centrifuged at 2,000 rpm for 15 min at 4°C. The supernatant (including the interface layer) with the late stage parasite-infected RBC was collected and washed three times in cold PBS. The parasite pellet was sonicated on ice for 2 min at 25 W and centrifuged at 2000 rpm for 10 min at 4°C. The protein concentration was determined by the Bradford method [Bradford, 1976]. The *P. falciparum* crude antigen was aliquoted and stored at -70°C until use.

Immunological analysis, the enzyme linked immunosorbent assay (ELISA) (I, II)

ELISA assays were performed for the determination of *P. falciparum*-specific IgG and IgE, total IgE and anti-CSP antibodies, as previously described [Hogh et al., 1991; Perlmann et al., 1994]. Microtiter 96-wells plates (Costar Corporation, USA) were coated with 50 μl of crude parasite antigen solution (20 μg/ml) for determination of the malaria-specific IgE and IgG, goat anti-human IgE (5 μg/ml) for the assay of total IgE and synthetic peptide (NANP)_{6} (10 μg/ml) to establish anti-CSP levels. The coated plates were incubated overnight at 4°C, and then blocked with 100 μl of 0.5% BSA in coating buffer for 3 h at 37°C. The test plasma, diluted 1:1000 for total IgE, anti-malarial IgG and anti-CSP antibodies, were added to the wells and incubated at 37°C for 1h. For determination of malaria-specific IgE the test plasma were diluted 1:100 and incubated at room temperature overnight for optimal binding.

Anti-malarial IgG and anti-CSP antibodies were detected using alkaline phosphatase (ALP) - conjugated goat anti-human IgG (Mabtech, Sweden), diluted 1:2000 and 1:1000, respectively. The secondary antibody for the assay of total and specific IgE was the biotinylated goat anti-human IgE (Vector Laboratories, USA) diluted 1:8000. ALP-conjugated Streptavidin (Mabtech, Sweden), diluted 1:2000 were added to the biotinylated antibody. All incubations were made for 1 h at 37°C, the plates were washed four times between each incubation step. The bound secondary antibody was quantified with p-nitrophenylphosphate (Sigma-Aldrich,
USA) substrate and the optical density (OD) at 405 nm was determined in a Vmax microplate reader (Molecular Devices, USA) and in a Multiskan EX reader (Thermo Electron, USA) for anti-CSP antibodies.

Antibody concentrations were calculated from standard curves. Sera from African donors with high antibody levels and from Swedish donors not exposed to malaria were used as positive and negative controls, respectively. All tests were done in duplicate.
RESULTS AND DISCUSSION

EPIDEMIOLOGY OF P. FALCIPARUM DIVERSITY

High endemic area (I, II)

P. falciparum genotyping was performed in blood samples collected from 873 individuals (1-84 yr, median 17 yr) in the longitudinally followed population in Nyamisati, Tanzania. The parasite prevalence was 27% by microscopy and 46% by PCR. Multiclonal P. falciparum infections, i.e. two or more \( msp2 \) genotypes, were detected in 70% of the PCR positive individuals. In asymptomatic individuals the mean number of \( msp2 \) was 2.4 (95% CI, 2.2-2.6), with a peak of 3.0 genotypes in children 6 to 10 years old. In the 108 individuals with fever and P. falciparum the diversity was relatively constant over age, presenting a mean of 3.4 (95% CI, 3.0-3.7) genotypes per infection (see paper I - Figure 3a & b). Similar diversity was observed when clinical malaria was defined according to a second definition, fever and >5000 parasites/\( \mu l \). The 3D7/IC allelic type of \( msp2 \) was more prevalent than the FC27 type, in both asymptomatic and symptomatic infections. However, parasites of the FC27 allelic genotype were more common in symptomatic adults. The genetic diversity of P. falciparum infections followed the age specific pattern reported in other holoendemic areas [Smith et al., 1999a; Konate et al., 1999; Engelbrecht et al., 2000]. The mean number of \( msp2 \) genotypes per infections was however lower than expected which can probably be explained by that the survey was performed before the rain season, which affects the prevalence and most probably also the diversity of P. falciparum infections in this population.

Mesoendemic area (V)

In a sahelian area in Mali the prevalence of P. falciparum by microscopy and/or PCR was 36% in the Fulani and 44% in the Dogon in 218 respective 208 asymptomatic individuals at survey (0-79 yr, median 8 yr - in both groups). Mean number of \( msp2 \) genotypes was highest in the youngest age group (0-4 yr) and decreased with age in both ethnic groups (see paper V - Table 2). Multiclonal P. falciparum infections were detected in 65% of infections in both the Dogon and the Fulani. There was no significant difference in the number of \( msp2 \) genotypes in asymptomatic individuals of the two ethnic groups, with a mean number of 2.25 in the
Dogon and 2.11 in the Fulani, respectively. The diversity of *P. falciparum* infections was higher in individuals with clinical episodes of malaria, i.e. concurrent fever, compared to asymptomatic individuals presenting mean values of 2.69 and 2.18, respectively (*P*=0.028). The frequency of the two allelic families (FC27 and 3D7/IC) was similarly distributed throughout different ages in the two ethnic groups and was independent of clinical status.

**Low endemic area (III)**

Genotyping was performed in 110 malaria patients (5-65 yr, median 27 yr), with microscopically confirmed *P. falciparum*, who attended the regional hospital in Chabahar, Iran, using both molecular markers *msp1* and *msp2*. For the *msp1* (analysis was performed in Iran with similar nested PCR with allele-specific primers [Snounou et al., 1999]) the frequencies of family-specific alleles were: 24% MAD20 only, 10% K1 only and 7% RO33-type only. Two allelic types were detected in 39% of infections whereas 19% harboured all three allelic types. For *msp2*, 39% of infections contained only 3D7/IC type, 6% only FC27 type, and 55% both types. At least two *msp1* and *msp2* genotypes were found in 77% and 87% of patients, respectively. The mean number of genotypes per patient was 2.5 (95% CI, 2.3-2.7) for *msp1* and 2.6 (95% CI, 2.4-2.8) for *msp2*, and 3.1 (95% CI, 2.8-3.3) by selecting the highest number detected for either *msp1* or *msp2*. No significant difference in diversity of infections was observed between temporary visitors (n=22, 16-47 yr, median 26.5 yr) compared with permanent residents in Chabahar (n=86, 5-65 yr, median 27 yr), nor between permanent residents who reported that they travelled prior to disease, compared with those who had not travelled.

The study provides the first estimate of the genetic diversity of *P. falciparum* infections in south-eastern Iran. Malaria transmission in this area is determined as low, however, we found a higher genetic diversity than expected which suggests that the level of transmission of *P. falciparum* may be higher than reported.
FACTORS AFFECTING *P. FALCIPARUM* DIVERSITY

**Parasite density (I, V)**

Number of *msp2* genotypes was investigated in relation to total number of the parasites, established by microscopy. In the Tanzanian population there was a correlation between high parasite densities and higher number of genotypes. A separate analysis of asymptomatic respective symptomatic *P. falciparum* infections did not found such an association within the respective groups, but parasite densities may explain differences in mean number of genotypes in the two groups. In the mesoendemic area in Mali an association between number of *msp2* genotypes and parasite densities was below the level of significance. The relationship between diversity and density was not tested in the 110 malaria patients in the low endemic area but may be possibly have partly affected the results since genotyping was performed in symptomatic patients with high parasitemias.

Previous studies reported significant correlation between parasite density and number of *msp2* genotypes in infants and young children [Smith et al., 1999a; Owusu-Agyei et al., 2002]. The impact of parasite density on number of genotypes may reflect higher sensitivity of detection but may also reflect unspecific PCR amplification, and has to be taken into consideration in the interpretation of genotyping studies.

**Age/immunity (I, V)**

In Tanzania, the age-specific distribution of the number of *P. falciparum* genotypes reflected the level of acquired immunity established for areas with high endemic of malaria [Smith et al., 1999a,b]. Diversity of *P. falciparum* infections was lowest in the immunologically most naïve age group (<3 yr) and increased with age in parallel with higher immunity (see paper I – Figure 3a). Diversity of infections in symptomatic individuals with fever and *P. falciparum* was independent of age. In the mesoendemic area in Mali with lower transmission of malaria, diversity of infections did not change significantly with age (0-15 yr).

**History of antimalarial treatment (I)**

Time to a previous antimalarial treatment was found to significantly influence the number of *P. falciparum* genotypes asymptomatic individuals although the parasite prevalence was not
markedly affected (see paper I - Figure 5). The increasing diversity of infections with increasing time to previous antimalarial treatment indicates an accumulation of parasite genotypes with time in the individual. The results may however also reflect that individuals with multiclonal infections have a lower incidence of clinical malaria.

**Exposure (I)**

Individual malaria exposure was investigated by measuring antibody levels against the CSP, major surface antigen of the infective sporozoites, in 662 plasma samples (1-84 yr, median 16 yr) in the Tanzanian population. Anti-CSP antibody levels increased with age and were higher in asymptomatic young children (1-10 yr) carrying *P. falciparum* than in children with no detected parasites. Multiple regression analysis, where adjustment was made for age, sex and clinical status at survey, found no association between anti-CSP antibody levels and number of infecting parasite genotypes, nor with the clinical status at survey. Our results are in line with studies which have reported that anti-CSP antibody levels are not associated with number of infecting *msp2* genotypes [Engelbrecht et al., 2000] nor with risk for clinical malaria [Burkot et al., 1989; Marsh et al., 1989]. Anti-CSP antibody levels have been proposed to reflect the rate of malaria transmission [Druilhe et al., 1986; Esposito et al., 1988], however, more recent investigations revealed intrinsic individual differences in the ability to produce anti-CSP antibodies [Del Giudice et al., 1987; Rosenberg and Wirtz, 1990] and lack of association with entomological inoculation rate [Burkot et al., 1989]. Although levels of anti-CSP antibodies may not be an optimal marker of malaria exposure, our results indicate that diversity of *P. falciparum* infections are influenced also by other intrinsic factors than exposure and/or that the number of infecting genotypes do not affect specific CSP antibody responses.

**IMPACT OF *P. FALCIPARUM* DIVERSITY ON MALARIA MORBIDITY**

**Clinical episodes with fever and *P. falciparum* (I)**

In order to further elucidate the role of *P. falciparum* infection diversity on malaria morbidity we assessed the risk for subsequent clinical malaria in the 320 asymptomatic children (1-16 yr) in the Nyamisati survey/Tanzanian population, by simultaneous multiple adjustment for
age, sex, number of *msp2* genotypes and malaria history in Cox regression analysis. Compared with having a single genotype, multiple *P. falciparum* genotypes as well as absence of detectable parasites were associated with reduced risk for subsequent clinical episode. The risk of subsequent episode was lowest in individuals with multiple *P. falciparum* infections i.e. 0.28 for 2-3 genotypes and 0.42 for \( \geq 4 \) genotypes, compared with those having one single genotype. The risk for PCR negative individuals, i.e. absence of detectable parasites was 0.53 (see paper I -Table 2).

In this study multiple *P. falciparum* infections were associated with reduced risk for subsequent malaria disease in concordance with previous reports in high endemic areas [al-Yaman et al., 1997b; Beck et al., 1997; Färnert et al., 1999; Smith et al., 1999b]. Earlier studies have not investigated how the history of clinical malaria and antimalarial treatment influence diversity and the prospective risk for a new clinical episode. Our results show that multiclonal infections are associated with reduced prospective risk for clinical malaria also after adjustment for malaria history, which supports that the presence of parasites *per se* is a marker of protection. Persistent low parasite density infections with multiple antigenically diverse parasites may continuously stimulate a broad range of immune responses which protect against clinical disease. The association between number of *P. falciparum* genotypes and risk of subsequent acute malaria was however not straightforward, with also parasite negative individuals being more protected than single genotype infection. These individuals have thus good antiparasitic immunity suppressing the parasitemia. Since antimalarial immunity however is not regarded to be sterilizing, also these individuals may have ongoing low grade, below detection level, infections which are not detectable. A prerequisite for antimalarial immunity may thus be controlled maintenance of low grade infections with multiple genotypes.

**Anemia (I)**

Persistent asymptomatic multiclonal *P. falciparum* infection may have a potential negative effect on hemoglobin (Hb) levels and contribute to anemia. Hemoglobin levels were therefore specifically assessed with regards to diversity of infections. Levels of Hb increased with age in the whole population. Children 1 to 10 years with clinical malaria had significantly lower Hb levels than asymptomatic children of the same age group, with mean Hb values of 96 g/l and 104 g/l, respectively \( (P=0.001) \). Parasite prevalence was associated with lower Hb in
asymptomatic adults but not in the younger age groups. In asymptomatic children 11 to 16 years the Hb levels were lower in individuals with more than three genotypes, compared to those with one genotype. However, in a multiple regression analysis, adjusted for age, sex and clinical status at survey, no association was found between number of genotypes and Hb levels in any age group. In individuals with fever and parasites levels of Hb decreased with increasing densities of the parasites (correlation coefficient -0.41) but not with number of infecting genotypes.

This study, which included only children older than one year, did not reveal any association between the diversity of asymptomatic infections and anemia. Continuous infections with multiple genotypes did thus not appear to have any adverse effects in these older children in this high endemic area which may argue against the use of IPT in older children since their protective immunity may benefit from stimulation from low grade asymptomatic infections.

**IMMUNE RESPONSES TO *P. FALCIPARUM* DIVERSITY**

**Antibody responses (II)**

Anti-*P. falciparum* (crude) IgG and IgE levels and total IgE were assessed in 700 asymptomatic individuals (1-84 yr, median 23 yr) living in a holoendemic area of Tanzania.

High levels (two highest fifths) of *P. falciparum*-specific IgE were – for the first time – found to be associated with reduced risk for subsequent clinical episodes, in all age groups. Several studies observed elevated IgE levels in severe malaria patients and indicated a pathogenic role of these antibodies [Perlmann et al., 1994, 1997], however a more recent study revealed higher IgE antibody levels in non-comatous patients compared with comatous patient [Calissano et al., 2003]. In our study, we specifically investigated antibody levels in asymptomatic individuals and found that those with the high levels of malaria-specific IgE at survey had a lower risk for subsequent disease than individuals with low antibody levels, independent of age. Further understanding of the role of IgE in malaria immunity is thus needed.

The levels of IgE and IgG antibodies, grouped into fifths of their distributions, were not linearly associated with number of *msp2* genotypes in a regression analysis where
simultaneous adjustment was made for the baseline measures, including age. In an age and total IgE-adjusted linear regression model with log-transformed anti-\textit{P. falciparum} IgE level as dependent variable, its association with number of \textit{msp}2 clones was assessed. Compared with a single \textit{msp}2 clone, no clones and more than one clone are associated with lower levels of malaria-specific IgE. The regression coefficients are: -0.07, -0.08 and –0.15 for no clones, two to three clones, and more than three clones, respectively. These findings were does opposite to those on diversity and morbidity and may rather reflect a controlled equilibrium in immune responses.

The reduced morbidity in high diversity infections could thus not be explained by total malaria-specific IgG or IgE levels. Antibodies are probably of high importance in the responses to antigenically diverse infections [Polley et al., 2005] but such specific responses were not reflected in the total \textit{P. falciparum}-(crude) antibody levels – nor in anti-CSP antibody levels (see above). We believe that mainly antigen variant specific antibody responses may be important for the ability to maintain antigenically diverse infections.

**Spleen enlargement (V)**

Spleen enlargement was more common in the Fulani and was apparent already in the youngest age groups (see paper V - Figure 1), with spleen rates in children aged 2-9 years of 75\% in the Fulani and 44\% in the Dogon, respectively (\(P<0.001\)). The number of \textit{P. falciparum} genotypes was associated with spleen enlargement in the Dogon, spleen rate being highest in individuals infected with 2-3 genotypes. The number of genotypes correlated with rate of splenomegaly but not the magnitude of enlargement, i.e. AES. In the Fulani no additional spleen enlargement, neither rate nor AES, was found with different number of genotypes. The genetic diversity of \textit{P. falciparum} infections could not explain the differences in susceptibility to malaria in these two ethnic groups. However, interesting patterns of spleen enlargement suggested a more acute response in the spleen to diverse parasites and clinical disease in the Dogon, whereas the continuously enlarged spleens in the Fulani may already be triggered to clear parasites without further responsiveness in size. The two groups thus appear to confer different patterns in coping with malaria infections.
A new method for DNA extraction was developed following repeated failures and non-consistent results of PCR-based detection of *P. falciparum* from blood spots on filter paper stored for up to 29 months in study V. In order to validate this new TE (Tris-EDTA) buffer-based DNA isolation method, a comparison was made with the two established methanol- and Chelex®- (Bio-Rad Laboratories, CA) methods. A total of thirty blood samples stored on two different filter papers, 3MM® Whatman (Brentford, UK) and 903® Schleicher & Schuell (Dassel, Germany) were analysed in duplicate with the *msp2* genotyping method. The new TE method was superior to the methanol and Chelex® methods both in sensitivity and reproducibility when performed on the two filter paper types, 3MM® Whatman and 3MM® Whatman, stored for 15 and 29 months, respectively (see paper IV - Table 1 and 2). This new method is simple, rapid and may be useful in studies with PCR amplifications from long term stored filter papers.
CONCLUSIONS

- *Plasmodium falciparum* infections are mainly composed of multiple *msp2* genotypes, reflecting diverse parasite populations, in areas of different malaria transmission.

- The level of diversity of *P. falciparum* infections in an individual is influenced by the time to previous antimalarial treatment.

- Exposure to malaria, assessed by anti-circumsporozoite protein antibody levels, is not alone affecting the number of infecting *P. falciparum* genotypes.

- Individuals, of all ages, with multiclonal *P. falciparum* infections have a reduced risk for subsequent febrile clinical malaria in high endemic areas.

- The risk for anemia is not increased in individuals with higher number of *P. falciparum* genotypes (but rather total parasite densities in symptomatic individuals).

- The differences in susceptibility to malaria in two ethnic groups in a mesoendemic area were not explained by the diversity of their asymptomatic *P. falciparum* infections but rather by their responses in spleen enlargement.

- Although antibodies are probably crucial in the immunity to malaria infection, it was not reflected in total anti-*P. falciparum* crude IgE or IgG antibody levels. High anti-*P. falciparum* IgE levels were, however, *per se* associated with reduced risk for subsequent clinical malaria in asymptomatic individuals of all ages, indicating - for the first time - that IgE antibodies may be clinically protective also in malaria.

- A new Tris-EDTA buffer-based DNA extraction method was effective for parasite-specific PCR typing from long term stored filter paper.

- The diversity of *P. falciparum* infections needs to be considered in the understanding of malaria immunity as well as in the design of effective malaria control interventions.
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