PURSUING THE FEVER TRAIL;

Pathogenesis of Blood-stage
P. falciparum malaria & Pregnancy

Niloofar Rasti
Abstract

The burden of the most virulent form of human malarias, *Plasmodium falciparum*, is concentrated among young children and pregnant women in sub-Saharan Africa. Excessive sequestration of parasite-infected red blood cells (pRBCs) in the capillary beds of different organs is the hallmark of *P. falciparum* infection contributing to pathology and severe outcomes. Adhesive interactions between parasite proteins at the pRBC surface and host receptors on vascular cell-linings or on immune cells in thought essential in this process. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the highly polymorphic *var* multi-gene family, is the principal virulence factor involved in both cytoadhesion and antigenic variation. The main goal of this thesis was to enhance our understanding of both of these processes in relation to severe disease, with a special focus on pregnancy-associated malaria.

**Paper I**

Histological evidence of placental *P. falciparum* infection was observed in 13.9% of the delivering mothers at Mulago hospital’s labour suite, in Kampala Uganda. Placental infection was associated with parity (P=0.039) and the main burden was concentrated among gravidae 1 to 3. Infection was also associated with adverse outcomes for the mother and the newborn baby with 3.3 times increased risk of maternal anaemia in all gravidities (OR: 3.3; CI: 1.6-6.9) and 200-300 grams of reduced birthweight (P=0.031).

**Paper II**

PRBCs eluted from infected placentas of a representative sub-fraction of the cases demonstrated adhesive capacity for a number of host receptors: chondroitin sulphate A (CSA), Hyaluronic acid (HA) and non-immune immunoglobulins (Igs). A majority of the isolates had a capacity to adhere to all three receptors (47%), whilst the remainder interacted with a single receptor or different combinations of two receptors. The data also implied that the same parasite subpopulation in each isolate co-binds CSA and Igs. The observed link between the two phenotypes was further established in a panel of laboratory isolates of diverse geographical backgrounds. The PfEMP1 variant, VAR2CSA, was dominantly expressed in the Ig-CSA binding isolates. Employing CHO-cells transfected with the six different domains of VAR2CSA, Ig-binding was mapped to three of the six domains. Two of the domains had also previously been reported to bind CSA. The same ligand may thus be involved in both adhesive events, which may explain the observation made in placental isolates. **Paper III**

By switching expression to new PfEMP1 variants the parasite alters its adhesive signature but at the same time evades the evolving immune responses. To understand the dynamics behind switching, *var* gene expression was systematically assessed over time. The parasites eventually converged to transcribe the same *var* gene, *var2csa*, matched by loss of PfEMP1 surface expression and host-cell binding. Albeit the high levels of spliced, full-length transcript, the relative abundance of intracellular translational product was sparse. In vivo, off-switching and translational repression may constitute one pathway, among others, coordinating PfEMP1 expression. **Paper IV**

Beside PfEMP1, the parasite exports an array of other proteins to the erythrocyte surface which may partake in host-recognition and pathology. Proteomic analysis of surface proteins is, however, a challenging task. To enable the elucidation of surface-associated pRBC proteins we developed a new workflow, combining mild surface trypsinization of live-infected erythrocytes and OFFGEL fractionation of the peptide mixtures followed by capillary LC-MS/MS analysis. Two highly abundant (GBP130, HSP70-1) and a number of low abundant proteins were identified. Further investigations are required to decipher the function of these proteins.
List of Publications

*Submitted* 2008 (under revision).


III. Bobo W. Mok, **Nilofar Rasti**, Ulf Ribacke*, Fred Kironde, Qijun Chen, Peter Nilsson, Mats Wahlgren. Default pathway of var2csa switching and translational repression in *Plasmodium falciparum*.

*Submitted* 2008.

* Shared authorship.
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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin-based combination therapy</td>
</tr>
<tr>
<td>ATS</td>
<td>Acidic terminal segment</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral malaria</td>
</tr>
<tr>
<td>CIDR</td>
<td>Cystein-rich inter domain region</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
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<tr>
<td>CSA</td>
<td>Chondroitin sulphate A</td>
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<tr>
<td>CSPG</td>
<td>Chondroitin sulphate proteoglycans</td>
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<tr>
<td>DBL</td>
<td>Duffy binding-like domain</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>HS</td>
<td>Heparan sulphate</td>
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<tr>
<td>Igs</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>IPT</td>
<td>Intermittent preventive treatment</td>
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<tr>
<td>IRS</td>
<td>Indoor residual spraying</td>
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<tr>
<td>ITN</td>
<td>Insecticide treated net</td>
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<tr>
<td>PAM</td>
<td>Pregnancy-associated malaria</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PRBC</td>
<td>Parasite-infected red blood cells</td>
</tr>
<tr>
<td>SIR</td>
<td>Silent information regulator</td>
</tr>
<tr>
<td>SMA</td>
<td>Severe malaria anaemia</td>
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<tr>
<td>SSA</td>
<td>Sub-Saharan Africa</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
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<tr>
<td>UPS</td>
<td>Upstreams promoter sequence</td>
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Part I
Background
Chapter 1

Prelude

1.1 The global health perspective

“One place for diseases to hide is among the poor, especially when the poor are socially and medically segregated from those whose deaths might be considered more important.” (Paul Farmer)

During the past century humanity has experienced major advances in global health and today, more than ever, we have the technology and know-how to ensure a life of health and well-being for each and every citizen on this globe. Since the WHO call for “Health for all by the year 2000” back in 1978 we have come a long way. However, for half the world’s population this still remains a fictitious goal and the disease burden is far from equally distributed. Whilst about six children per 1000 live births and eight mothers per 100,000 live births are dying in the healthiest developed countries, sub-Saharan Africa is losing 160 young lives and 920 mothers, per 1000 and per 100,000 live births respectively.

Mortality in children under five accounts for a major proportion of the global disease burden. Of the 60 million deaths in the world in 2004, 10.6 million were among children below five years of age. More than 90% of the child deaths occur in low-income countries or in poorer parts of middle-income countries and the vast proportion are caused by a handful of diseases: malaria, pneumonia, diarrhoeal diseases, perinatal conditions, HIV/AIDS, and measles. Alongside communicable diseases and perinatal conditions, maternal conditions represent a major contributor to the disease burden in developing countries and in sub-Saharan Africa in particular. More than 500,000 women die each year as a result of pregnancy or childbirth. Moreover, pregnancy renders women susceptible to the adverse outcomes of malaria infection, especially in combination with HIV. About 25 million pregnancies are at risk of malaria infection each year and approximately 61% of the people living with HIV/AIDS in sub-Saharan Africa are women.
Beyond the general ecology of many developing countries, the major impediment to the elimination of communicable diseases and poor child and maternal health is poverty\textsuperscript{6}. The global trend of improving health has been accompanied by a steady global economic growth. However, some developing countries were left behind and there is a widening gap between rich and poor citizens both within and between developed and developing countries\textsuperscript{7}. Poverty not only characterizes the circumstances in which communicable diseases thrive, but the cycle of poverty is exacerbated by lost productivity, missed educational opportunities, and high health-care costs for the affected and their families. Diseases such as malaria and HIV/AIDS affect those who are in the prime productive stages of life, while pneumonia, malaria and diarrhoeal diseases more often cut short the lives of children before their fifth birthday. Moreover, if one considers that of the world’s billion poorest people 60\% are women and girls\textsuperscript{8}, the relationship between poverty and gender surely represents one of the most important risk factors to be addressed in efforts to arrest communicable diseases.

### 1.2 Global burden of malaria

Malaria is today recognized as a communicable disease caused by protozoa of the genus \textit{Plasmodium} and is transmitted between humans by the female \textit{Anopheles} mosquito. Four species of \textit{Plasmodium} naturally infect humans: \textit{P. falciparum}, \textit{P. vivax}, \textit{P. ovale} and \textit{P. malariae}. Other members of the genus \textit{Plasmodium} are parasites of various species of birds, reptiles, amphibians and mammals. \textit{Plasmodium} can also be zoonotic. Recent studies in Malaysia, using molecular genetic techniques, revealed that humans can also be infected with the monkey parasite \textit{P. knowlesi}\textsuperscript{9,10}. \textit{P. falciparum} and \textit{P. vivax} are the main causes of the disease in humans. Whilst \textit{P. vivax} infections mainly contribute to the morbidity of malaria infection by acute recurrent febrile episodes and chronic anaemia, almost all mortality and severe disease manifestations (cerebral malaria, multi-organ failure, severe anaemia, pregnancy-associated malaria) occur as a result of infection with \textit{P. falciparum}. Malaria was reported endemic in 107 countries in Africa, Asia and America between 1990 and 2002\textsuperscript{11}(Figure 1A). Most estimates suggest that malaria directly causes 350-500 million clinical cases and about 1-3 million deaths each year\textsuperscript{12,13}, its main victims being children below five years of age and pregnant women.
Around 60% of the clinical cases and over 80% of the deaths occur in sub-Saharan Africa. The distribution of disease burden is partly due to the presence of unique ecological factors in tropical Africa such as the dominance of the most virulent parasite species *P. falciparum* and the most efficient vector *Anopheles gambiae*. After adjustments for parasite species and transmission level, however, the risk of death after a clinical episode of *P. falciparum* is still tenfold higher in Africa than in areas of similar endemicity in Southeast Asia and the western Pacific, reflecting the lack of basic public health services and the economical constraints in SSA (Figure 1B). The impact of malaria on a society extends far beyond the actual mortality. Evidence suggests that the disease can impair the cognitive development...
of children\textsuperscript{16}. Malaria reduces attendance at school and productivity at work. Moreover, high child mortality blocks the demographic transition to low fertility rates contributing to rapid population growth and large families which exacerbate poverty. The disease is estimated to cause an average annual reduction of 1.3\% in economic growth for countries with the highest burden\textsuperscript{17}, costing Africa US$ 12 billion each year. The result is a poverty trap where poverty and disease are mutually reinforcing (Figure 1 A-B). Because of malaria’s pervasiveness, combating malaria is also an important poverty reduction strategy.

### 1.3 Malaria throughout history

If we consider the impact of diseases on populations over time, as measured by the greatest harm to the greatest number, malaria has been the most devastating disease in history. Scientists and historians generally agree that malaria has been a significant force in human evolution and in determining the success or failure of settlement patterns and colonial ventures throughout the world\textsuperscript{18}. Malaria was probably introduced in Europe from Africa via the Nile valley and the contacts made between ancient Egypt and Greece. From the era of Imperial Rome to the Renaissance, malaria remained endemic in the southern tier with \textit{P. falciparum}\textsuperscript{18}. North of the Alpes and all the way up to Sweden \textit{P. vivax} was the major disease causing species. The last case of endemic malaria in Sweden was recorded as late as in the 1930's. For many years malaria and other murderous diseases kept Europeans from penetrating the vast African continent. The discovery of quinine, the active ingredient in cinchona (also known as Peruvian bark), by the Indians and the elucidation of its anti-malarial effects has been one of the great achievements of medical science. However, the spread of this New World remedy during the seventeenth century throughout Europe also became one of the tools that made European exploitation of Africa, and much of Asia, possible.

Owing to its unique symptomatic profile with periodic fevers, malaria can be traced to as early as the time of the first written medical accounts of the Sumerians in Mesopotamia\textsuperscript{19}, through Chinese Nei Ching (2700 B.C.)\textsuperscript{20}, the Indian Vedic writings (1600 B.C.)\textsuperscript{21} and the papyri of Egypt\textsuperscript{22}. Despite the existence of these older sources of information, the Greek Hippocrates (460-360 B.C.), also referred to as the founder of Western medicine, is credited for the first accurate description of the febrile stages of malaria accompanied by splenomegaly\textsuperscript{23}. He also noted a distinct relationship between malaria and complications in pregnancy. Because anopheline mosquitoes prefer to lay their eggs in stagnant waters, malaria typically becomes endemic in marshy areas. This association was already suspected by the time of Hippocrates who claimed that the intermittent fevers were caused by Miasma
(harmful atmosphere or influence; from Greek *miasma*, “pollution”), which put the four “humors” of the body in imbalance. The “marsh miasma” theory stood unchallenged as the etiological explanation for malaria for centuries, and the Romans eventually adopted the name *mal aria*, literally meaning “bad air”, for the disease.

It was not until 20th October 1880, that the true causative agent of malaria was discovered by Alphonse Laveran, a French military physician. When stationed in Algeria, Laveran examined a drop of blood from a soldier suffering from intermittent fever. Using a light microscope he noted crescent formed bodies (now known as the sexual form of *P. falciparum*, gametocytes) as well as mobile filaments emerging from spherical bodies (the exflagellation of the male gametocyte). He realized that these bodies were alive and that he was looking at an animal parasite, not a bacterium or a fungus. Laveran’s findings were at first not embraced by the medical community, partly because of the scientific fashion at that time claiming all diseases of infectious nature to be bacterial. Also a few years earlier Klebs and Tomasi-Crudeli had isolated “Bacillus Malariae” from rabbits injected with marsh water. And since other investigators had examined heat-fixed blood preparations, they could not observe the parasite movements as described by Laveran. It would take six years before Laveran’s findings were finally accepted. In 1886, Camillo Golgi discovered that the parasites could also reproduce asexually by multiple fission and showed that the fever coincided with erythrocyte lysis and parasite release. The true route of malaria transmission via mosquitoes would however take yet few more years to resolve. Ronald Ross, a surgeon-major in the Indian Medical Service provided the first proof in 1897 when he discovered cysts on the exterior stomach wall of mosquitoes fed on malaria patients. Before he could complete his work on human malaria Ross was posted to Calcutta, India, where the number of malaria cases was low. He thus pursued his studies on birds and could complete the life cycle of the malaria parasite by demonstrating the migration of parasites to mosquito’s salivary gland and their subsequent transmission to healthy creatures subjected to the infectious bite of the mosquito. Independently, the Italian professor Giovanni Battista Grassi together with Amico Bignami and colleagues followed the same lead as Ross and completed the life cycle of human malaria. Grassi also recognized *Anopheles* mosquito as the vector. Ross and Laveran were, however, the only two malarialogists awarded with the Nobel Prize for their findings, in 1902 and 1907 respectively.
1.4 Eradication versus control

After World War II, strenuous efforts were made to eradicate malaria. Application of DDT as part of the indoor residual spraying (IRS) programme, coupled with the effectiveness of anti-malarial treatments such as chloroquine formed the cornerstones of the WHO malaria eradication programme launched in 1955. Although these efforts were successful in many areas, they did not succeed in sub-Saharan Africa and in many parts of Asia. The optimism raised by the anti-malaria campaigns of the 1950s and 1960s ended in the 1970s as the resurgence of malaria became obvious. By the 1980s the hope that malaria could be eradicated by insecticides and drugs had thus been abandoned. Deterioration of the malaria situation, especially in Africa, may be explained by a number of factors: emergence of insecticide-resistant mosquitoes and drug-resistant malaria parasites, climate instability, civil disturbances, population movements, disintegrating health services and HIV epidemics. Although the effort has been regarded a failure, it did provide some valuable lessons: whereas eradication may not be a realistic short-term goal, sustained control is essential to the economic development and thus poverty reduction in endemic areas.

During the past years malaria has again attracted more attention with the establishment of new international initiatives such as the Roll Back Malaria Partnership (RBM), launched in 1998 and funded by a consortium of WHO, World Bank, United Nations Development Program, and United Nations Children’s Fund, with the overall aim of halving the burden of malaria by 2010. The Global Fund for AIDS, TB and Malaria (GFATM) provides hundreds of millions of dollars for malaria prevention and treatment programmes. Moreover, Medicines for Malaria Venture (MMV), a joint public-private partnership, initiated in 1999, promotes the development of new anti-malarials and drug combinations for distribution in poor countries. A number of consortia have also been established to accelerate the pre-clinical and clinical development of promising vaccine candidates, e.g. Malaria Vaccine Initiative (MVI), launched in 1999 through grants from the Bill and Melinda Gates Foundation and the European Malaria Vaccine Initiative (EMVI), mainly funded by agencies/departments under the Ministries of Foreign Affairs of Sweden (SIDA), The Netherlands (DGIS), Republic of Ireland (IA) and Denmark (DANIDA).

Owing to advances in molecular biology at the end of the twentieth century, parasitology has become an attractive and challenging area of biomedical research. Basic research in the fields of malaria biology and immunology combined with the decoding of the \textit{P. falciparum} and \textit{A. gambiae} genomes, have provided new insights into the parasite biology and raised hopes for the future development of vaccines, new drugs, insect repellants and mosquito traps. Although eradication is still far away, and likely to rely on the future development of an effective vaccine, there are
tools available to control malaria thus reducing the burden of severe disease and the loss of lives (Panel 1).

There are currently three main global initiatives which provide a framework with key principles and goals guiding the control of malaria in malaria-endemic countries (Panel 2). Among the three, the Millennium Development Goals (MDGs), adopted under the Millennium Declaration in 2000 by all the member states in the UN General assembly, constitute the principal initiative. MDGs are a set of time-bound goals/targets to be achieved by the year 2015 and were launched in an attempt to improve the global health status and to reduce poverty. Malaria control is one of the top priority targets for disease and poverty reduction in sub-Saharan Africa (Panel 2).

1.4.1 ACT

Due to widespread parasite resistance, chloroquine, the hitherto cheapest and most effective anti-malarial, had to be abandoned as the first-line treatment in many countries. Instead, many African countries adopted the use of sulphadoxine-pyrimethamine (SP) compounds. Unfortunately, there is also a growing resistance to SP and the use of combination therapies of two or more compounds with different modes of action are currently the recommended strategy to increase drug efficacy and delay resistance development. Artemisinin, a medicine derived from the sweet wormwood plant, is the most powerful drug at hand today. It has a short half-life and as yet no in vivo resistance has been recorded. Artemisinin-based combination therapies (ACT), although effective, are very expensive. By the end of the year 2004, at least 40 countries had adopted ACT as their national drug policy but these countries depend on international support to cover the high cost, which is tenfold higher than former regimens. Recent surveillance studies from Zanzibar, where ACT has been provided free of charge to all malaria patients since 2003, reported a dramatic decrease, by as much as 75%, in malaria-attributable mortality. To avoid wide-spread resistance problems adequate structures have to be in place for regular monitoring of medication efficacy. There are currently six regional drug efficacy networks in Africa supported by RBM.
1.4.2 ITN & IRS

Control measures against mosquito bites have beneficial impacts on malaria morbidity and mortality. In Africa, insecticide-treated nets reduce all-cause mortality for children below 5 years of age by 18%\textsuperscript{26}. ITNs have also recently been shown to have beneficial effects for pregnant women and their newborn babies\textsuperscript{27}. The risk of placental malaria and low birthweight is substantially reduced by ITNs and distribution to pregnant women has the added value of protecting the newborn babies during infancy, as they sleep with their mothers. ITNs are thus one of the main strategies of the RBM partnership. ITNs will only have an impact when nets are retreated and used consistently. Thus, besides the ambitions of reaching high coverage levels in endemic areas, logistics are required to ensure the re-treatment of nets on a regular basis. ITNs are thought to contribute the most in reducing infections in areas where vectors have late biting habits (\textit{A. gambiae} & \textit{A. funestus}).
Indoor residual spraying with insecticides is currently mainly recommended in areas that are epidemic-prone or have low malaria transmission. There are currently 12 insecticides recommended for IRS including DDT\textsuperscript{28}. There are concerns regarding the possible long-term toxic effects of DDT as it accumulates in the environment through food chains in tissues of exposed organisms including the residents of endemic areas. The reason for DDT still being recommended today is simply due to the lack of equally efficacious alternatives.

1.4.3 IPTp & IPTi

Whilst transmission may be prevented by the use of ITNs and IRS, disease may be prevented by prophylactic anti-malarial drugs. Studies have demonstrated that the use of intermittent preventive treatments with anti-malarials during pregnancy (IPTp) or infancy (IPTi) reduce the incidence of maternal anaemia, improve birthweights and lower malaria-attributed mortality and morbidity in children\textsuperscript{27,29,30}. Until its widespread resistance, weekly chloroquine prophylaxis was given to pregnant women in sub-Saharan Africa. The discovery that sulphadoxine-pyrimethamine administered only on two or three occasions during pregnancy was effective in preventing placental infection as compared to chemoprophylaxis with chloroquine was a breakthrough \textsuperscript{31}. IPTp with at least 2 doses of SP is currently recommended by WHO and has been introduced into national malaria control programmes of many countries in sub-Saharan Africa\textsuperscript{32,33} but levels of coverage are still modest. Furthermore, its effectiveness is being hampered by increasing levels of resistance to SP across Africa\textsuperscript{34}. Combination therapies have been suggested as a strategy to maintain the effectiveness of SP-IPTp. Three IPTp clinical trials are currently underway in Benin, Malawi and Tanzania to evaluate alternative regimens namely SP \textit{vs} mefloquine\textsuperscript{35}, SP alone \textit{vs} SP plus artesunate\textsuperscript{36} and SP alone \textit{vs} SP plus azithromycin\textsuperscript{37} respectively.

IPT with SP or amodaquine during infancy has in two previous studies in Tanzania shown to protect children\textsuperscript{29,30}. A recent study based on two parallel trials of IPTi in Tanzania and Mozambique, however, reported varying efficacy in the two settings. Whereas IPTi reduced the risk of clinical malaria with 53\% in Tanzania, no effect was observed in Mozambique\textsuperscript{38}. One plausible explanation provided in the study was the high ITN-coverage in Ifakara, Tanzania, suggesting that combination of IPTi and ITNs may constitute the most cost-effective malaria control tool.
Chapter 2

*P. falciparum* & pathophysiology

2.1 Life cycle

Malaria parasites are obligate intracellular parasites of the phylum Apicomplexa and have a complex life cycle alternating between an arthropod vector (anopheline mosquito) and a vertebrate host. The life cycle of *P. falciparum* (and other *Plasmodium* species) comprises several developmental stages in both human and mosquito hosts (Figure 2).

Following the bite of an infected female *Anopheline*, the mosquito injects saliva containing the sporozoite forms of the parasite. A small proportion of the sporozoites enter the bloodstream while most remain in the dermis. The sporozoites target and invade the liver hepatocytes within minutes and mature in the liver over
the ensuing 6 to 16 days. After their first passage through Kupffer cells, hepatocyte cell death is halted by the parasites until merosomes mature. At this stage the parasite induces cell death to release tens of thousands of merozoites into the bloodstream which is the onset of the asexual erythrocytic stage of infection. Merozoites rapidly invade circulating erythrocytes by a receptor-ligand mediated mechanism. Within the erythrocyte, the merozoite undergoes an approximately 48-hour maturation process through ring-stage to pigmented trophozoites and finally multiplies into daughter merozoites at the schizont stage. Hereby, the parasitized red blood cell (pRBC) ruptures and releases the new brood of merozoites into the circulation to resume further cycles of asexual reproduction. Symptoms accompany the rupture of erythrocytes (hence, the periodicity of malaria fevers) and severe disease and death occur solely during the erythrocytic stages of the infection.

Occasionally, some ring-stage pRBCs differentiate into sexual stages (male and female gametocytes), which are central to malaria transmission. The gametocytes may be ingested by a new feeding mosquito and initiate the sexual reproduction phase of the life cycle in the mosquito gut. Developmental time in the mosquito varies depending on ambient temperatures, but is typically between 7 and 14 days.

Upon erythrocyte invasion the parasite matures within a vacuole, parasitophorous vacuolar membrane (PVM). During the course of the 48 hour cycle the erythrocyte is extensively remodeled by the parasite in order to satisfy its need. An array of proteins involved in nutrient import, waste product export, antigenic variation, immune evasion and sequestration are exported beyond the confines of the PVM across the erythrocyte cytosol to the erythrocyte surface. For this purpose the parasite have to establish it own transport machinery. For example several membrane bound compartment, the so called Maurer’s clefts (MCs), extend or bud from the PVM into the erythrocyte cytoplasm. Mounting evidence suggest that MCs are secretory organelles established in order to route parasite proteins across the RBC cytoplasm to the surface membrane. Moreover, a conserved host-targeting signal bearing a five- amino acid core motif (RXLXE/D/Q) together with an upstream signal sequence is predicted to target \( P. falciparum \) proteins for export across the PVM 39.

### 2.2 Clinical manifestations

The majority of \( P. falciparum \) infections in endemic areas cause clinical symptoms such as fever, malaise and headache and which in some cases may resolve spontaneously, even without drug therapy. A minor proportion of all cases lead to severe disease with a range of clinical manifestations of variable severity (Panel 3). However, the greatest disease burden results from a handful of distinct syndromes: cerebral malaria (coma/convulsions), severe malarial anaemia and acute respiratory distress in young children and placental malaria in pregnant women. One or several
syndromes can be present in one subject. Hence, the disease may be accompanied by single-organ, multi-organ and/or systemic involvement. Apart from administration of anti-malarial drugs to treat the infections, organ complications in severe disease require management in their own right. The majority of deaths in hospitals occur in the first few hours before anti-malarial therapy can be expected to have any impact. Supportive therapy is thus of immediate importance until tissue and organ dysfunctions are corrected, whilst appropriate anti-malarial therapy should also be administered as promptly as possible.*

Individuals who recover from complicated infections usually have no residual disease, yet children recovering from coma/convulsions may have neurological sequelae40,41. Some may be afflicted by long-term brain damage with manifestations such as epilepsy, motor or cognitive defects41. Further studies are needed to assess the extent of these long-term effects.

2.3 Determinants of clinical manifestations

Complex interactions between the host, parasite, and mosquito vector lead to wide variability in the risk of malaria and its clinical manifestations, ranging from asymptomatic parasitaemia to severe disease. In areas of stable malaria transmission, severe malaria is usually confined to children <5 years, mainly manifesting itself as severe malarial anaemia (SMA), cerebral malaria (CM), respiratory distress or a combination thereof42-44. In contrast, in areas of unstable transmission, severe malaria may occur at all ages. Although vectorial capacity may determine the level of malaria transmission, the relationship between malaria susceptibility and age reflects

* For detailed information on supportive and anti-malaria treatment procedures please refer to WHO’s “Guidelines for the treatment of malaria”, 2006.
the natural acquisition of protective immunity in response to protracted episodes of infectious bites. Population studies on the incidence of infection and severe disease in endemic areas suggest that following the initial susceptibility of young children to severe malaria, protective immunity develops in three sequential phases: first immunity to severe malaria disease, then clinical immunity to symptomatic uncomplicated disease and finally immunity to parasitisation which limits parasite quantity within the host\textsuperscript{14,45} (Figure 3). Clinical immunity thus seems to develop with greater ease than anti-parasite immunity, which may take a life-time and is only partially efficacious. How fast and how much the immunity develops ultimately depends on the level of exposure. Moreover, anti-malarial immunity is not of the sterile type meaning that ongoing exposure to the pathogen is required to maintain immunity. Even short periods of interruption of exposure lead to loss of immunity\textsuperscript{46}. The reasons for this may be manifold including antigenic diversity and variation\textsuperscript{47,48}, redundancy in parasite invasion and cytoadherence strategies\textsuperscript{49,50}, active immunosuppression and immune-dysregulation\textsuperscript{51}.

Despite the life-long acquired clinical immunity, women become susceptible to malaria upon pregnancy\textsuperscript{52}. The major complications of infection are maternal anaemia, which in turn may cause maternal deaths, and reduced infant birthweight due to intrauterine growth retardation or premature delivery leading to excess infant

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Acquisition of natural immunity in malaria endemic areas as a function of age. Protection from severe manifestations are acquired first followed by clinical immunity to uncomplicated disease and partial anti-parasite immunity. Women become susceptible to severe malaria upon pregnancy. Susceptibility decreases with successive pregnancies. The diagram represents the pattern of immunity acquisition in high transmission areas. With decreasing transmission levels, the immunity acquisition is shifted to higher ages.}
\end{figure}
Malaria-induced low birthweight is estimated to be responsible for up to 360,000 infant deaths every year in Africa. In some areas pregnancy-associated malaria (PAM) may also cause spontaneous abortions or stillbirths. PAM may also bring about other indirect consequences such as limitations in antibody transfer to the fetus, which may result in limited protection of the new-born from other diseases e.g. measles, pneumococcal infections and tetanus. In high transmission areas, primigravidae are at greater risk of infection, whereas the gravidity effect is less marked in areas of low transmission. Younger maternal age has also been identified as an independent risk factor for PAM reflecting the fact that beside the parity-dependant immunity acquired with consecutive pregnancies, age-dependant immunity also plays an important role in controlling the infection in areas of stable transmission. Hence, the clinical outcomes of malaria during pregnancy vary with the degree of immunity women have acquired by the time they become pregnant and thus by the epidemiological setting.

While the level of acquired immunity of the individual is an important determinant for clinical manifestations, some host genetic factors may also influence the risk of an infection leading to severe disease. The best documented of these are the protection afforded against severe malaria by the heterozygous state for HbS (the sickle cell trait) and by the α+thalassemias. Many other genetic polymorphisms have been shown to affect risk of severe malaria to a lesser degree, some of these effects being inconsistent between regions. Currently multi-center studies are in progress to improve our understanding of host genetic factors that may confer resistance or susceptibility to malaria.

Co-existence of other diseases and their interaction with malaria may also influence disease severity. HIV and malaria co-exist at high intensity in many of the malaria-endemic regions. Available evidence suggests that immuno-suppression due to HIV conveys an increased risk of malaria infection, and is associated with higher circulating parasite densities. The clearest evidence for interactions between HIV and malaria has been obtained from studies of placental malaria. In pregnant women, HIV infection has been shown to increase the prevalence of peripheral and placental malaria, induce higher parasite density, more febrile illness, and more severe anaemia. Moreover, HIV infection seems to diminish the parity-specific immunity development, typically observed in multigravidae.

Setting aside all other factors, the parasite itself, armed with a repertoire of virulence factors, constitutes a critical determinant in malaria disease processes. Among the four human malaria parasite species, P. falciparum is the sole species capable of endothelial adhesion and sequestration in the deep vascular beds. It is generally agreed that this unique ability to withdraw from the peripheral circulation by adhesive events is a major contributor to the pathogenesis of severe disease.
role of parasite factors in disease initiation and progression will be further illuminated in the following chapters.

2.4 Sequestration and pathogenesis

Sequestration of parasite-infected erythrocytes in capillary beds is the hallmark of *P. falciparum* infections and is a process that occurs in all infections, including immune asymptomatic parasitaemia in immune individuals and severe illness in non-immune patients. Excessive sequestration is, however, believed to be central in the pathogenesis of severe malaria. As early as in 1884, Alphonse Laveran, discoverer of the malaria parasite, noted the relationship between organ-specific *P. falciparum* malaria syndromes (e.g. cerebral malaria, placental malaria) and the sequestered mass of parasites in the affected tissue. Later in-depth quantitative histological studies have confirmed the described relationship. The two previously proposed hypotheses on pathological mechanisms associated with sequestration are i) the mechanical obstruction theory and ii) the immunopathology theory. Although previously emphasis was placed on one or the other theory as the explanatory basis for malaria pathogenesis, there is today an understanding that the two views are not contradictory (Figure 4). Excessive parasite sequestration via adhesive interactions in processes such as rosetting and cytoadhesion are necessary for concentrating large numbers of parasites in focal sites causing mechanical blockage of blood flow and tissue hypoxia, but additional downstream events such as the local or systemic action of toxins released by the parasite on host tissue and the recruitment and intravascular infiltration of inflammatory mediators also contribute significantly to the final disease state. Appropriate immuno-regulation hence seems to be a pre-condition for healthy outcomes. The importance of immunological processes to severe malaria pathogenesis in humans is also exemplified by clear associations of genetic polymorphisms in immune loci, such as CD36, CD40L, TNF2, IFNγ, IL4 and IL12B, with altered risk of disease.

2.4.1 Toxic mediators in pathogenesis

The existence of a malaria toxin that partakes in bringing about severe disease is an attractive hypothesis that has led to the identification of *P. falciparum* glycosylphosphatidylinositol (GPI) as a candidate malaria toxin. The interaction of pathogen-associated molecular patterns (PAMPs) with host pattern recognition receptors (PRRs) is thought to control innate immune responses to infectious agents.
In the context of malaria infection, GPI seems to act as a PAMP and toxin. GPI purified from cultured parasites induces pro-inflammatory cytokine release (TNF, IL-12) from macrophages and dendritic cells and causes fever and hypoglycemia in mice. Immunization against GPI reverses susceptibility to CM, as demonstrated in a *P. berghei* ANKA mice model for severe malaria disease, where cerebral vascular occlusion by leucocytes, pulmonary oedema and acidosis, which are the disease characteristics of the CM murine model, were reversed. Acidosis and hypoglycemia have also been associated with the worse outcomes in humans with severe malaria infections. Acidosis is largely metabolic, attributable to increased lactate production by the host and parasite biomass and hypoglycemia may arise from increased glucose consumption by large parasite biomass, hepatic gluconeogenesis or may be triggered by parasite toxins such as GPI. Moreover, GPI can, directly or via the action of proinflammatory cytokines, induce the expression of inducible NO synthase (iNOS) in macrophages and vascular endothelium and cause an upregulation of cell adhesion molecules such as ICAM-1 on the surface of leucocytes and endothelial cells. This may result in positive feedback cycles with increased pRBC cytoadhesion, elevated GPI and proinflammatory cytokine and chemokine release and a cascade of enhanced activation and intravascular recruitment of immune effector and regulatory cells, leading to local vascular and organ derangement (Figure 4).

**Figure 4.** Schematic representation of mechanical and inflammatory events likely to lead to severe malaria disease. The overall disease spectrum in humans might depend on whether all or some of these processes occur.
2.4.2 Immunopathology

Cerebral malaria

Postmortem examination of individuals who die of CM reveals accumulation of mature stage pRBCs in brain capillaries and post-capillary venules\textsuperscript{66,81}. In histological analyses of murine CM but also, to a limited extent, of human CM, pRBCs have either been observed alone or accompanied by leucocytes and platelets\textsuperscript{82,83}. Case-control studies comparing non-malarial encephalopathy with CM reveal a higher abundance of sequestered monocytes and macrophages in the latter group\textsuperscript{84}. The pathological role of immune effector cells in CM pathogenesis has mainly been investigated in murine models of CM. Although the experimental models provide a valuable tool to study the dynamics of disease, the animals are not susceptible to the human-infecting \textit{P. falciparum} species. Hence, precautions should be taken in direct extrapolation of the observations to the human situation. The models are, however, still useful in providing insights on possible disease mechanisms.

IFN\textsubscript{γ}, a proinflammatory Th1 cytokine, becomes elevated very early during malaria infection and seems to be the most important cytokine in the pathogenesis of murine CM. \textit{In vivo} neutralization of IFN\textsubscript{γ} in \textit{P. berghei} ANKA-infected mice prevents CM\textsuperscript{85}, which has also been confirmed by the use of IFN\textsubscript{γ} knock-out mice\textsuperscript{86,87}. There is now considerable evidence that non-conventional lymphoid populations capable of rapid responses may be the source of elevated IFN\textsubscript{γ} levels. CD1d-restricted iNKT cells are at the interface of innate and adaptive immunity and play a pivotal role in regulating the differentiation of CD4+ T-cells into Th1 or Th2 cells. iNKT cells can be activated by GPI\textsuperscript{88} and determine cytokine levels, the pro-inflammatory cascade, pathogenesis and fatality in murine CM\textsuperscript{89}. The regulatory role of the CD1/NKT pathway relies on the differential expression of polymorphic natural killer complex (NKC) loci on NK and iNKT, controlling the Th1/Th2 cytokine production\textsuperscript{89}. BALB/c mice with a genetical bias towards Th2 or Th1 responses are resistant versus susceptible to CM\textsuperscript{90,91}, reflecting the contribution of polymorphic NKC loci. The NKC thus seems to be a genetic determinant of malaria pathogenesis, at least in murine models. There is, however, data suggesting that human pRBCs stimulate IFN\textsubscript{γ} production from γδ T-cells rather than NK cells\textsuperscript{92}.

Moreover, cytotoxic CD8+ T-cells are found in elevated numbers in brain eluates during murine CM\textsuperscript{93}, where they may induce perforin-mediated lesions in the endothelium. In humans, the risk of CM is higher in individuals with high T-cell responsiveness\textsuperscript{94}.
Placental malaria

Similar to murine and human CM, severe placental malaria is also associated with massive pRBCs sequestration combined with prominent infiltrates of monocytes and macrophages and high chemokine expression\(^{68,69,95,97}\), suggesting that adhesive pRBC phenotype and chemokine-driven cellular infiltration may be the key determinants in organ-specific disease syndromes. Dense monocyte infiltrates are commonly found in the placentas of primigravidae women in endemic areas and are associated with low birthweight caused by in-utero growth retardation\(^{98}\). Interestingly, \textit{P. vivax}, generally accepted not to sequester like \textit{P. falciparum}, may also cause PAM with low birthweights\(^{99}\), which raises questions on the nature of different parasite-induced changes leading to pathogenic outcomes.

Severe malarial anaemia

Severe malarial anaemia appears to arise principally from two processes: i) increased destruction of non-parasitized RBCs, and ii) decreased RBC production due to erythropoietic suppression. Prevailing data suggest both of these processes to be regulated by the innate and acquired immune systems.

Accelerated RBC clearance in SMA is proposed to result from acquired changes to the uninfected RBC surface and structure, such as IgG binding to non-specifically adsorbed parasite antigens\(^{100}\), reduced RBC deformability\(^{101}\), phosphatidylserine externalization\(^{102}\) and complement binding\(^{103}\), all of which may target the cells for immunologically-mediated destruction by intravascular hemolysis or reticuloendothelial (RES)-mediated clearance. RES clearance of RBCs is mainly mediated by splenic red pulp macrophages, thus an upregulation of their activity may accelerate the process\(^{104,105}\). In agreement with this theory, indications of higher macrophage activity have been found associated with SMA and phagocytosis of uninfected RBCs have been documented in humans\(^{106}\). The appearance of distended spleens in individuals with SMA is also consistent with hyperactivation of RES\(^{107}\). Data from murine SMA model have further established the above observations and suggest that SMA is regulated by factors controlling hypersplenism and splenic macrophage activation, such as CD4+ T-cells and chemokines\(^{108}\). If allowed to speculate, perhaps parasite PAMPs such as GPI are the initiating factors of this cascade and similar to placental and cerebral malaria, SMA has organ-specific features, with the spleen being the effector site of SMA.

Data from both human and murine infections also suggest a role for erythropoietic suppression in SMA\(^{70}\). Suppressed proliferation, differentiation and maturation of erythrocyte precursors is evident in murine SMA model\(^{70}\). The elevated TNF and IFN\(\gamma\) levels in the early acute phase of infection are thought to mediate erythropoietic suppression as they decrease the responsiveness of erythroid...
precursor population to erythropoietin, resulting in a decreased production of new RBCs\textsuperscript{109}, but the critical requirement for these cytokines still remains unproven. Moreover, the cellular sources and triggers for their release in SMA have yet to be deciphered. Besides the spleen, the bone-marrow thus appears to constitute an additional site of cellular inflammation during SMA.

### 2.4.3 Cellular adhesive phenomena

The observed association between clinical symptoms and sequestration of pRBCs in the deep vascular beds of various organs has led to intense investigations on adhesive interactions between pRBCs and host cells, amassing a body of evidence on host molecules and parasite ligands implicated in adhesion. Electron-microscopy examination of post-mortem tissue suggests that pRBCs directly adhere to endothelial cells\textsuperscript{110} via electron-dense knobs on the pRBC surface\textsuperscript{66,81} and consistent with the histological observations that sequestration is mainly confined to pRBCs during the later stages of the erythrocytic cycle\textsuperscript{66,111}, in vitro observations of receptor adhesion are also predominantly restricted to trophozoites and schizonts. Moreover, late-stage parasites are seldom observed in the peripheral blood of malaria infected patients\textsuperscript{112}. As the parasite matures, it initiates an extensive remodeling of the pRBC such as the expression and export of variant antigens to the cell surface that engage in host-cell interactions\textsuperscript{113}. Parasites failing to develop an adhesive phenotype are believed to be entrapped and cleared from the circulation by the spleen. Although sequestration at distal sites is an important survival strategy for the parasite, it is, as described earlier, deleterious to the host causing organ-specific pathology. The principal cellular adhesive events in asexual stages described to date are:

- **Cytoadhesion** - adhesion of pRBCs to vascular endothelial cells in various organs such as the brain, intestine, lung, liver, skin and the syncytiotrophoblast cell-lining of the placenta. Via cytoadhesion the microaerophillic parasite gains access to a relatively hypoxic environment thus improving its ability to reinvade and proliferate. This phenomenon appears to be a critical virulence factor, especially in placental malaria, but does not seem to be sufficient in bringing about other severe malaria syndromes in humans. Studies in laboratory animals have, however, demonstrated that isolates with lost adhesive phenotype only cause mild infections\textsuperscript{114}.

- **Rosetting** - the adhesion of two or more uninfected RBCs around one pRBC, or the adhesion of several pRBCs and RBCs to each other forming a “giant
Rosetting parasites are generally associated with more severe clinical disease and rosetting-disrupting antibodies seem to comprise an important part of the protective immunity as sera from adults in endemic areas can inhibit rosettes whereas children with severe disease lack anti-rosetting antibodies. The role of rosetting in vascular occlusion is not fully understood but the force of the pRBC-RBC interactions have been measured and shown to withstand flow stresses typical to those in arteries and artificial introduction of rosettes into perfused rat mesocacemum can impede the microcirculatory flow significantly. Moreover, using transmission electron microscopy, the membrane of pRBCs and the surrounding uninfected RBCs have been demonstrated to be in close association in isolates from P. coatneyi and P. fragile infected rhesus monkeys and in autopsy material from a CM patient. Other possible virulence functions of rosetting stipulated, but not further dissected, are masking of pRBCs from immune cells and increased efficiency of merozoite invasion imparted by the proximity of newly ruptured schizonts to uninfected RBCs.

- **Autoagglutination** - the adhesion of pRBCs to each other. This is a common phenomenon in clinical isolates cultured in non-immune plasma and has been associated with severe malaria in children. Rosetting and autoagglutination are not completely overlapping phenomena reflecting differences in the underlying molecular mechanisms.

- **Platelet-mediated clumping** - adhesion of pRBCs to each other mediated by platelets. Interestingly, platelets express high levels of CD36, a molecule that most P. falciparum isolates adhere to (see next section). It is noteworthy that platelets can also directly interact with activated endothelium (via CD40-CD40L interactions) which is one of the earliest events in inflammation or tissue injury. These events can perhaps explain the common clinical finding of thrombocytopenia (reduced circulating numbers of platelets) in human malaria infections.

- **Adhesion to cells of the immune system** - pRBC interaction with the cells of the immune system. Chronic infection with P. falciparum malaria leads to a severely disregulated immune system. The parasite can subvert the immune system employing mediators of immunosuppression and hyperactivation simultaneously. Immunodevation can thus occur at multiple fronts. PRBC binding to dendritic cells (DCs) have been shown to impair DC maturation thus inhibiting T-cell activation. CD36 and CD31 on the DC-surface are suggested
to interact with parasite ligands on pRBC surface or toxins released from the parasite\textsuperscript{130}. Another example is the hyperactivation of B-cells following interactions with pRBCs, as illustrated in \textit{in vitro} and animal studies\textsuperscript{131,132}. Malaria infection, in residents of endemic areas, is characterized by the presence of high titers of Igs specific for various self-antigens\textsuperscript{133,134}, a sign of non-specific polyclonal B-cell activation. Moreover parasite surface ligands exhibiting domains with non-immune Ig binding potential are capable of polyclonal B-cell activation \textit{in vitro}\textsuperscript{135-137}.

\section*{2.4.4 Host receptors in adhesive events}

\subsection*{Cytoadhesion}

An array of \textit{in vitro}, \textit{ex vivo} and \textit{in vivo} studies have led to the identification of a heterogeneous repertoire of receptors, expressed on vascular endothelial or placental syncytiotrophoblast cell-linings, capable of specific interactions with late-stage pRBCs. For many of the receptors the precise binding site has been mapped to specific protein domains harbored by different variants of \textit{P. falciparum erythrocyte membrane protein 1} (PfEMP1), a pRBC surface-exposed parasite ligand encoded by the highly polymorphic \textit{var} multi-gene family. Although adhesion to some of the receptors appears to be correlated with severe malaria (e.g. CSA, HS, non-immune Igs and HA), the relative importance of most interactions in malaria pathogenesis \textit{in vivo} is still largely unclear.

\subsection*{A. Endothelial cytoadhesion}

- **CD36**- is an 88 kDa surface glycoprotein with broad endothelial distribution. It is also expressed on platelets, monocytes and dendritic cells. It is absent from placenta and sparsely expressed in cerebral blood vessels\textsuperscript{138}. CD36 was one of the first endothelial receptors proposed to be involved in pRBC cytoadhesion\textsuperscript{139,140}. The majority of wild isolates analyzed to date, except for placental isolates, can bind CD36\textsuperscript{141-143} and the binding has been shown to be stable under \textit{in vitro} flow conditions\textsuperscript{144}. However, no association has been found between the CD36 binding capacity of the isolates and severe disease manifestations\textsuperscript{119,143,145}.

- **CD31/PECAM1**- is a 130 kDa protein and a member of the immunoglobulin superfamily. The receptor is expressed on the surface of platelets, monocytes, neutrophils and dendritic cells and can mediate binding between endothelial cells and
pRBCs\textsuperscript{146}. CD31 is normally confined to tight junctions between endothelial cells and thus absent from the luminal side where pRBCs would potentially bind. The receptor is, however, up-regulated and redistributed to the luminal face of the endothelium upon IFN\textgreek{g} stimulation. Pre-treatment with IFN\textgreek{g} has been demonstrated to yield a dramatic increase in pRBC binding to human umbilical vein endothelial cells (HUVEC)\textsuperscript{146,147}. The receptor is also commonly recognized by clinical isolates\textsuperscript{119}, but has not been associated with any specific syndrome.

- **TSP**- Thrombospondin is a 450 kDa extracellular matrix glycoprotein which is secreted by endothelial cells, platelets and monocytes. TSP can specifically interact with pRBCs\textsuperscript{148} but the binding appears to be of low affinity character and unstable under flow conditions\textsuperscript{144}. However, TSP can also interact with heparin and CD36 and may perhaps act as a co-receptor promoting firm interactions between pRBCs and CD36 or heparin/heparansulphate. As for CD36, most isolates analyzed to date adhere to TSP but no correlation to specific disease syndromes has been discerned\textsuperscript{148}.

- **ICAM1**- or CD54 is a 80 - 115 kDa member of the Ig superfamily expressed on endothelial cells and is upregulated by cytokine stimulation and interactions with pRBCs\textsuperscript{149-151}. Whilst the receptor can support binding to pRBCs in static \textit{in vitro} assays, it cannot support stable pRBC binding under flow conditions and has been suggested to act in synergy with CD36, supporting the initial rolling phase of pRBC attachment\textsuperscript{152,153}. One autopsy study has suggested a role for ICAM1 in cerebral malaria\textsuperscript{138}. However, whereas a large study in Kenya documented a tendency between ICAM1 binding and clinical disease\textsuperscript{143}, a study in Malawian children reported a negative correlation\textsuperscript{145}. This is believed to reflect the receptor polymorphisms prevailing in the different populations\textsuperscript{154,155}.

- **HS**- Heparan sulphate is a 10 - 70 kDa heparin-like glycosaminoglycan (GAG) which has been ascribed a role in both cytoadhesion and rosetting events\textsuperscript{156-158}. HS is produced by all cells, although different tissues show distinct molecular characteristics i.e. sulphation and epimersation level\textsuperscript{159,161}. Both laboratory and wild isolates from children with clinical disease can specifically interact with HS expressed on endothelial cells\textsuperscript{156}.

**B. Syncytiotrophoblasts & cytoadhesion**

- **CSA**- Chondroitin sulphate A, which constitutes the polysaccharide part of chondroitin sulfate proteoglycans (CSPGs), is a 450 kDa GAG capable of pRBC
adhesion under flow conditions\textsuperscript{162}. Placental isolates commonly bind CSA but not CD36 whereas non-placental isolates rarely bind CSA\textsuperscript{163-165} (paper II). Placental isolates are thus proposed to be functionally distinct from other isolates. Several CSPG types are present in the placenta but not all are equally important for pRBC adhesion. Placental pRBCs preferentially interact with low-sulphated CSPGs, which are predominantly expressed in the intervillous spaces of the placenta and the adhesion relies on the occurrence of 4-sulphated disaccharide clusters\textsuperscript{166-168}. A recent study has also demonstrated a substantial increase in the level of low-sulphated CSPGs in \textit{P. falciparum}-infected term-placentas from Cameroonian women as compared to uninfected placentas\textsuperscript{169}. Elevated CSA levels induced by the parasite may \textit{in vivo} result in a positive feedback cycle exacerbating pRBC adherence and placental pathology. Moreover, over successive pregnancies, women acquire antibodies that block binding to CSA. Both antibody responses and cellular responses to CSA-binding laboratory isolates have been reported to increase in a parity-dependant manner, which in addition has been linked to improved clinical outcomes such as increased birthweight and maternal haemoglobin levels\textsuperscript{170-173}.

- **HA-** hyaluronic acid is the only GAG that is not negatively charged due to the lack of sulphation. HA has been reported as a typical receptor for placental isolates with a majority of the isolates having dual binding specificity for HA and CSA\textsuperscript{174} (Paper I & II). The expression of HA in placentas is however a matter of controversy, with a recent study reporting a lack of HA in the placenta after removing umbilical cord tissue\textsuperscript{169}, whereas numerous earlier studies have reported HA to be expressed on the placental lining\textsuperscript{175-177}. HA is also expressed on endothelial cells\textsuperscript{178} and may thus act as a sequestration receptor in other organs. HA adhesion is, however, a rare phenotype among parasite isolates from children with mild or severe malaria and if present, the level of adhesion level is much lower than that of other receptors (CD36, ICAM1)\textsuperscript{174}. Of note, pRBC binding to HA appears to be shear-dependent and high adhesion levels occur at shear stresses lower than those existing in post-capillary venules\textsuperscript{128}. The blood flow in the placenta is much slower than in the rest of the body, which may constitute a valid explanation for the preferential binding of placental isolates to HA.

- **None-immune Igs-** The important role of Igs from malaria-naïve human serum was at first illustrated by the presence of human Igs in fibrillar strands connecting infected and uninfected RBCs\textsuperscript{124}. Binding of non-immune IgM and to a certain degree IgG on the pRBC surface has been reported to be a common phenotype of children’s isolates and associated with severe disease\textsuperscript{119,179} and have mainly been implicated in isolates with rosetting phenotype (please refer to the next section
“rosetting and receptors” under the subheading “serum proteins”). However, our recent work on parasites eluted from the placentas of Ugandan women illustrates that non-immune Ig-binding is, in addition to CSA and HA binding, also a feature of placental isolates (paper I & II). Of note, rosetting has not been reported as a prominent phenotype of placental isolates\textsuperscript{(180)} (Paper II). Ig-binding may thus, in addition to its role in rosetting, promote cytoadhesion\textsuperscript{(181)} (paper II). Non-immune Igs, in particular IgG, may bridge pRBCs to IgG-binding receptors on the syncytiotrophoblast cell-lining\textsuperscript{(182)}. More discussion on the subject will follow in the “Results & Discussion” chapter.

**Rosetting and receptors**

As in cytoadhesion, a number of receptors are implicated in rosette formation. In contrast to cytoadhesion, however, rosetting has repeatedly been found to be associated with severe disease. Both receptors on uninfected RBCs and human serum factors seem to be required for rosetting.

- **HS**- in addition to its presence on endothelial cells, HS has also been identified on the surface of uninfected RBCs\textsuperscript{(157)}. HS can block rosetting in both laboratory and clinical isolates, although not all isolates are HS-sensitive. Rosetting and heparin binding phenotypes have also been correlated with severe malaria in several studies\textsuperscript{(119,158,183-186)}.

- **CR1**- complement receptor 1 or CD35 is present in varying numbers on erythrocytes and leukocytes. The observation that erythrocytes from CR1 deficient donors failed to form rosettes in a study involving a number of rosetting laboratory parasites illuminated for the first time the importance of this receptor in rosetting\textsuperscript{(187)}. The finding has further been confirmed by the use of soluble CR1 or monoclonal antibody against CR1 which was shown to disrupt rosettes in both laboratory and clinical isolates\textsuperscript{(188)}. Moreover, polymorphisms in CR1 which confer reduced rosetting, were found to be prevalent in malaria endemic areas of Papua New Guinea and associated with protection from severe disease\textsuperscript{(189)}.

- **ABO**- blood group antigens are another group of glycans present on erythrocytes implicated in rosetting. Both laboratory and clinical parasite isolates display enhanced rosetting when cultured in erythrocytes of blood group A or B (but not O)\textsuperscript{(185,190)}. The trisaccharide of blood group antigens A and B but not the disaccharide of blood group O can disrupt rosettes formed in corresponding blood\textsuperscript{(185,190)}. Increased rosetting has been reported in isolates from patients with
blood groups A/B/AB relative to those with blood group O and blood group A has also been correlated with severe malaria manifestations\textsuperscript{191-193}. Of note, blood group O is more common than blood group A among Africans\textsuperscript{194} and has in a recent study been shown to protect against severe malaria via the mechanism of reduced rosetting\textsuperscript{195}.

- **Serum proteins**- fibrinogen, albumin and non-immune Igs, in particular IgM, have all been reported to support rosette formation\textsuperscript{119,124,196-200}. Binding of non-immune Igs on the pRBC surface has been reported to be a common phenotype of children’s isolates and associated with severe disease\textsuperscript{119,179} and the ability of anti-human immunoglobulin serum to inhibit rosetting in a range of clinical isolates pinpoints the importance of Igs in natural infections\textsuperscript{188}. Despite a suggested role for serum factors other than Igs in rosetting, it is still not clear whether they interact directly with the pRBC surface via adhesion to surface exposed parasite ligands or only function as secondary factors increasing the propensity of cell aggregation\textsuperscript{201}. At least in one study Ig-mediated rosetting has been demonstrated to require complementary serum factors\textsuperscript{199}.

*In vivo* microvascular heterogeneity in receptor expression probably contributes to differential homing of pRBCs throughout the body and under physiological flow conditions, it is more likely that organ-specific pRBC sequestration, mediated by the rosetting and cytoadhesion events, involves multiple host-receptors acting in a cooperative fashion. After the primary establishment in a distal site, the parasite can also induce local changes in receptor expression which can result in a positive feedback cycle with increased adhesion and excessive pRBC sequestration and pathology. Indeed, a number of studies have illustrated that pRBCs from clinical isolates have the ability to interact with various combinations of receptors\textsuperscript{19,174,202} (Paper II), which in some cases have been related to different clinical outcomes and recent findings further support the notion that parasites may induce elevated receptor levels at the sequestration site\textsuperscript{169}. To summarize, receptor preference and adhesive capacity of the parasite and the accompanying immunopathological events as well as the genetic predisposition and immunological status of the host are all important interactive factors, decisive for the final clinical outcome.
2.5 *P. falciparum* in the postgenomic era

The entire genome sequence of the first *P. falciparum* parasite clone (3D7) was completed in 2002 and the complete genome sequences of yet other *P. falciparum* strains (HB3, Dd2, IT, Ghanaian isolate) are under way (Broad Institute of Harvard & MIT; Wellcome Trust Sanger Institute). In addition, we now have access to an array of genome sequences originating from additional human and non-human *Plasmodium* species allowing for comparative genomics analyses to improve our understanding of the evolution of genome organization and gene functions across and within species (Wellcome Trust Sanger Institute). Combined with data from a number of genome-wide microarray expression studies and mass-spectrometry based proteomic studies across the various *P. falciparum* life cycle stages we now possess a comprehensive set of tools to dissect the parasite biology. Data analysis is further facilitated via the availability of an integrated database (www.plasmoDB.org) which compiles data on apicomplexan parasites and provides tools for bioinformatic exploration of the emerging genomic and functional genomic datasets.

The haploid genome of the *P. falciparum* clone 3D7 has an approximate size of 22.8 mega bases. The nuclear genome comprises fourteen chromosomes harboring at least 5400 genes. According to comparative genomic analyses about 85 percent of the predicted *P. falciparum* genes have orthologs in at least one of the rodent *Plasmodium* parasites, with an overwhelming majority being located in the central chromosomal regions. Inter-species conservation breaks down at the chromosomal ends where predominantly species-specific genes are clustered. Many of these subtelomeric genes participate in host-parasite interactions e.g. the polymorphic *var* multi-gene family in *P. falciparum*, which encodes variants of the pRBC surface exposed parasite ligand PfEMP1.

2.5.1 PfEMP1- the polymorphic adhesin

Mounting evidence supports the notion that PfEMP1s constitute the principal adhesive and antigenic ligands on the pRBC surface. Whilst surface exposure of PfEMP1 licenses the parasite for adhesive interactions, it also renders it vulnerable to antibody recognition. To evade the host immune responses the parasite thus undergoes clonal antigenic variation by switching expression to a different PfEMP1, hence ensuring the chronicity of infection (for discussions on the mechanisms behind antigenic variation please refer to section 2.6).

PfEMP1 was first discovered in the 1980s by radio-iodination of viable pRBCs followed by electrophoretic analysis of the labeled surface proteins. The size of the
protein (>200 kDa) was found to vary between parasite strains and the protein could be immunoprecipitated in a strain-specific manner and was thus termed variant surface antigens (VSAs)\textsuperscript{205}. The presence of VSAs on the pRBC surface was also demonstrated to correlate with the ability of pRBCs to adhere to host cells\textsuperscript{206}. Early on, it was also evident that PfEMP1 is a target of protective immune responses and the acquired immunity developed in response to protracted infections with pRBCs expressing different PfEMP1 variant types\textsuperscript{207,208}. Many studies since have discerned the role of this protein family in pathogenesis and induction of protective immunity.

### 2.5.2 Var gene organization and adhesive traits

The overall organization of var genes appears similar between the three \textit{P. falciparum} isolates 3D7, HB3 and IT4, despite their distinct geographical backgrounds, representing Africa, Central America and Southeast Asia respectively\textsuperscript{209}. Each \textit{P. falciparum} haploid genome encompasses approximately 50-60 different var genes. In 3D7 var genes are distributed across the fourteen chromosomes, with two thirds clustered in the subtelomeric regions (Figure 5), along with members of two further multigene families, \textit{rif} and \textit{stevor}. All var genes share a basic two-exon structure. The first exon encodes the hypervariable extracellular portion, whilst the second smaller exon, following a transmembrane domain, serves as a conserved cytoplasmic tail encoding a 450-500 amino acid long acidic terminal segment (ATS) (Figure 5). The adhesive function of PfEMP1s resides in the first exon which comprises an N-terminal segment (NTS) and a number of Duffy-binding like (DBL) and cystein-rich inter-domain regions (CIDR)\textsuperscript{210}. Despite the related basic architecture, different PfEMP1 species may differ in sequence, domain composition and host-receptor binding properties. Between the three \textit{P. falciparum} isolates 3D7, HB3 and IT4, most PfEMP1s have overall amino acid identities of less than 50 percent in individual domains. The only exceptions are the three isolate-transcendent vars (var1csa, var2csa and type-3-var), with identities greater than 75 percent across multiple domains.

Superimposed on the organization described above, var genes can, based on similarities in the 5′promoter regions, be classified into four major “upstreams sequence (Ups) groups” (UpsA, UpsB, UpsC, UpsE), which are linked to specific chromosomal locations and transcriptional orientations\textsuperscript{209} (Figure 5). This overall conservation in Ups promoter regions indicates some evolutionary pressure and has been proposed to restrict recombination between limited subset of var genes\textsuperscript{211}. The physical clustering of \textit{P. falciparum} telomeres in the nuclear periphery, forming four to seven clusters of chromosome ends, may promote recombination between vars with
the same promoter type and gene orientation. The three atypical var genes, for example the UpsE-linked var2csa, share little sequence identity with other vars and supposedly undergo self-self recombination. Hence, although the var repertoire may be diversified via high rates of meiotic and/or mitotic recombination rates, the conserved promoter hierarchy is a reflection of possible recombinational and/or host selection pressures. The notion of separately recombining var groups would make sense from the parasite perspective as it would allow the parasite to establish in a sequestration site whilst exhibiting new adhesion combinations. To fully appreciate the functional significance of recombination hierarchies for adhesive traits and disease outcomes, comprehensive datasets on expressed var types, in clinically and phenotypically well-defined isolates of diverse geographical origin, are needed. So far, accumulating data from a number of recent studies are in favor of the proposed hypothesis. UpsA vars are for instance more related to each other than to other var genes and all encode non-CD36-binding CIDR domains. UpsA vars have instead been implicated in rosetting, which is commonly associated with severe syndromes\textsuperscript{212,213} and so far, available data implies that predominantly transcription of UpsA vars is correlated with severe malaria in children and in non-pregnant adults\textsuperscript{212,214,215}. UpsB and UpsC vars mainly harbor CD36-binding CIDR domains and CD36-binding is, as discussed earlier, a prevalent phenotype in mild infections. The transcription of UpsC vars was indeed in a recent study found to predominate in children with asymptomatic infections\textsuperscript{216}. UpsB vars consist of a more divergent group of genes with a subset located close to UpsA vars in the subtelomers and the other subset located more centrally with UpsC var genes. In a recent study the expression of both UpsA and UpsB vars was correlated with severe malaria cases in Tanzanian children\textsuperscript{217}.

The idea of recombination hierarchies and adhesive traits is currently perhaps best exemplified by the isolate transcendent UpsE-linked var2csa which is implicated in pregnancy-associated malaria\textsuperscript{218}. Isolates expressing var2csa interact with CSA but not CD36 which is one of the distinctive placental adhesion phenotypes (see section 2.4.4) (Figure 5). Var2csa does not harbor the classical DBL\(\alpha\) and CIDR domains and besides possessing an exclusive promoter (UpsE), it also harbors a unique upstream open reading frame (uORF) 5’ of the promoter region\textsuperscript{219,220}, which has been suggested to participate in translational regulation\textsuperscript{221}. Of note, an orthologue of var2csa is present in the chimpanzee malaria parasite P. reichnovi which presumably diverged from P. falciparum about 5 to 7 million years ago\textsuperscript{220,222}. The ancient nature of this gene implies that it may be under specific selection to be maintained in the parasite population.
Figure 5. A) Chromosomal distribution of var genes. B) Var gene classification according to upstream promoter type, subtelomeric/internal chromosomal location and transcription direction is denoted. C) General domain organization of PFE1P1s. Receptor binding mapped to specific domains and possible involvement in cytoadhesive events are illustrated. D) The unique structure of VAR2CSA, the PFE1P1 implicated in pregnancy-associated malaria and receptors mapped to various domains are outlined.
2.6 Antigenic variation

Pathogens such as the unicellular protozoans *Plasmodium* and African trypanosome, the bacteria Borrelia sp. and Neisseria sp., the human immunodeficiency virus (HIV) and the fungi Candida sp., have all employed antigenic variation as a key survival strategy. Equipped with this ability infectious agents can avoid the evolving immune responses, establish persistent infections and maximize their chances of being transmitted to a new host. In general, the process of antigenic variation involves the variable expression of genes encoding immunodominant surface antigens and most often the same genes actively partake in the virulence of disease hence linking antigenic variation to pathogenicity.

The *P. falciparum* genome harbors an extensive repertoire of multi-copy, hypervariable gene families, var, rif, stevor and Pfmc-2TM genes, all of which could potentially be involved in antigenic variation. However, except for var genes, the exact role of the other gene families for the pathology of disease still remains elusive. In each clonal parasite population, periodical transcriptional switches between different members of the var gene family alter both the antigenic and functional signature of the parasite ensuring that only a small portion of the antigenic repertoire is exposed to the host at any given time. The repeated switches in gene expression pattern underlie the waves of parasitaemia typical of chronic *P. falciparum* infections. What induces the switching process in the first place is still not fully comprehended. Whilst spontaneous switching has been observed *in vitro* in the absence of immunological pressure, data from several *in vivo* studies suggest that the immune system, in particular the spleen and antibodies, may constitute one of many possible pathways modulating antigenic variation (Figure 6).

![Figure 6. Suggested switching pathways of var genes & their PfEMP1.](image-url)
2.5.1 Control of var gene transcription and switching

Given the relatively limited var repertoire (≈60) in any haploid clonal parasite population, the rate of antigenic variation is a balancing act for the parasite. It has to be sufficient to maintain infection in face of the mounting immune responses but must be tightly regulated in order to avoid exhaustion of the repertoire before the parasite is ready for transmission via a new mosquito bite to the next host.

The control of var gene expression is complex and incorporates several molecular processes: temporal regulation of gene expression during the intraerythrocytic cycle, mutually exclusive expression of only one var variant per pRBC and transcriptional switch to a new var variant in the ensuing erythrocytic cycles.

Within each 48-hour cell cycle, one full-length var transcript is dominantly transcribed from early ring stages reaching a transcriptional peak at around 12-16 hours post invasion. The corresponding PfEMP1 protein is translated and exported to the pRBC surface at the trophozoite stages. The temporal transcriptional pattern is controlled at the level of transcription initiation. One var variant, var1csa, is exceptional in that it is constitutively transcribed in all parasites across the erythrocytic cycle, and thus falls outside the controls of mutually exclusive gene expression. Whilst, var1csa is transcribed as a truncated pseudogene in 3D7, it is not known whether it encodes a surface exposed protein in other parasite isolates and its role in antigenic variation remains elusive.

The regulatory mechanisms behind mutually exclusive gene expression are still poorly understood. Accumulating data, however, indicate that multiple layers of control are involved in var gene expression with an evident role for epigenetic mechanisms, including non-coding genetic elements, repositioning of var loci in subnuclear compartments and chromatin modifications.

Non coding DNA elements and var gene expression

Unlike the variant surface glycoproteins of Trypanosoma brucei that are activated by rearrangement or gene conversion into a restricted number of expression sites, var genes are activated in situ. Each gene has been shown to harbor two promoters, one upstream of exon 1 (Ups A-E) responsible for the mRNA expression and a second one in the intron generating non-coding or “sterile” RNA. While a single var gene is transcribed from the upstream promoter and the reminder of the var repertoire is basically silent, most of the intron promoters seem to be active simultaneously. Efforts in understanding var gene silencing and mutual expression and the interactions of these promoters were recently facilitated using transfection technology, where parasites were transfected with plasmid constructs harboring 5′
promoters or introns of various \textit{var} genes driving the expression of drug resistance markers\textsuperscript{231,232}. These studies illustrated the cooperative nature of upstream and intronic \textit{var} sequences where the active \textit{var} gene was silenced by the addition of a \textit{var} intron. The silencing effect of the intron was however not observed until after the parasites had passed through the S-phase of the cell cycle, which is a feature of gene regulation based on modifications in the chromatin structure.

**Role of perinuclear location in \textit{var} gene regulation**

Regardless of chromosomal location or transcriptional states, all \textit{var} genes are physically located at the nuclear periphery, usually in four to seven telomeric clusters\textsuperscript{233}. Employing fluorescence in situ hybridization (FISH) and electron microscopy it was recently shown that whilst a silenced \textit{var} gene primarily co-localizes with the telomeres within the perinuclear heterochromatin, upon activation the gene dissociates away from the telomeric clusters to subnuclear locations that are transcriptionally competent\textsuperscript{234,235}. The data suggests that once such site is occupied by the active \textit{var}, transcription at other \textit{var} loci cannot occur. Although plausible, perinuclear repositioning does not provide a universal explanation for mutually exclusive regulation of \textit{var} genes. Any active \textit{var} gene is linked to silent \textit{var} genes, in some cases within several kilobase pairs. Hence, additional layers of control would need to operate to prevent the upregulation of adjacent \textit{var} genes.

**Chromatin structure**

Gene expression can be influenced by histone modifications (acetylation, phosphorylation, methylation, ubiquitination) which alter the chromatin context of the gene by affecting DNA accessibility or recruiting other proteins to the site\textsuperscript{236}. While there is a paucity in transcription factors in the \textit{P. falciparum} genome, an array of genes involved in chromatin modification and assembly has been identified\textsuperscript{237} and histone hypoacetylation has been found to be correlated with \textit{var} gene silencing. In yeast, transcriptional repression is mediated by the histone deacetylase, Silent Information Regulators (SIR), which are necessary for the assembly and maintenance of silent telomeric chromosomes confined to the nuclear periphery\textsuperscript{238}. Recently, a \textit{P. falciparum} homologue of the yeast SIR2 (PfSIR2) was found to be associated with silent \textit{var} 5' promoters of UpsE and B type but not UpsC\textsuperscript{239}. This finding was further complemented by knock-out studies of the PfSir2 gene which resulted in the activation of a subset of subtelomeric \textit{var} genes (UpsA and UpsE)\textsuperscript{234}, suggesting that the UpsB-\textit{vars} are subjected to additional layer of silencing. Both PfSir2 and perinuclear repositioning seem to be confined to subtelomeric \textit{var}s. Internally located UpsC-\textit{vars} may thus require alternative regulatory mechanisms, such as interactions with different nuclear factors\textsuperscript{232}. 
Chapter 3

The context- Uganda

3.1 Country profile & health status

Uganda is located in Eastern Africa at the Equator and inhabited by a population of 29.4 million people (2008 census). Life expectancy at birth is around 50 years and with a population growth of about 3.2%, Uganda has one of the fastest growing populations in the world. The country is ranked as number 154 among the 177 nations of the world with an estimated GDP per capita of US$300. According to the World Bank, poverty declined rapidly in Uganda from 1992 to 2006 as a result of high and broad-based economic growth, largely owing to macroeconomic adjustment and structural reform programs. Nevertheless, Uganda remains one of the poorest countries in the world with unequal distribution of welfare gains across regions, sectors and social/economic groups. Rural populations and Ugandans living in the northern and eastern part of the country are the most afflicted groups. Uganda’s health status is characterized by a high level of disease burden predominated by infectious diseases (malaria, pneumonia, HIV, diarrhoea), prenatal and maternal conditions. Despite the improving trends in the recent past, health indicators such as maternal, prenatal and infant mortality rates remain unacceptably high: 435 in 100,000; 29 in 1000 and 76 in 1000 live births respectively.

† Represents the human development index (HDI) which looks beyond GDP to a broader definition of well-being. The HDI provides a composite measure of three dimensions of human development: living a long and healthy life (measured by life expectancy), being educated (measured by adult literacy & enrollment at the primary, secondary and tertiary level) and having a decent standard of living (measured by purchasing power parity (ppp) income).
3.2 The malaria situation

In Uganda, stable *P. falciparum* transmission occurs in 95% of the country. The lowland plains of the northern and eastern parts of the country have very high transmission, whereas transmission is moderate in the central areas around the capital Kampala. The highland area of southwestern Uganda has a low transmission level and is prone to epidemics. Transmission rates range everything from 4 infective bites per person per year in the southwestern parts of the country to >1500 in the swampy areas of the north (Figure 7)\(^{243}\). These values cover the wide spectrum of transmission intensity observed across all of Africa.

Malaria is the leading cause of attendance at health facilities accounting for 30-50% of all outpatient attendances in health units and around 35% of hospital admissions\(^{244}\). An estimated 70-100,000 deaths occur per year among children below five years of age and 10 to 12 million clinical cases are treated in public health systems alone\(^{244}\). It is however highly probable that these numbers underestimate the true burden of malaria. Owing to health system constraints, a high proportion of
fever cases may be managed at home and many children may suffer or die from malaria-related causes without any prior contact with the formal health care system. Moreover, only 42% of pregnant women in Uganda make the required four antenatal clinic visits during pregnancy and fewer than 40% deliver at health units. Early access and adherence to IPT is thus low, with fewer than 40 percent receiving the recommended 2 doses of SP. Care-seeking most often includes self-medication with anti-malarial drugs from drug shops or use of herbs; visiting a health unit may be a last resort if the illness does not improve. Apart from discouraging care-seeking behavior and limited health system resources, Uganda is in parallel experiencing a widespread resistance to the commonly used anti-malarials which is mirrored by the progressive increase in malaria-related mortality ratio in all ages from 20.2% in 1988 to 32.1% in 2004. To address the overwhelming burden of malaria, Uganda’s Ministry of Health (MOH) has formulated a “Malaria control strategic plan” which is based on principles and aims of the global Roll Back Malaria partnership, the Abuja Declaration of African Heads of State and the Millennium Development Goals (Panel 2). The progress made between year 2000 and 2006 is outlined:

<table>
<thead>
<tr>
<th></th>
<th>2000/2001</th>
<th>2006</th>
</tr>
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<tbody>
<tr>
<td>% households with mosquito net</td>
<td>12.8</td>
<td>34.3</td>
</tr>
<tr>
<td>% households with ITN</td>
<td>-</td>
<td>15.9</td>
</tr>
<tr>
<td>% children &lt;5 sleeping under ITN</td>
<td>0</td>
<td>9.7</td>
</tr>
<tr>
<td>% children &lt;5 with fever receiving anti-malarials within 24 hours</td>
<td>7.3</td>
<td>10</td>
</tr>
<tr>
<td>% pregnant women receiving 2 doses of IPT</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>% pregnant women sleeping under ITN</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Major achievements in Uganda’s fight against malaria during recent years include the development of control policies, enhanced monitoring of anti-malaria drug efficacy and an increase in mosquito net coverage. The coverage and use of ITNs are however still unacceptably low among children and pregnant women as is the use of IPT during pregnancy. The success of control programs should ultimately be assessed by measurements of disease burden and clinical outcomes among populations at risk, as opposed to the simplistic notion of amount of resources expended and level of coverage. Moreover, there is a need to consider the effects of such interventions in relation to the transmission intensity of the area since it is known that disease patterns vary with varying transmission and thus the level of acquired clinical immunity. In Uganda, as in the rest of Africa, there is a paucity of
robust baseline data on malaria burden, especially in pregnancy and from urban areas. Sub-Saharan Africa currently has the highest rate of urbanization in the developing world. In the year 2000, 38% of Africa’s 784 million inhabitants were urban dwellers and an increase to 55% is expected by the year 2030\(^{247}\). Urban areas are usually characterized by low transmission intensity with focal mosquito larval breeding sites and variable parasite prevalence rates\(^{248}\). Moreover, studies have demonstrated malaria incidence to be centered in micro-environments in these areas. Given the existing infrastructure and accessibility to health care facilities, urban settings provide a unique opportunity for integrated and cost-effective malaria control strategies. A better understanding of the underlying epidemiology of malaria in each setting is however required to maximize the impact of control measures. Hence, efforts to assess the malaria burden in densely populated urban settings should constitute a future priority.
Part II
The Investigation
Chapter 4

Scope of the thesis

1) To investigate the burden of pregnancy-associated malaria at Uganda’s national referral hospital, Mulago, an urban area of lower malaria transmission.

2) To assess the adhesive nature of parasites sequestered in the placentas of Ugandan women directly upon elution, with specific focus on adhesion capacities for CSA, HA, non-immune Igs and a combination thereof.

3) To study the dynamics of var gene switching in vitro and phenotypic changes induced by switching.

4) To exploit the possibility of a direct proteomic approach for elucidation of surface exposed protein moieties on live-infected erythrocytes.
Chapter 5

Results & Discussion

Since protective immunity to clinical malaria is gradually acquired by increasing age, severe *P. falciparum* malaria is predominantly a childhood disease in endemic areas. There is however one exception to this general rule: pregnancy-associated malaria (PAM), which is detrimental both to the mother and the fetus. It has been speculated that the increased susceptibility of pregnant women to infection, despite the previously acquired immunity, is a consequence of maternal immuno-suppression to protect against fetal rejection\textsuperscript{249}. However, such immuno-modulation mainly affects the cellular arm of immunity, whereas humoral immunity, which seems to be an important component of protective malarial immunity, is unaffected\textsuperscript{250} and the notion that PAM mainly afflicts women of low parity indicates the specific absence of protective immunity to a particular subset of parasites which is acquired with increasing parity (Figure 3). Moreover, the selective sequestration of pRBCs in the placenta and the fact that pregnancy-associated parasitaemia is usually resolved after the expulsion of placenta post-delivery\textsuperscript{251}, supports the hypothesis that PAM is caused by functionally and immunologically unique subset of parasites. This hypothesis is further strengthened by a number of key observations made during past years: i) the finding that placental pRBCs have a distinct adhesion phenotype. They commonly bind CSA, a receptor rarely exploited by non-PAM parasites\textsuperscript{163,164} (Paper II) ii) over successive pregnancies women acquire antibodies that can block pRBC binding to CSA\textsuperscript{170} iii) the antibody responses to both placental and CSA-binding isolates have been reported to increase in a parity-dependant manner and seem to be directed to a subset of surface exposed variant surface antigens unique to PAM (VSA\textsubscript{PAM})\textsuperscript{171,252} iv) elevated levels of anti-VSA\textsubscript{PAM} IgG have also been associated with improved clinical outcomes such as increased birthweight and maternal haemoglobin level\textsuperscript{172}. Taken together the aforementioned observations are generally accepted as the explanatory basis for why and how pregnancy allows a subset of parasites to circumvent the previously acquired immunity and why susceptibility to PAM depends on parity.
5.1 Paper I

Burden of malaria in pregnancy at Mulago hospital, Uganda’s national referral hospital. The data presented in paper I and partly in paper II originate from a hospital-based exploratory cross-sectional study conducted at Mulago hospital in Kampala to investigate the burden of malaria in pregnancy. The investigation was, in part, prompted by the urgent lack of baseline data from the country’s national referral hospital but also by the paucity of such information from urban settings of low transmission in general (Paper I). The study also provided a unique platform to compile data on the adhesive nature of sequestered pRBCs in placenta in a previously unexplored epidemiological setting (Paper II).

Between October 2004 and January 2005, a total of 399 women attending the labor suite at Mulago hospital were recruited consecutively to the study based on a number of inclusion/exclusion criteria. A questionnaire was used to record pregnancy history and additional background information of relevance (see paper I, Material & methods). Peripheral and placental blood was analyzed for *P. falciparum* infection by blood film microscopy. Placental infection was in parallel assessed by histological examination of paraffin-embedded biopsies deriving from the maternal facing side of the term-placentas.

We recorded histological evidence of *P. falciparum* infection in 13.9% of the placenta. Histological assessments have repeatedly proven more sensitive in providing estimates of the burden of PAM and have the additional value of providing detailed pathological data. Infections were histologically graded into four categories: no infection, acute, chronic or past infection, based on the presence/absence of pRBCs, monocyte infiltrates and hemozoin deposits in fibrin. Whilst acute infection predominated (64.1%), only 3.8% of the cases were classified as being chronic. The classification is believed to reflect the natural progression of infection and immunity and have been linked to various pregnancy-outcomes. Unfortunately, in basically all previous studies conducted in Uganda, only peripheral blood parasitaemia have been assessed and used as the principal indicator for PAM, which may have provided a partial picture of the disease burden. Since individuals in endemic areas can harbor circulating parasites asymptomatically, the mere presence of peripheral parasites is not a proof of their involvement in placental infection and adverse outcomes.

The majority of cases were concentrated among gravidae 1 through 3. Active infections (parasites present) predominated among primigravidae, whilst past infections (no parasites, only hemozoin in fibrin deposits) were more prevalent among gravidae 2 and 3. The observed pattern indicates a faster clearance of placental pRBCs in the high parity groups which probably mirrors the acquisition of
protective immunity upon consecutive pregnancies to the distinct subset of PAM parasites. However, in contrast to previous reports from high transmission areas, the difference between primigravidae and the higher parity groups was not as marked, which could be a reflection of the lower transmission level in this setting but may also reflect a confounding effect of HIV.

Both maternal anaemia (Hb<11 g/dL) and reduced birthweight were found to be associated with placental infection. Anaemia was associated with active placental infection and irrespective of gravidity infected women were 3.3 times more at risk of anaemia. One positive aspect was that basically all of the anaemic cases were of moderate nature. Severe anaemia (Hb<7 g/dL) was very rare, with an overall prevalence of 0.8 per cent in the studied population.

Women with placental malaria delivered on average 200-300 g lighter babies, however, we found no correlation to low birthweight (birthweight < 2500 g). After data stratification for age and parity status the mean birthweight of babies born to infected mothers tended to be lower in, in particular, young primigravidae but the differences were no longer statistically significant. Exhaustive advanced analysis to exploit the interactive nature of all factors for the outcome measure was, however, hampered by the limited sample size.

The worst birthweight outcomes e.g. LBW have previously been associated with active chronic infections, which are characterized by both pRBC and monocyte infiltrations of the placental intervillous spaces. The lack of association between LBW and placental infection in our study may thus be explained by the fact that only a minor proportion of the actively infected placentas were chronically infected. The majority of the women had thus contracted the infection close to delivery. This may, again, reflect the lower transmission level prevailing in the area. Urban populations have also better access to treatments and preventive measures which might prevent the establishment of stubborn chronic infections. Nevertheless, a reduction of 200-300 g in birthweight still constitutes an adverse pregnancy outcome.

A study from Malawi has previously reported that malaria contracted late during pregnancy is associated with pre-term delivery (<37 gestational weeks) and chronic malaria with in-utero growth retardation. We did observe a significantly shorter mean gestational age in mothers with acute placental infections (38 weeks, IQR: 37-39) as compared to non-infected ones (39 weeks, IQR: 38-40) (P=0.004) (see Paper II, supplementary results), we could, however, not find an association with pre-term birth. The total prevalence of pre-term deliveries in the study population was quite low (3.1%).

One of the limitations of the study was the small sample size. As no previous baseline data was available it was difficult to perform sample size estimations at the planning stage of the study. We overestimated the prevalence of infection in our calculations which limited the extent of our data analysis. In the future it would be
interesting to assess the contributing role of additional parameters such as HIV, which has been shown to compromise malaria immunity and essentially eliminate the typical gravidity-pattern of malaria risk in pregnancy.

5.2 Paper II

Cooperative receptor-adhesion phenotype of Ugandan placental isolates. Although CSA-binding has repeatedly been identified as the discriminatory adhesion phenotype of placental isolates, a few studies have also proposed an additional role for other receptors such as HA and non-immune Ig. Prior to the initiation of the study in Paper II, no attempts had been made to investigate the cooperative role of all the proposed receptors in parallel in fresh placental isolates. Laboratory isolates and clinical isolates from children had, however, been reported to have multiple adhesive capacity. We thus reasoned that placental parasites may also have adopted additional adhesive mechanisms. At delivery, women can present with one or many parasite genotypes in the infected placenta, indicating that they may be exposed to parasite variants of differential binding phenotype during the course of the pregnancy.

PRBCs sequestered in the placentas of Ugandan women (n=24) delivering at Mulago hospital were eluted post-delivery and analyzed immediately for their receptor-adhesion profile. Binding to IgG, HA, CSA and a combination thereof was assessed employing ex vivo placental adhesion/inhibition assays. Fresh cryosections originating from an uninfected Swedish term-placenta were used for this purpose. PRBCs from each isolate were allowed to adhere to the sections in the presence or absence of inhibitory soluble receptors (IgG, CSA). The HA-binding ability of the isolates was assessed by enzymatic removal of the receptor subset from the sections prior to pRBC binding.

The placental isolates were found capable of interacting with all three receptors. Specific interactions of varying degree to IgG, CSA and HA was observed in 65, 82, and 73 per cent of the isolates respectively, indicating the presence of overlapping/mixed receptor-binding pRBC sub-populations per isolate. Six different consensus adhesion profiles were delineated with a majority of the isolates exhibiting adhesive capacity for all the three receptors (47%). The remainder of the isolates interacted with different combinations of two receptors or a single one. Experiments were also performed with soluble inhibitors (IgG, CSA) and enzymatic treatment (HA) in combinations. The approach revealed that the main bulk of the pRBCs in the multi-adhesive isolates (HA+CSA+IgG) contributed to the observed adhesion profile. Another interesting finding was that in a majority of the isolates
preincubation of pRBCs with IgG and CSA in combination did not yield higher inhibition levels as compared to adhesion profiling for each receptor in isolation, suggesting that the same subset of parasites harbors dual receptor binding capacity.

Binding of the placental isolates to non-immune IgG and IgM were further investigated using live surface immunofluorescence assays with antibodies directed against human Ig, IgM and IgG. All possible combinations of binding profiles were recorded: IgG (19%), IgM (24%), IgG+IgM (29%) and Ig- negative (29%).

Placental isolates can thus interact with other receptors than CSA. Whilst, low-sulphated CSA may allow for pRBC selection and accumulation in the placenta, additional adhesive interactions could ensure the firm binding of parasites on site or provide alternative adhesion pathways if the tissue tropism changes over time, pregnancy is indeed a long process of constant changes. Questions that warrant further investigations are: do surface-bound immunglobulins interact with Ig-binding receptors on the syncytiotrophoblast surface, which receptor(s) in that case? Apart from the mechanistic notion of adhesion, does the receptor-ligand interaction exert any other downstreams effects? A recent study demonstrated that pRBCs selected for binding to cultured syncytiotrophoblast induced tyrosine phosphorylation in the syncytiotrophoblast. The use of such dynamic systems to explore the consequences of cytoadhesion on the function of syncytiotrophoblast constitute important future tools and has the ability to provide a deeper insight on host-parasite crosstalks.

**The link between CSA and non-immune Ig adhesion phenotypes.**

To dissect further the potential correlation between the CSA versus non-immune Ig-binding phenotypes, a panel of laboratory isolates with distinct geographical backgrounds were chosen for analysis. Each isolate was enriched for CSA adhesion. Whilst the non-CSA binding parental-lines were similarly recognized by serum IgG of multigravidae women and men in endemic areas, the CSA-binding sub-lines were differentially recognized in a sex-specific manner, hence justifying the use of these parasites as models for CSA-binding PAM isolates. We performed non-immune IgG and IgM profiling of each set of CSA-selected and unselected parasite-lines and observed a striking pattern. Upon CSA selection a concomitant increase of Ig-binding was observed in all the tested isolates, hence endorsing our observations on the co-binding of Ig and CSA in fresh placental isolates.

Having established a correlation between the two phenotypes, we now turned our attention to the potential parasite surface ligand involved in these interactions. As a vast number of studies have established the central role of PfEMP1 variants in pRBC adhesive events and since one species of PfEMP1 is expressed and displayed at the pRBC surface at any given time, it seemed reasonable to suggest that the link
between the two phenotypes may be mediated by the same surface ligand. Accumulating data also suggested that a unique var2CSA PFEEMP1 variant was dominantly transcribed in PAM and CSA-binding isolates, which was also the case with some of the CSA–binding isolates used in our phenotyping study. Moreover, CSA binding had been mapped to specific domains of the VAR2CSA protein. Encouraged by the data, we decided to investigate the Ig-binding potential of various VAR2CSA domains. Stable transfectants expressing the various domains of VAR2CSA on the surface of Chinese Hamster Ovary cells (CHO-745) were used for Ig-binding profiling by FACS. We identified three potential domains for IgM (DBL2X, DBL5e and DBL6e) and two for IgG (DBL2X, DBL6e) binding. Two of the domains (DBL2X, DBL6e), had also previously been demonstrated to interact with CSA.

5.3 Paper III

The dynamics of var gene switching. Var genes and PFEEMP1s have a central role in pathogenesis and immunity development. During the recent years we have gained critical insights into some aspects of var gene regulation (see section 2.6). But our understanding of the dynamics of the switching phenomenon is poor. How does the parasite manage to coordinate the expression of its variable antigens to ensure its establishment in the host? What is the initiating factor for a switch in expression from one variant to another? Does the parasite have an inherited switching pattern? Then how do we reconcile with the notion of an exclusive association of var2csa with pregnancy isolates? Does it rely on a selection mechanism where minute subpopulations of circulating var2csa expressing parasites gain access to an optimal growth niche and expand clonally or is a switch induced in var expression from for example a CD36-binding var to the CSA-binding var? A number of possible general switching pathways have previously been suggested for P. falciparum (see figure 6). Studies on splenectomized humans and monkeys have previously suggested a possible role for the spleen in coordinating the antigen expression and parasites in vitro have been shown to undergo spontaneous switching.

To increase our understanding on var gene switching we performed a systematic analysis of the transcriptional profile of var genes over time in two phenotypically distinct 3D7 parasite clones, exploiting quantitative real-time RT-PCR and a set of primers covering the var gene repertoire of 3D7 parasite. The interesting outcome of this study was the finding that the parasites eventually converged to transcribe var2csa. To assess the populational homogeneity of the late generation parasites an
array of single cell clones were generated from one of the \textit{var2csa} expressing parasites. Sixteen out of the 17 generated clones showed dominant transcription of \textit{var2csa}. Using primers covering the intronic region of \textit{var2csa} we confirmed that the transcript was spliced and northern blots confirmed the full-length feature of the transcript. Whilst the early generation parasites had clear adhesive phenotypes (CD36 binding versus rosetting), the late generation \textit{var2csa} transcribing clones had no adhesive phenotype. At least binding to CSA had been the expected outcome. We thus investigated whether the parasites expressed PfEMP1 on the surface of the pRBCs using a panel of sera from malaria endemic areas. Whilst the early generation adhesive parasites were similarly recognized by IgG from both the male and multigravidae female sera, no surface recognition was observed in the late generation parasites. To further confirm the absence of \textit{var2csa} PfEMP1 on the pRBC surface, we used specific set of sera generated in rabbits against different domains of VAR2CSA. But none of the parasites displayed surface recognition by the specific sera. We thus decided to investigate whether there is any intracellular expression of \textit{var2csa} and were hit by yet another striking pattern. Despite the presence of abundant \textit{var2csa} transcripts, the intracellular protein production was sparse suggesting the involvement of post-transcriptional mechanisms. In \textit{Plasmodium}, translational repression has previously only been reported as a regulatory mechanism for transcripts of \textit{P. berghei} gametocyte origin.

What are the \textit{in vivo} implications of our findings? Off-switching as observed here could occur \textit{in vivo} allowing for a temporal expansion of parasites which will eventually be removed by the spleen, whilst a minute subpopulation of other variants are allowed to subsist. The finding of \textit{var2csa} transcription as such is intriguing and raises the possibility that \textit{in vivo}, parasites may switch to transcribe \textit{var2csa} even in non-pregnant individuals. In fact, there are studies reporting on the occasional transcription of \textit{var2csa} in children with malaria and in experimentally infected humans, although the transcription is low\textsuperscript{217,262,263}. Is it possible that \textit{in vivo} \textit{var2csa} is transcribed but translationally repressed in non-pregnant individuals but the suppression is released upon pregnancy by placenta specific factors/conditions? Future studies will have to resolve whether the hypothesis holds and whether translational repression is a feature of \textit{var2csa} solely. Apart from a unique promoter (UpsE), the unique upstream open reading frame (uORF) 5’ of the promoter region of \textit{var2csa} could perhaps participate in translational regulation\textsuperscript{221}. 
5.4 Paper IV

The challenge of developing a direct approach to decipher surface exposed pRBC proteins. Due to their fundamental role in host immune responses and pathogenesis, surface proteins such as the well-known PfEMP1s are potential drug and vaccine targets. However, studies aiming at direct characterization of membrane-associated proteins are often challenging owing to the low abundance and poor solubility of these proteins. Rapid advances made in mass spectrometry and the completion of the \emph{P. falciparum} genome enabled the first assessments of global protein expression profiles in \emph{P. falciparum} life cycle stages in the year 2002\textsuperscript{264,265}. These studies provided the malaria community with valuable catalogs of expressed proteins. However, since less abundant proteins in highly complex mixtures are likely to escape detection, a number of subsequent targeted studies have attempted the exploration of proteome subsections including pRBC surface proteins\textsuperscript{266-268}. Previous work has mainly relied on the sequential extraction of membrane proteins with detergents followed by tryptic digestion, separation of the peptides by liquid-chromatography and tandem mass spectrometric analysis (LC-MS/MS) of the eluting peptides.

In paper IV, we set out to develop a more direct strategy by performing surface trypsinization of live-infected erythrocytes. Two 3D7 parasite clones (3D7S8.4 & 3D7AH1S2) with distinct phenotypes were used for this purpose. Our work was at first hampered by the high abundance of hemoglobin peptides which masked the detection of low abundant parasite proteins. We thus included an additional separation step where tryptic peptide mixtures were fractionated by an in-solution isoelectric focusing approach (OFFGEL fractionation) prior to LC-MS/MS analysis (see figure 1 in Paper IV). In 3D7AH1S2 a total of 29 spectra distributed between 19 peptides, identified 14 proteins and in 3D7S8.4 a total of 19 spectra distributed between 12 peptides identified 8 proteins (Table 1 & 2 in paper IV). Although a number of more abundant and less abundant proteins were identified, no members of the variable low abundant PfEMP1 family were detected. One of the abundant proteins identified was the glycophorin-binding protein 130 (GBP130). Specific sera were raised against one of the recurrent GBP130 peptides, as detected by LC-MS/MS. Localization studies were performed on pRBCs using the antisera in immunofluorescence assays. The protein was found to be abundantly expressed in late-stage trophozoites and schizonts, and was localized to the parasite, the RBC cytoplasm and the RBC membrane (figure 2 in paper IV). The protein was also occasionally associated with the surface of uninfected RBCs juxtapositioned with late-stage trophozoites. The possibility is raised that the protein may be shed from the trophozoite infected RBCs. Previous studies have indeed identified GBP130 in
culture supernatants and a few studies have shown that the protein or peptides thereof are capable of interactions with receptors on uninfected RBCs. The role of the protein, however, remains elusive. Does it function as a decoy to distract the immune system? The protein is ubiquitously expressed and appears to be antigenically conserved (similar molecular weight) in all the isolates tested (see figure 2, Paper IV) and it is restricted to the \textit{P. falciparum} species.

In summary, the inclusion of the pI-fractionation step proved to be a good strategy for unmasking some of the low abundant parasite proteins from the abundant hemoglobin peptides but surface exclusiveness of the identified proteins could not be ascertained, as a few peptides belonging to non-surface exposed proteins were also identified. The approach is still in its infancy and cannot stand on its own. Confirmatory studies are thus required to elucidate the surface association of the identified peptides.
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