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Parasite Virulence and Disease Severity in *Plasmodium falciparum* Malaria

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ABSTRACT

Malaria stands out as one of the most important infectious diseases and one of the world's leading causes of death. *Plasmodium falciparum* is the parasite responsible for the great majority of severe disease syndromes and mortality, and affects mainly children and pregnant women. Despite intensive research efforts, the understanding of *P. falciparum* virulence is limited. Infections with the parasite cause everything from asymptomatic parasitemia to severe disease and death, and the reasons for the different disease outcomes are not fully understood. Here we approached this issue by comparing several molecular aspects of parasites with different phenotypic traits as well as clinical isolates from children with severe and uncomplicated disease. Doing so, we first identified a substantial number of gene duplications and deletions in parasite genomes from all over the world. The genes found variable in copy numbers encode molecules with a wide variety of functions, and some of these were shown to have a direct effect on the parasites chances of survival. Apart from suggesting that the parasite regularly duplicate and delete genes to adapt to environmental changes, this also indicates that gene duplications and deletions could render parasites more or less virulent. To increase the understanding of how the virulence-associated adhesion of *P. falciparum* infected erythrocytes to endothelial cells and uninfected erythrocytes is achieved and regulated, we investigated the entire transcriptomes of parasites with distinct adhesive phenotypes. In a maze of transcriptional differences, receptor preferences for certain PfEMP1 proteins could be elucidated, as well as candidate genes for explorations of new molecules possibly involved in adhesive events or regulation of adhesion mediating proteins. The *var* genes encoding the original PfEMP1 in these parasites were subsequently shown to switch off when the parasites were cultivated continuously *in vitro*. Instead, another *var* gene was turned on, accompanied by low levels of expressed protein and with loss of the adhesive phenotypes. Apart from signifying that a constant selection or immunological stimuli is needed to maintain adhesive traits in *P. falciparum*, the results also suggested that exposure and adhesion mediated by the maternal malaria associated VAR2CSA protein is regulated on a post-transcriptional level. The gene encoding this protein had previously been reported duplicated in one particular parasite. Using a sensitive allelic discriminative approach we showed that these two gene copies were simultaneously transcribed in single parasites. This contradicts the principle of mutually exclusive expression of *var* genes in *P. falciparum*, and adds another layer of complexity upon the understanding of antigenic variation. To identify potentially underlying differences in parasites causing different disease outcomes we also analyzed *var* gene transcription in clinical isolates from children with severe and uncomplicated malaria. Using a novel analysis approach, we identified small degenerate amino-acid motifs that were over-represented in parasites causing severe disease and in parasites with high rosetting rates. Multiplication rates were analyzed for the same isolates and revealed a higher multiplication potential among severe disease causing parasites. The ability to multiply was also shown to correlate to the rosetting rates of the parasites, and was decreased when rosettes were disrupted with various reagents, suggesting rosetting to facilitate merozoite invasion of erythrocytes. In conclusion, we have identified specific parasite differences that besides increasing the understanding of virulence mechanisms in *P. falciparum* also present potential candidates for future intervention strategies.

LIST OF PUBLICATIONS

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

I. **Ribacke U**, Mok BW[*], Wirta V[*], Normark J, Lundeberg J, Kironde F, Egwang TG, Nilsson P and Wahlgren M. Genome wide gene amplifications and deletions in *Plasmodium falciparum*.
Mol Biochem Parasitol 2007, 155;33-44

II. Mok BW, **Ribacke U**, Winter G, Yip BH, Tan C-H, Fernandez V, Chen Q, Nilsson P and Wahlgren M. Comparative transcriptomal analysis of isogenic *Plasmodium falciparum* clones of distinct antigenic and adhesive phenotypes.
Mol Biochem Parasitol 2007, 151;184-192

III. Mok BW, **Ribacke U**[*], Rasti N[*], Kironde F, Chen Q, Nilsson P and Wahlgren M. Default pathway of *var2csa* switching and translational repression in *Plasmodium falciparum*.
PLoS ONE 2008, 3(4);e1982

IV. Brolin K[*], **Ribacke U**[*], Nilsson S, Ankarklev J, Moll K, Wahlgren M and Chen Q. Allelic discrimination of sequence variable gene copies in the haploid genome of *Plasmodium falciparum.*
Manuscript

V. Normark J, Nilsson D, **Ribacke U**, Winter G, Moll K, Wheelock CE, Bayarugaba J, Kironde F, Egwang TG, Chen Q, Andersson B and Wahlgren M. PfEMP1-DBL1α amino acid motifs in severe disease states of *Plasmodium falciparum* malaria.
Proc Natl Acad Sci U S A 2007, 104;15835-15840

VI. **Ribacke U**, Moll K, Normark J[*], Vogt AM[*], Chen Q, Flaberg E, Szekely L, Hultenby K, Egwang TG and Wahlgren M. Merozoite invasion in *Plasmodium falciparum* malaria is facilitated by PfEMP1 mediated rosetting.
Manuscript

[*] These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
ATS	Acidic terminal segment
CGH	Comparative genomic hybridization
CNP	Copy number polymorphism
CIDR	Cysteine rich interdomain region
CM	Cerebral malaria
CR1	Complement receptor 1
CSA	Chondroitin sulphate A
DBL	Duffy binding like protein or domain
GAG	Glycosaminoglycan
GPI	Glycosylphosphatidylinositol
HA	Hyaluronic acid
HS	Heparan sulphate
ICAM-1	Inter cellular adhesion molecule 1
IDC	Intraerythrocytic developmental cycle
KAHRP	Knob-associated histidine rich protein
LT	Lymphotoxin
MESA	Mature parasite infected erythrocyte surface antigen
NCAM	Neural cell adhesion molecule
NTS	N-terminal segment
PECAM-1	Platelet endothelial cell adhesion molecule 1
PEXEL	*Plasmodium* export element
PFA	Paraformaldehyde
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfEMP3	*Plasmodium falciparum* erythrocyte membrane protein 3
PfRh	*Plasmodium falciparum* reticulocyte binding protein homologue
RBC	Red blood cell
RIFIN	Repetitive interspersed protein
STEVOR	Subtelomeric variable open reading frame protein
SURFIN	Surface associated interspersed protein
TNF	Tumor necrosis factor
TSP	Thrombospondin
VCAM-1	Vascular cell adhesion molecule 1
VTS	Vacuolar transport signal

1 INTRODUCTION

1.1 THE GLOBAL BURDEN OF MALARIA

With an estimated 247 million cases and close to one million deaths annually (1), malaria stands out as one of the most important infectious diseases and one of the world's leading causes of death. Approximately half of the world's population, concentrated to relatively few countries and regions, is at risk of acquiring the disease. The burden is greatest on the African continent, and vulnerability is highest among children and pregnant women, which portraits the malaria parasite as especially cruel. Other afflicted populations are refugees, migrant workers and non-immune travelers. As with many other plagues affecting humans, malaria imposes most suffering and sorrow on populations in monetarily poor parts of the world. It is obvious that the burden of malaria extends well beyond morbidity and mortality, as the disease closely correlates to economic underdevelopment and paucity of life maintaining resources in endemic countries (2). Just as poverty can prevent efficient strategies for treatment, prevention and eradication of malaria, malaria itself hampers social and economical development through charging tolls of high socioeconomic costs. Examples are medical costs, loss of production and income, negative impact on trade, foreign investment and tourism as well as negative impact on education through absence from school and reduced cognitive development and learning ability (3). As a striking example of this vicious circle is the epidemiological picture of how the geographical distribution of malaria has changed over the years, with successful eradication in wealthier countries while obstinately remaining present in the developing world (Figure 1).

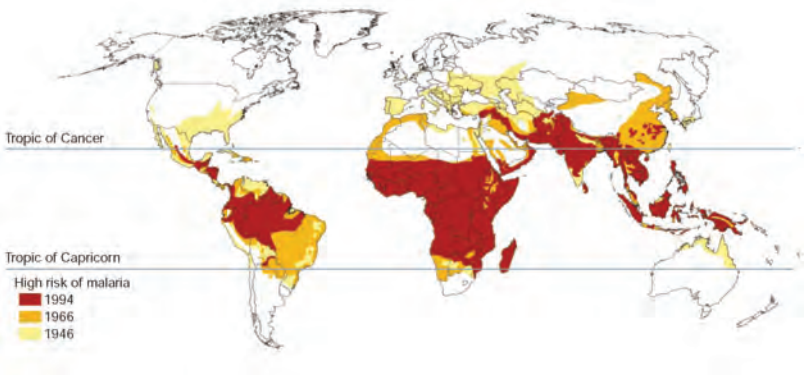

Figure 1. The change in global distribution of malaria risk from 1946 to 1994.
(Adopted from Sachs et al., 2002 and published with permission from Nature Publishing Group)

Even though the malaria situation is still alarming, recent progress has been made with relatively simple and inexpensive means to improve the situation in endemic countries (1). The progress can be attributed to better provision of antimalarials, insecticide sprays and treated bed nets in conjunction with an increased economical strength in

Asia and more resolute efforts invested by the global community. Yet, despite the recent progress, it is unlikely that malaria can be eradicated without the invention of novel diagnostics and treatment regimens. The parasites that cause the disease quickly acquire resistance to antimalarials, the transmitting mosquitoes develop resistance to insecticides and it is problematic to maintain good treatment compliance in poor countries. This opts for development and use of long lasting effective treatments such as vaccines, long lasting enough to eventually free afflicted populations from the heavy burden of malaria.

1.2 THE *PLASMODIUM* PARASITES

Malaria is caused by unicellular, eukaryotic protozoan parasites of the *Plasmodium* genus. The possession of an apical complex, a cone-shaped structure containing the machinery needed for host cell invasion, places the *Plasmodium* parasites in the Apicomplexan phylum in company with other protozoan parasites of human importance. Over a hundred distinct *Plasmodium* species, infecting a wide range of definite hosts (reptiles, birds and mammals) have been identified. Out of these, five have been shown infective to man. *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* have for long been known as causative agents of human malaria. The fifth, *P. knowlesi*, historically thought to only infect macaques, have recently attracted profound attention since proven to also infect and cause disease in man (4-9).

1.2.1 Life cycle of *Plasmodium* species

The life cycle of all *Plasmodium* parasites is of great complexity. A wide variety of morphologically and physiologically distinct stages, involving both sexual and asexual forms, are presented to its insect vector and vertebrate host. The human host is infected when female *Anopheles* mosquitoes bite and gorge themselves on blood, at the same time introducing *Plasmodium* sporozoites to the host circulation. Ejection of sporozoites from the salivary glands occurs while the mosquito probes the skin for blood. The sporozoites are consequently deposited in the dermis, from where they actively migrate to the circulation, which brings them to the liver. Recent studies, performed in rodents, have established that sporozoites can reside in the skin for a considerable period of time before they reach a blood vessel and enter the circulation (10-15). Not all sporozoites make it to the blood and liver. Instead, some remain in the skin and some enter the lymphatic circulation and reach the lymph nodes where they are degraded (15, 16). The sporozoites that successfully reach the blood circulation rapidly flow to the liver where they invade hepatocytes after traversing the Kupfer cell lining of the sinusoids (17-19). Each sporozoite differentiates and divides into thousands of merozoites, which are believed to be released from the infected hepatocytes as merosomes containing hundreds of cells surrounded by host cell membrane (20-22). Merozoites released into the circulation invade erythrocytes, and thereby initiates the intraerythrocytic development cycle (IDC), the part of the life cycle during which clinical manifestations are observed in the host. During the development in the erythrocyte (24-72 hours depending on *Plasmodium* specie) the parasite develops

from a ring stage to a mature pigmented trophozoite before mitotic nuclear division in the schizont stage. After complete schizogony new merozoites are released, an egress dependent on orchestrated release of an arsenal of proteases (23) that ruptures the erythrocyte. Released merozoites in turn invade new erythrocytes to initiate a new IDC, leading to an exponential increase in parasite load for every cycle. This exponential increase will continue either until the human host succumbs to the disease or the parasite is controlled by the host immune system or chemotherapy.

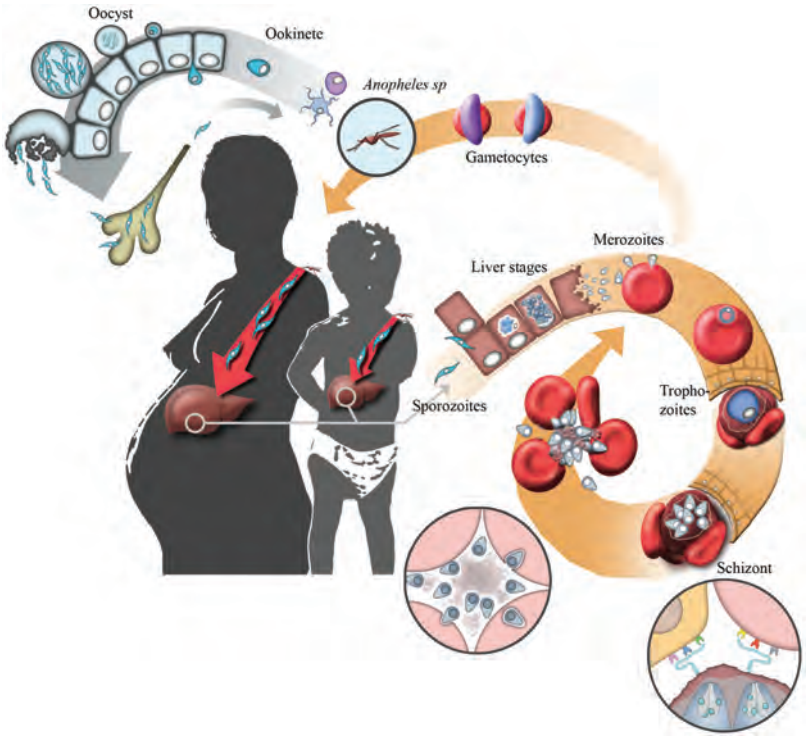

Figure 2. The *Plasmodium falciparum* life cycle.
(Published by courtesy of Dr. Johan Normark)

The *Plasmodium* life cycle continues through a developmental switch into sexual gametocytes. The timing and mechanism behinds this switch is not fully understood and has been suggested to occur at various times in the IDC (24, 25) involving various inducing factors (26, 27). Gametocytes can survive for prolonged periods of time in the circulation, believed in part to be due to immune evasion through sequestration, until ingested by a feeding mosquito. In the mosquito, both the male and female gametocytes develop into gametes before the male microgametes fertilize the female macrogametes. Resulting motile ookinetes penetrate the mosquito midgut wall, and when outside they encyst in bodies known as oocysts. Yet again does the parasite turn asexual, expands mitotically into thousands of sporozoites, which upon rupture of the oocyst migrate to the salivary glands where they become infective (28, 29). When the mosquito feeds again, sporozoites are injected into the next host to complete the transmission cycle.

1.3 DISEASE CHARACTERISTICS

Out of the five species of *Plasmodium* infective to man, *P. falciparum* is almost exclusively the one causing severe malaria disease, the form, which is attributable to almost all severe symptoms and mortality. *P. ovale*, *P. malariae*, *P. vivax* and *P knowlesi* cause mainly benign malaria, even though exceptions have been reported for all, but in particular the two latter (5, 30, 31).

1.3.1 General clinical manifestations

Infections by all five species share many common clinical features. The symptoms and pathology are mainly restricted to the stage of infection when parasites have reached the blood circulation (8-15 days post infection), invade the red blood cells and propagate in the IDC. Uncomplicated malaria is characterized by rather nonspecific symptoms, initially often described as flu-like manifestations. Most malaria patients report symptoms such as headache, weakness, muscular discomfort and malaise. Even though not specific to infections by *Plasmodium*, a few symptomatic features are still referred to as classical to uncomplicated malaria. Fever, with either regular or irregular onsets, is such a classical clinical finding in patients. The fever is believed to be caused by release of pro-inflammatory cytokines such as TNFα (32-34) as response to erythrocyte destruction and parasite-derived pyrogens. The regularity and timing of onset is thought to be a result of synchronous schizogony, thus presented differently depending on *Plasmodium* specie and time required to complete the IDC. *P. malariae* with an IDC of 72 hours (referred to as quartan) therefore results in fever peaks every 72 hours, *P. vivax*, *P. ovale* and *P. falciparum* every 48 hours (tertian) and *P. knowlesi* every 24 hours (quotidian or semi-tertian). A lack of regularity is however often seen, mainly for *P. falciparum* and *P. knowlesi*. Another typical feature of infection is splenomegaly, where the spleen enlarges as response to the acute infection by all human malarias. Upon repeated number of infections the spleen enlarges even further and can result in a secondary hypersplenism. Hemolytic anemia, assumed to result from erythrocyte destruction and failure of the erythropoesis to compensate for theses losses (35, 36), is often also noted, sometimes accompanied by a mild jaundice.

1.3.2 Severe malaria

Complications and severe manifestations due to *P. falciparum* are numerous and diverse. The progression from uncomplicated malaria disease with minor non-specific symptoms to severe disease with grave symptoms and high risk of fatal outcome can be rapid. Among the severe manifestations are severe anemia, acute respiratory distress syndrome (ARDS), pulmonary edema, unrousable coma (cerebral malaria), multiple convulsions, renal failure, circulatory collapse, abnormal bleeding, hypoglycemia, acidosis and hyperlactatemia. The appearance of these, either alone or in combinations, does at this stage present the patient with a bad prognosis and vouches for hospitalization. Even if the severe malaria is treated the mortality rate is high, but if left untreated it is nearly always fatal. In particular severe anemia, cerebral malaria and

ARDS (or combinations thereof) constitute the major clinical findings in severe malaria, and it is also for these that most work has been done to characterize the underlying pathogenic mechanisms. A succinct review of these and a special case of severe malaria, the maternal type, follows below.

1.3.2.1 Severe anemia

Anemia of varying degrees is often an adjunct to severe malaria. Severe anemia, diagnostically defined as having a hemoglobin level lower than 5g/dl or hematocrit beneath 15 %, is possibly the malarial complication causing most deaths in children in endemic regions (37). The reasons for the malaria mediated severe anemia are not fully understood, but research findings presented in recent years has shed considerable light on the molecular mechanisms potentially underlying this life-threatening manifestation. An obvious contributor to the anemia is the destruction of RBCs upon infection and rupture by *Plasmodium* parasites. Similarly obvious is the opsonization and clearance of intact infected erythrocytes by the host immune system (38). However, considering the relatively low numbers of infected versus uninfected RBC in the host circulation, the destruction of infected RBC alone cannot be held responsible for the severe anemia. Evidently, also uninfected RBCs are cleared during an infection. Using mathematical modeling, Jakeman et al (35) estimated a figure of 8.5 cleared uninfected per infected RBC and suggested phagocytosis of uninfected erythrocytes an important contributor to anemia. The latter was recently supported using a rodent model, where depletion of the phagocytic monocytes and macrophages as well as CD4+ T lymphocytes alleviated the anemia in malaria infected mice (39). The accelerated turnover of uninfected RBCs could partly be due to failed merozoite invasions, during which parasite antigens such as the rhoptry protein RSP2 seems to be deposited on uninfected RBCs. The RSP2 protein has in addition been suggested to cause dyserythropoesis through tagging of erythroid precursor cells in the bone marrow (40). Another factor suggested important in the clearance of uninfected RBCs is hemozoine (41). The hemozoine, which is produced in large quantities by the parasites when acquiring amino acids through degradation of hemoglobin, is released during schizont rupture. A resulting increase in rigidity of RBCs has been observed in the presence of heme-products, making the less deformable cells more prone to be cleared in the spleen. Suppression of erythropoietin response and synthesis are other suggested mechanisms of dyserythropoesis (42), with increased production of cytokines such as TNFα and IFNγ in the bone marrow possibly responsible for the latter (43, 44).

1.3.2.2 Respiratory distress

Respiratory distress, the failure of the respiratory system to perform adequate gas exchange, is a rather common finding among patients with *P. falciparum* malaria. The acute respiratory distress syndrome (ARDS) is however a manifestation with poor prognosis and high mortality rate. ARDS due to malaria is most often encountered among adults, where it serves as an important predictor of mortality (45), and in particular pregnant women with severe malaria seem prone to develop this syndrome (45, 46). Even though ARDS is considered rare in the pediatric population, respiratory distress accompanied by metabolic acidosis is common and concur with high case

fatality rates (47, 48). Increased respiratory rate, abnormally deep breathing and coughing are the initial manifestations seen in patients. The progression into life-threatening hypoxia can be fast, and can occur even after several days of treatment when the patients appear to be improving and the peripheral circulation is cleared from parasites (45, 46, 49). As with most of the clinical manifestations in severe malaria, the pathogenesis of ARDS is not fully understood. It is however known that *P. falciparum* infected RBCs sequester within capillaries of the lungs, together with monocytes and with adjunct endothelial cell cytoplasmic swelling and interstitial edema (50-52). The edema is also seen mainly in regions where infected erythrocytes sequester (53). Thus seems parasite sequestration to be an important factor behind the pulmonary complications seen in severe malaria. Recent findings observed using mice infected with *P. berghei* ANKA supports this idea (54). Upon infection, the mice displayed alveolar capillary membrane disruption and marked increase in pro-inflammatory and anti-inflammatory cytokines such as TNFα, IFN-γ, IL-10 and IL-6. Observations were shown to correlate positively with higher parasite loads, and much less pronounced in CD36 deficient mice, suggesting that abundant parasite sequestration through interaction with CD36 are of importance for lung complications.

1.3.2.3 Cerebral malaria

Possibly the most feared manifestation of *P. falciparum* infections is the cerebral malaria (CM). CM is defined as unrousable coma (inability to localize a painful stimuli, i.e. scoring less than three on the modified Blantyre coma scale (55)) after other causes of encephalopathy have been excluded (56). The onset of coma may be gradual after an initial stage of confusion (most often seen in adults) or may be abrupt after seizures (in children). As one of several neurological complications seen in association with *P. falciparum* malaria, CM is without doubt the most severe and associated with a mortality rate of 15-20% (57). In addition, a substantial proportion of patients that rise from the coma and survive develop neurocognitive sequelae. The prevalence and severity of these neurological abnormalities (including ataxia, aphasia, cortical blindness and hemiplegia) is higher in children than adults (58, 59), most often transient but may cause permanent disability (60-62). In recent years, investigations of more subtle neurological sequelae have revealed that as many as one out of four children surviving CM develop long-term cognitive impairment (63, 64). As with the other clinical manifestations reviewed in this chapter, the pathogenic mechanisms behind CM are not completely resolved. Micro-vascular sequestration of *P. falciparum* infected RBC in the brain has been observed in several post-mortem examinations (51, 52, 65). The obstruction of the blood flow caused by the sequestration could lead to hypoxia, reduction of metabolite exchange as well as release of inflammatory mediators, thus resulting in cerebral edema and raised intracranial pressure (66), but the direct link between sequestration and the damages seen has not yet been proven. Elevated levels of pro-inflammatory cytokines such as TNFα and IFN-γ have long been implicated in the pathogenesis of CM in both murine models and humans (67-73), but their role as mediators have been debated. For example, more recent data has suggested lymphotoxin α (LTα) to be the true mediator instead of TNFα (74, 75). Anti-inflammatory cytokines, such as IL-10, have instead been proposed to have a protective role against CM (74), suggesting the pathogenesis development in part dependent on an

intricate balance of pro- and anti-inflammatory cytokines. Several other factors have in addition been identified as potential players in the pathogenesis of CM, including perforin (76) heme oxygenase-1 (77), quinolinic acid (78), *P. falciparum* glycosylphosphatidylinositol (GPI) (79) and nitric oxide (NO) (80). NO, which has been argued to be protective against severe malaria requires extracellular arginine for its synthesis. In agreement with this has associations between hypoargininaemia and CM been found (81), as well as proof of parasites being responsible for this depletion through uptake and conversion of arginine to ornithine (82).

1.3.2.4 Maternal malaria

Even though adults and adolescents in general develop protective immunity against severe disease in endemic regions, women become susceptible to severe malaria during pregnancy. The maternal malaria is associated with a high risk of mortality in mothers and infants and often leads to premature deliveries and abortions. Approximately 300,000 infant and fetal deaths and 1,500 maternal deaths per year are attributed to malaria (83). The reason for the increased susceptibility to malaria is thought to be dependent on the appearance of the placenta, which presents a new niche for the parasites to dwell in. *P. falciparum* infected erythrocytes have repeatedly been found to sequester and accumulate in the intervillous space of placentas, a sequestration phenomenon shown strongly linked to particular parasite derived proteins that interact with receptors such as chondroitin sulphate A (CSA) on syncytiotrophoblasts (further discussed below) (84-87). Besides the damage caused by parasite sequestration through obstruction of blood flow and flow of nutrients, oxygen and maternal protective antibodies to the fetus, inflammatory responses in the placenta appear to be harmful as well. In particular, massive infiltration of monocytes into infected placentas and raised levels of TNFα appears to be linked to fetal growth restriction and maternal anemia (88-94). Interestingly, primigravidae are at highest risk of developing maternal malaria, a risk that drops drastically with successive pregnancies (95-98), suggesting a rather quick development of immunity towards the maternal malaria causing parasites.

1.3.3 Known determinants of severe disease development

Despite the complex picture of potential factors influencing the pathogenesis of severe malaria, there are apparent common denominators among all severe manifestations. Parasite load, sequestration of infected RBC in the microvasculature of various organs, age, immune status and involvement of pro- and anti-inflammatory responses are all recurrent topics. Variations in these factors can be either protective or be associated with an elevated susceptibility of infection and development of severe disease. Apart from the impact of *Plasmodium* specie on disease severity, which will be further discussed below, other examples of determinants that have been identified will here be reviewed.

1.3.3.1 Age, gender, rate of transmission and acquired immunity

There are considerable differences in the manifestations of severe malaria between adults and children, sometimes between male and female adults and adolescents, as well as between areas with different rates of transmission. In areas of high malaria transmission, severe malaria is usually confined to children under the age of five where displayed as severe anemia, respiratory distress, CM or combinations thereof. Interestingly, in areas with lower transmission the mean age of clinical cases is higher and severe malaria, morbidity and mortality may occur at all ages (99-102). This is thought to be dependent on the development of protective immunity, which is acquired faster in areas of high transmission as a consequence of earlier and more frequent exposure to the parasites. The acquisition of protective immunity to malaria (nicely reviewed in (103)) is thought to develop in three sequential phases. First immunity is developed against severe disease, second to symptomatic but uncomplicated disease and third to the asymptomatic carriage of parasites (101, 104). Thus seems clinical immunity develop with greater ease than anti-parasite immunity, which may take a lifetime to develop. The main reasons for the exception of adults developing severe malaria are lack of immunity and pregnancy, in which gender plays an obvious role in the latter. Immunity towards malaria is not sterile, and requires a continuous exposure to the parasite in order to be protective. Even short periods of interrupted exposure lead to loss of the acquired clinical immunity (103). Despite developed clinical immunity, women become susceptible upon pregnancy (see above). Apart from the new niche that the appearance of the placenta presents for the parasites, it has also been suggested that pregnant women are more attractive to mosquitoes and thereby exposed to malaria at higher levels than non-pregnant individuals (105, 106).

1.3.3.2 Host genetic factors

Several genetic factors have been identified in humans that alter the susceptibility to infection and severe disease, many of which correspond well with suggested factors underlying the pathogenesis of the severe manifestations described above. Malaria appears to be the evolutionary driving force behind several Mendelian diseases in endemic regions, including sickle cell disease, thalassemia and glucose-6-phosphatase dehydrogenase deficiency that despite being harmful to humans are selected for due to decreased susceptibility to malaria infection. The best described hemoglobinopathy shown protective against malaria is the hemoglobin S (HbS) variant of the *HBB* gene (which encodes β-globin), where homozygotes suffer sickle cell disease and heterozygotes have a 10-fold reduced risk of severe malaria (107, 108). Protection is also achieved through regulatory defects of *HBA* and *HBB*, which cause α and β thalassemia (109, 110), polymorphisms in the *G6PD* gene, which causes glucose-6-phosphatase dehydrogenase deficiency (111-114) and variation in the chemokine receptor *FY*, responsible for the Duffy-negative blood group (115-118) among many others. Alterations as these renders the erythrocytes less suitable as host-cells for the parasite to invade, reside and proliferate in. Genetic variability in genes encoding host erythrocyte and endothelial cell receptors, such as CD36, ICAM1, PECAM1 and CR1 (reviewed in (119)), used by the parasite in order to sequester have also been observed. Their potential roles in conferring protection against severe disease is however not completely clear (further discussed in chapter 1.4.1.2). A wide range of polymorphisms

in genes encoding molecules of the host immune system has been described protective though. The use of massive sequencing approaches has recently identified new or confirmed already known protective polymorphisms in for example TNFα, LTα and interferon regulatory factor-1 (IRF-1) (120-122). With the use of massive sequencing and microarray approaches for genome wide association studies, it is likely that the already large panel of host genetic factors will expand and thereby also the understanding of the mechanisms behind severe malaria.

1.3.3.3 Co-infections

Susceptibility of infection and progression of severe malaria disease may also be influenced by simultaneous presence of other pathogens in the human host. In particular co-infections with HIV have been studied and shown associated to an increased risk of malaria infection and elevated numbers of parasites in the circulation (123, 124). Pregnant women seropositive for HIV have a higher prevalence of maternal malaria, higher parasite densities, display more severe anemia and do not develop equally effective disease protection with successive pregnancies as their seronegative counterparts (125-128). To date, HIV is the only clear co-infectious determinant of severe disease progression in malaria.

1.4 *PLASMODIUM FALCIPARUM* VIRULENCE

Maybe the most important determinant for development of severe disease is the infecting *Plasmodium* specie. *P. falciparum* differs from the other *Plasmodium* species infective to humans in various aspects. Examples of the special features are the ability to invade erythrocytes of all ages, multiply asexually at high rates and efficiently evade the host immune system through sequestration and antigenic variation (129). All these features result in high parasite loads in the host, which has been shown to correlate to severity of the disease (130). The ability to sequester, which is considered being the major virulence trait of *P. falciparum* will here be reviewed.

1.4.1 Sequestration

Only erythrocytes infected with early stages of *P. falciparum* parasites are seen in the peripheral circulation whereas infected RBCs containing mature parasites are not. The latter instead accumulate in the microvasculature and are first released into the circulation after completion of schizogony and invasion of new erythrocytes. Upon maturation they are yet again removed from the circulation. The sequestration phenomenon occurs due to drastic modifications of the infected RBC, orchestrated by parasite-derived proteins. These modifications alter the rigidity of the infected RBC and adhesive parasite proteins transported to the infected RBC surface mediate binding to endothelial receptors (cytoadhesion) and to uninfected and/or other infected RBC (rosetting and autoagglutination respectively).

1.4.1.1 Erythrocyte membrane modifications

Normal erythrocytes display a tremendous deformability. This changes abruptly upon infection by *P. falciparum* when the rigidity is gradually increased as the parasite matures in the erythrocyte. From slightly impaired deformability when infected by a ring-stage trophozoite, the erythrocyte turn practically impossible to deform when infected with mature trophozoites or schizont stages (131). The rigidity has been suggested a result of membrane modifications by parasite proteins, the physical presence of a less deformable parasite, oxidative stress through released hemozoin or combinations thereof (132-136). In recent years, several proteins synthesized by the intracellular parasite have been shown transported towards the erythrocyte surface (137). Already during invasion of the erythrocyte, the merozoite releases proteins from the rhoptries and dense granules that end up in the cytoplasm of the RBC cytoplasm (138, 139). At least one of these, the ring-infected erythrocyte surface antigen (RESA), is destined to the surface of the infected RBC, where it stabilizes spectrin tetramers and suppresses further invasion by other merozoites (140, 141). Following this early change in host cell membrane the parasite synthesize and transport a vast number of proteins through the cytoplasm to the surface of the erythrocyte upon maturation. A subset of these was recently shown to alter the rigidity of the infected RBC through a reverse genetics screen (142). Others had before this been identified, and among them the well studied knob-associated histidine-rich protein (KAHRP), the mature parasite-infected erythrocyte surface antigen (MESA), *Plasmodium falciparum* erythrocyte membrane protein 3 (PfEMP3). KAHRP (143-145), MESA (146, 147) and PfEMP3 (132, 148) are all known to alter the rigidity of the infected RBC and the two former have also been shown involved in the appearance of electron dense protrusions (knobs) on the infected cell. Thus, a large number of proteins affecting the erythrocyte rigidity are produced by the parasite. The loss in deformability of infected RBCs, resulting in an augmented hemodynamic resistance, seem to be of importance for the pathogenesis of severe disease (149, 150). The rigid cell will however be destined to the spleen and cleared if remaining in circulation, but this is counteracted by the use of adhesive parasite proteins interacting with the endothelial cell lining causing the infected RBC to cytoadhere.

1.4.1.2 Cytoadherence

The term cytoadhesion encompasses all the infected RBC binding events to vascular endothelium seen in various organs such as brain, intestine, liver, lung, skin and the syncytiotrophoblast cell lining of the placenta. Via the cytoadhesion the infected RBC is not only removed from the circulation and thus prevented from clearance by the spleen, but it also gains access to a relatively hypoxic environment preferred by the parasite for proliferation and RBC invasion. While often proposed, the importance of adhesion to specific endothelial receptors as well as the whole concept of cytoadhesion to severe disease remains unclear. This is particularly clear considering that all RBCs infected with mature trophozoite stage parasites cytoadhere, irrespective of displayed clinical manifestations of the patients. A number of endothelial receptors have been

identified as targets of the infected RBC, with different roles suggested in the pathogenesis of the disease.

CD36

CD36 is a surface glycoprotein expressed on the endothelium of various organs, platelets, monocytes and dendritic cells, that was early recognized as a receptor of infected RBC (151, 152). The receptor seems to be widely used by the parasites, since clinical isolates analyzed for their adhesive phenotypes *in vitro* has revealed that almost all bind CD36, a binding shown stable under flow (153-155). This indicates that CD36 binding is of fundamental importance for cytoadhesion, which is further supported by the high abundance of polymorphisms found in the *CD36* gene in malaria endemic regions suggesting strong selection (156-158). However, no clear disease protective associations of these polymorphisms have been made. Neither has CD36 binding among parasites been shown associated to severe disease manifestations (159-162).

TSP

Thrombospondins (TSP) is a family of multifunctional glycoproteins secreted by endothelial cells, platelets and monocytes. TSP binds to various receptors, including integrins, CD47 and CD36. Similar to CD36, TSP has been shown widely used as receptor of infected RBCs but no clear association between binding by clinical isolates and severe disease has been made (159, 163, 164).

ICAM-1

The immunoglobulin superfamily member, inter cellular adhesion molecule 1 (ICAM-1), also known as CD54, is present on the surface of endothelial cells and monocytes. ICAM-1 is a receptor that has been associated to severe disease, since found abundantly expressed in the vascular endothelium in brains from patients deceased with CM (162). The expression of the receptor has also been shown upregulated by the severe disease manifestation-linked pro-inflammatory cytokines TNFα and IFNγ (165, 166) and by binding of infected RBC to endothelial cells (167). ICAM-1 has also been shown to act synergistically with CD36 in increasing the binding to endothelial cells (168, 169) and that this binding triggers intracellular signaling cascades which appear to be parasite strain dependent (170). A few reports have also suggested stronger binding of ICAM-1/CD36 by severe disease causing isolates (160, 171). Even though implicated as an important receptor in progression of severe disease there appears to be no compensatory polymorphisms in the *ICAM1* gene associated with protection (172).

PECAM-1

The platelet endothelial cell adhesion molecule 1 (PECAM-1) is another member of the immunoglobulin superfamily shown to mediate binding of infected RBC to endothelial cells (173). PECAM-1 is normally confined to tight junctions between endothelial cells and thus not present on the luminal side where infected RBCs would potentially bind. The receptor is however upregulated and redistributed to the luminal side upon IFNγ stimulation. Although not associated to any particular clinical manifestation, PECAM-1 binding is a common feature of clinical isolates (159). Polymorphisms in the encoding gene have however been identified protective against development of severe disease (174, 175).

CSA and other placental receptors

The glycosaminoglycan (GAG) chondroitin sulphate A (CSA) may be the cytoadherence receptor with the strongest link to severe disease. Infected RBC from placental isolates commonly bind to CSA whereas non-placental isolates rarely bind this receptor (176-179). The role of CSA as an important mediator of adhesion in maternal malaria is further supported by the demonstration of sera from multi-gravid women able of blocking adhesion to CSA (180, 181). The level of antibodies and blocking of CSA-binding has in addition been shown to be parity-dependent (182) and to improve the clinical outcomes of the maternal malaria (183). Apart from CSA, hyaluronic acid (HA) has also been identified as a receptor for infected RBCs (184, 185) and non-immune immunoglobulins suggested acting as bridges between parasite derived proteins and cytoadherence receptors in the placenta (185, 186)

Other endothelial receptors

Vascular cell adhesion molecule 1 (VCAM-1), E-selectin and P-selectin are other potential endothelial receptors reported to mediate cytoadherence. They seem however poorly recognized by clinical isolates (187, 188), questioning their relevance *in vivo*. The GAG heparan sulphate (HS) is produced by all cells and has been ascribed roles in both cytoadhesion and rosetting (therefore discussed in more detail below). The neural cell adhesion molecule (NCAM) was recently identified as yet another potential cytoadherence receptor (189), but its role in this potentially severe disease-causing phenomenon needs further characterization.

1.4.1.3 Rosetting

P. falciparum infected RBCs can adhere to uninfected RBCs to form spontaneous rosettes (190, 191). Defined as one mature trophozoite infected RBC binding two or more uninfected RBCs, this phenomenon was early on identified *in vitro* both in laboratory strains and clinical isolates examined directly after sampling (154, 192, 193). The interactions between cells in rosettes were also shown to be strong enough to withstand the shear forces experienced *in vivo* (194-196). Early on it became clear that rosetting is a variable phenotype, with big differences in individual isolates capacity to form rosettes (193). Since then, and in contrast to cytoadhesion, rosetting has repeatedly been found clearly associated with severe disease in the human host (159, 197-202), with higher rosetting rates seen in isolates from severely ill patients. In addition, this correlation was observed independent of the severe clinical manifestations displayed, signifying rosetting as a potentially universal virulence mechanism in severe malaria. What the parasites gain from cytoadhesion seems obvious, but the gain of forming rosettes has not been clearly resolved. It has been speculated that the rosetting allows for more efficient cytoadhesion through decreased blood flow, protection of the infected RBC from immune cells and opsonizing antibodies as well as more efficient erythrocyte invasion by merozoites (203). That rosetting does present the parasites with improved chances of survival and proliferative advantages could be supported by observations made in both humans and primates. Rosetting parasites have been shown to generate higher parasitemias in experimentally infected primates than non-rosetting parasites (204) and positive correlations have been observed between peripheral parasitemias in patients and the corresponding isolates

rosetting rates (200). Rosette disruption experiments have been shown highly valuable for the identification of RBC receptors and serum factors involved in this interaction (205-207).

HS

In addition to its presence on endothelial cells, HS has also been identified on the surface of normal erythrocytes (208). Heparin and a wide range of other sulphated glycans have been shown able to inhibit rosette formation and to disrupt rosettes (205, 209-211), though at variable levels for different *P. falciparum* strains and isolates. Depolymerized heparin, devoid of anticoagulant activity, has also been shown to efficiently disrupt rosettes (212) and is currently evaluated as a potential de-sequestration and anti-rosetting drug target.

CR1

Complement receptor 1 (CR1) is an immune regulatory molecule expressed in various levels on the surface of erythrocytes, some peripheral blood leukocytes, podocytes and dendritic cells. CR1 was identified as a receptor through the use of erythrocytes from CR1 deficient donors, which impaired rosetting in otherwise highly rosetting laboratory *P. falciparum* strains (213). This was confirmed by showing that soluble CR1 could inhibit rosetting in laboratory strains, and by demonstrating that a monoclonal antibody towards CR1 was capable of reversing rosette formation in both laboratory strains and clinical isolates (214). The binding was further mapped to a particular domain of the parasite-derived protein PfEMP1 (further discussed below). Importance of CR1 and the mediated rosetting in the pathogenesis of severe disease has been supported in a number of population studies. A polymorphism in the *cr1* gene has been shown highly frequent in populations in endemic regions of Papua New Guinea and been linked to protection of severe disease (215). Several studies in Africa have also suggested variable levels of CR1 on erythrocytes to influence the disease outcome (216-218).

Blood group ABO antigens

Rosetting levels and sizes of rosettes have been shown to vary with different blood groups of erythrocytes. Both laboratory strains (205, 207) and clinical isolates (199, 205, 219-222) have been demonstrated to prefer in particular blood group A, but also B and AB in front of blood group O, forming larger rosettes that can withstand higher shear force (206, 223). Interestingly, there appears to be strain specific preferences for different blood groups, most likely reflecting the adhesive ligand being produced and exposed by different parasites (206, 219), and the sensitivity of rosette disruption using various GAGs is drastically diminished when strains are cultured in their preferred blood group (206, 219). Successful rosette disruptions using blood group tri-saccharides have also confirmed the blood group antigens as receptors involved in rosetting. In particular blood group A has been associated to severe disease (172, 221, 224, 225), whereas blood group O seems to be protective and is instead over-represented in uncomplicated cases of malaria (221, 226).

Serum proteins

Serum proteins are sometimes essential for rosette formation, most likely acting as bridging molecules between the parasite ligand and the RBC receptor. Fibrinogen, von Willebrand´s factor (207) and non-immune IgM (223, 227, 228) and IgG (186) have

been shown to partake in the interactions of both laboratory strain and clinical isolate infected RBC to uninfected RBC (207, 223, 229). Binding of non-immune Igs has in addition been reported as a common phenotype among clinical isolates from patients suffering from severe disease (159, 230). The role of IgM in rosetting seems generally accepted, and is also the most thoroughly studied. The interaction with infected RBC was recently mapped to the Cμ4 domain of the heavy chain, and showed that IgM polymerization is essential for binding (231). The role of IgG has been more controversial, which could in part be dependent on the fact that different strains/isolates display different binding patterns and that the binding of IgM is a more abundant and strong phenotype.

1.4.1.4 Adhesive parasite ligands on the P. falciparum infected erythrocytes

Apart from altering the rigidity of the infected RBC, some of the proteins described above (KAHRP and MESA) also serve as anchors for other parasite-derived proteins that are transported and exposed on the erythrocyte surface. At least one of these surface exposed proteins is known to mediate the cytoadhesion and rosetting through interactions with host cell receptors. This protein, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), is considered the most important virulence factor of *P. falciparum*.

PfEMP1

The erythrocyte surface exposed adhesion protein PfEMP1, is the main protein responsible for the sequestration of infected erythrocytes in the deep tissues. It is a multi-domain protein of 200-350 kDa, encoded by the hyper-variable *var* gene family that undergoes antigenic variation and thereby allows for the generation of diverse adhesive phenotypes (160, 232-236). There are approximately 60 *var* genes per parasite genome, located mainly in the highly polymorphic subtelomeric regions but also in central parts of the 14 chromosomes (236-239). All *var* genes share a basic two-exon structure, with exons separated by a conserved intron (Figure 3C). Exon I encodes the hypervariable extracellular part of PfEMP1, which comprises the N-terminal segment (NTS), multiple adhesion domains named Duffy binding like (DBL) and cysteine rich interdomain region (CIDR) sometimes interspersed with C2 interdomains (240). Seven types of DBL domains exist (α, α_1, β, γ, δ, ε and x) and four types of CIDR domains (α, α_1, β and γ). The smaller exon II encodes the semi-conserved acidic terminal segment (ATS), which has been proven to contain a C-terminal transmembrane region (236, 240). Even though

The chromosomal location and transcriptional orientation of *var* genes have been shown to correspond to similarities in the 5' upstream open reading frame of the genes (Figure 3A). Based on this conservation, the *var* genes are sub-divided into five major upstream sequence (Ups) groups (UpsA, UpsA2, UpsB, UpsC and UpsE) (241), groups that interestingly also have been shown to be of clinical relevance (see below). The overall conservation of the groups, in conjunction with their physical location, indicates that there is an evolutionary pressure imposed and that recombination is restricted to occur in between limited subsets of *var* genes. Based on the Ups and the domain composition architecture yet another, but very similar grouping is also used, with three

major (A, B and C) and two intermediate (B/A and B/C) groups (Figure 3B) (242-244). Two unique types of *var* genes have been shown remarkably conserved in between all sequenced parasite genomes to date, and do not to fit in this classification system. The *var1csa* carries the UpsA2 flanking sequence and was initially named due to experimentally proven CSA binding capacity of its DBL3γ domain (245, 246). It has later been shown to be truncated, hyper-conserved, transcribed in almost all clinical isolates and with unusual transcription pattern (244, 247, 248), but the function remains elusive. The second, *var2csa*, is flanked by a 5' UpsE and have been intimately linked to CSA binding and therefore to maternal malaria.

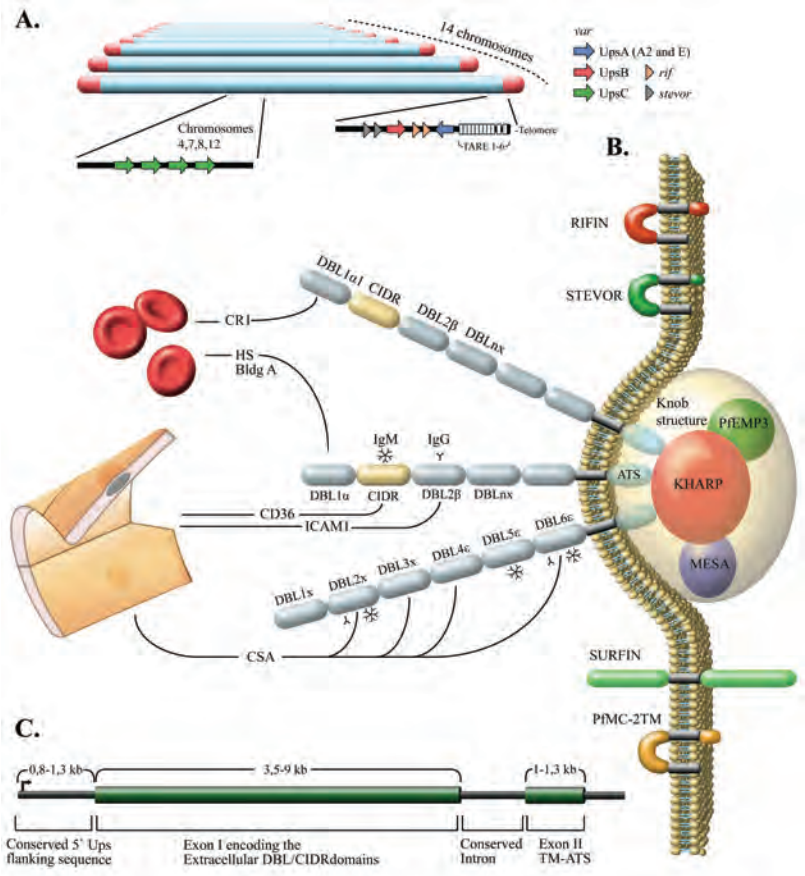

Figure 3. A) The organization of *var* genes in the *P. falciparum* genome. Ups A and Ups B *var* genes are located in the subtelomeric regions of the chromosomes whereas UpsC locate to the central regions. **B)** Schematic representation of PfEMP1 and other surface molecules in the infected erythrocyte membrane. Binding preferences for group A, group B/C and for VAR2CSA PfEMP1proteins are exemplified from top to bottom. **C)** The common configuration of *var* genes. All memebers conatin one exon encoding the polymorphic extracellular part, one exon encoding the internal semi-conserved ATS, and the two promotor regios (Ups-type specific conserved 5' flanking region and the conserved intron).
(Modified and published by courtesy of Dr. Johan Normark)

The expression of PfEMP1 on the infected RBC surface coincides with the disappearance of mature stage infected erythrocytes from the peripheral circulation. Binding specificities of various PfEMP1 variants have been mapped to the different adhesion domains of the proteins and have revealed that DBL1α binds blood group A, CR1 and HS on both endothelial cells and erythrocytes (209, 213, 219, 249, 250). CIDR1α has been shown to bind CD36 and IgM (249, 251, 252), and DBLβ-C2 binding to CD36 and ICAM-1 (163, 253) for example. Patterns have also been observed between different PfEMP1s and disease severity. The PfEMP1 with the strongest connection to severe disease is VAR2CSA, which mediates maternal malaria through sequestration to CSA in the placenta (85, 87, 254, 255). Recurrent observations have been the association of group A *var* genes transcribed in highly rosetting isolates and isolates from patients with severe disease, in particular if a the DBL1α domain contained few cysteines (256-260).

Other surface exposed parasite antigens

In addition to PfEMP1, other parasite-derived proteins have been suggested exposed on the infected RBC surface. The family of repetitive interspersed proteins (RIFINs) is encoded by the *rif* genes and has been demonstrated expressed on the surface of infected RBC in a clone dependent manner (261, 262). The function of RIFINs is elusive, but high gene copy numbers and clonal variation clearly imply exposure to the immune system. In fact, antibodies towards RIFIN are thought to be part of the acquired immunity to the disease (263, 264). The closely related family of subtelomeric variable open reading frame (*stevor*) genes is similar to the *rif* family in chromosomal location, gene structure and sequence homology (265). As for the RIFINs, the function is unknown, but recent evidence has located the STEVORs to the infected RBC surface (266, 267) as well as to the apical end of merozoites (266). This dual localization has previously been shown for another protein family of *P. falciparum*, the surface associated interspersed protein (SURFIN) family (268). Yet another example of a suggested gene family encoding surface associated molecules and potentially mediating adhesion is *pfmc-2tm* (269, 270).

1.4.2 Antigenic variation

Expression of antigens on the surface of host cells presents a grave danger to the parasite since revealing its presence in this way makes it a target of the host immune system. In order to cause a persistent infection and thereby increase its chances of transmission, the parasite needs to avoid being cleared. This is achieved by altering the antigens they display on the surface; a strategy employed by a wide variety of pathogens including African trypanosomes (271), *Borrelia* species (272), *Giardia lamblia* (273) as well as many others. Even though several gene families have been suggested to undergo antigenic variation in *P. falciparum*, it is mainly for the *var*/PfEMP1 family where distinct molecular mechanisms have been determined.

1.4.2.1 Antigenic variation and PfEMP1

Mutually exclusive antigen expression and var gene switching

While multiple transcripts of the *var* gene family can be seen in the early stages of intraerythrocytic parasite development, only one dominant transcript is present in the later stages. In conjunction, only a single variant of PfEMP1 that determine the adhesive properties of the parasite is expressed at the surface of the trophozoite-infected erythrocyte at a time (274, 275). This phenomenon is referred to as mutually exclusive expression, and recent studies have revealed this to be regulated on the transcriptional level and independent of antigen production (276, 277). This implies that changes in *var* gene transcription are independent of external stimuli, and that the parasite instead employs an intrinsic regulation of transcription to create switch variants. The rate of switching requires an intricate balance of high enough rate in order for parasites to escape the human immune response and at the same time avoiding exhaustion of the *var* gene repertoire. In addition, a switch in transcription must be coordinated so that activation of a new gene is accompanied with a simultaneous silencing of the previous. Variable *var* gene switch rates and switch orders have been observed in parasites cultivated *in vitro* devoid of immune pressure, and so far has no consensus been seen (278-280).

Molecular mechanisms behind antigenic variation

The understanding of the molecular mechanisms that control *var* gene transcription and antigenic variation in *P. falciparum* have in recent years been drastically expanded, but are still far from fully understood. The regulation is to date believed to occur at different levels, where the first level involves the two promotor regions found in virtually all *var* genes, the 5' upstream region (UpsA-E) and the intron (281). The upstream promotor is responsible for the mRNA transcription, whereas the intron promotor produces non-coding, sterile RNA (236, 282). While a single *var* gene is transcribed from the upstream promotor at a time, and thus mutually exclusive, most of the intron promotors seem to be active at the same time (283). The introns are believed to function as transcriptional silencing elements through promotor pairing, and therefore controlling antigenic variation (277, 281, 284-286). Other factors potentially regulating the antigenic variation through the upstream promotor sequences have recently been proposed. The ApiAP2 family of transcription factors in *P. falciparum* (287) could be of importance for regulation of *var* gene transcription after a member of this family were shown to bind the regulatory upstream regions of UpsB *var* genes (288), although its exact role has not yet been defined.

Another suggested level of regulation involves perinuclear repositioning of *var* genes upon activation. The nucleus of *P. falciparum* has been shown to contain two distinct chromatin environments, with mainly electron dense heterochromatin in the periphery and loose euchromatin in the internal part (289). There are however apparent gaps in the heterochromatin in the periphery, possibly indicating the presence of transcriptionally active zones. Using parasites with known transcriptional state of the *var2csa* gene, Ralph et al showed that *var2csa* reposition away from telomeric clusters upon activation and suggested the location of the active gene to be in the euchromatic portion of the periphery. Contradictory findings were however observed using transgenic parasite lines with drug inducible *var* gene promotors. When selected for activation, the transgenes were shown to co-localize with telomeric clusters independent of transcriptional status (277). The most recent addition to the debate of

whether transcriptional active zones exist in the nucleus or not, was provided by Dzikowski et al (284, 290). Using parasites with at least two simultaneously active *var* promotors (generated through incorporation of constitutively expressed episomal *var* promotors) it was noted that the properly regulated transcriptionally active *var* gene and the constitutively active episomal *var* always co-localized. Virtually no co-localization of silent chromosomal *var* genes and the constitutively active episomal *var* was observed, strongly suggesting the existence of a *var*-specific subnuclear expression site. However, since the suggested site can accommodate more than one active *var* gene at a time, this suggests that mutually exclusive transcription of *var* genes is regulated at a different level.

The third considered level of regulation involves chromatin modifications. Expression of genes can be influenced by histone modifications (acetylation, phosphorylation, methylation and ubiquitination), which alter the chromatin surroundings of the genes through alterations in DNA accessibility or recruitment of various proteins to the site (291). The *P. falciparum* genome contains a range of genes encoding molecules involved in chromatin modification and assembly (237, 292) and these have recently been shown important for the regulation of *var* gene transcription. Extensive amounts of research findings regarding chromatin modifications and their role in *var* gene regulation have in recent years been presented. Among these modifications have acetylated histone H2 and H3 and methylated H3K27 been found at active genes, whereas tri-methylated H3K9 has been observed at silent loci (293-296). Two paralogues of the histone deacetylase SIR2 (PfSIR2A and PfSIR2B) have been shown involved in the silencing of *var* genes. Knockout of any of these two results in loss of transcriptional control, and collapsed mutually exclusive expression (294, 297). Perhaps the most interesting histone modification identified to date, is the di- and trimethylated H3K4, which has been shown enriched in active *var* genes even after the gene is no longer transcriptionally active. This could indicate that the gene is ready to be active again in the consecutive cell cycle and suggests H3K4 to play a role in epigenetic memory (296).

2 SCOPE OF THIS THESIS

The general objective of the investigations presented in this thesis was to increase the understanding of *Plasmodium falciparum* virulence and the contribution of parasite virulence factors to progression of severe malaria disease. The role of surface antigens, the regulation and adhesion mediated by these, as well as the potential contribution of proliferative advantages and other genetic modifications were particular targets in this context.

Specific aims:

The specific aims of the presented papers were as follows

I. To investigate the abundance of gene copy number variations in genomes of both laboratory strains and clinical isolates of different origin and phenotypes.

II. To compare the transcriptomes and adhesive phenotypes of distinct isogenic parasite clones generated by micromanipulation.

III. To characterize *var* gene switching patterns and corresponding adhesivity in isogenic clones grown continuously *in vitro*.

IV. To elucidate transcriptional activity of duplicated genes through allelic discrimination, both on population level and in single cells.

V. To characterize and compare *var* gene sequences transcribed by parasites from patients with different disease states.

VI. To compare *in vitro* proliferative capacity in parasites from patients with severe and uncomplicated malaria disease.

3 EXPERIMENTAL PROCEDURES AND CONSIDERATIONS

Materials and methods are detailed in each respective study included in this thesis (paper I-VI). Experimental procedures and considerations of extra importance, and experiments that had to be developed or modified from standard methodologies previously reported, are succinctly reviewed here.

3.1 PARASITE *IN VITRO* CULTURE CONDITIONS

A successful *in vitro* cultivation method for *P. falciparum* was in 1976 (298), a report that has had a tremendous impact on malaria research. The method is still extensively used and is referred to as the candle-jar technique, since open flasks containing parasite infected erythrocytes and culture medium (RPMI1640 supplemented with glutamine, gentamicin and 10 % human serum or Albumax) are incubated at 37°C in air-tight desiccators where lit candles are used to consume excess oxygen. However, even though successful for cultivation of already adapted laboratory strains, the method has been shown unsatisfactory for adaptation of recently collected clinical isolates to grow *in vitro*. Poor outgrowth, low multiplication rates and complete growth failures have traditionally been observed in many trials. A systematic evaluation of more suitable culture conditions was therefore undertaken using a panel of field isolates. Each isolate was sub-cultivated into four and subjected to different growth conditions involving the use of gas (5% O_2 and 5% CO_2 in N_2), classical candle-jar, growth in static manner, growth in suspension on an orbital shaker (50 revolutions per minute) or combinations thereof. Medium constituents were in all cases kept identical to the original methodology. The differently treated cultures were evaluated for outgrowth, the rate of multiple parasitized erythrocytes, multiplication rates and rosetting rates (see below for details). Growth in suspension with the use of fixed gas composition to maintain a stable microaerophilic environment was proven superior, with all monitored parasite features significantly improved (paper VI). All clinical isolates, as well as most laboratory strains and clones, used in the studies included in this thesis were therefore cultivated in this way.

3.2 SINGLE CELL CLONING OF PARASITES

Three methods are currently used in the malaria community to achieve single cell cloning; limiting dilution, micromanipulation and sorting by flow cytometry. The micromanipulation technique, where single cells are picked using a micromanipulator MN-188, sterile micropipettes (glass capillaries with ~3-5 µm internal diameter) and an inverted Diaphot 300 microscope, presents an important advantage compared to the other methods. Due to the microscopical monitoring during the whole procedure one can be directly ascertained that single infected (or uninfected) erythrocytes are indeed selected. Limiting dilution in particular and sorting by flow cytometry do not present

the same advantage. The sorting by flow cytometry depends on the use of staining in order to differentiate infected from uninfected erythrocytes or a subsequent investigation of randomly sorted unstained cells. Even though the sorting itself is quick, the time consuming microscopical confirmation of single infected erythrocytes as well as unsuitability of staining for subsequent parasite propagation, it is still not an optimal method. Limiting dilution, where cultures are diluted so that a certain culture volume theoretically contains only a single cell requires the same time consuming microscopical confirmation. The described advantage of micromanipulation prompted us to use this method to pick single 3D7 parasites with rosetting and CD36 binding phenotypes for transcriptome comparisons and to study *var* gene switching (paper II & III). HB3CSA parasites were similarly selected for the investigation of *var2csa* allele specific transcription in single cells (paper IV).

3.3 BINDING PHENOTYPE CHARACTERIZATION

Binding phenotypes of infected erythrocytes can be characterized in numerous ways using a wide variety of reagents, cell-lines and techniques. The rosetting rate, a rather crude binding phenotype, is monitored regularly for all parasites cultivated in the Wahlgren laboratory. A rosetting infected erythrocyte is defined as an erythrocyte infected with a mature trophozoite (24-30 h p.i.) binding ≥ 2 uninfected erythrocytes. Rosetting rates (of vital importance in papers I-III and V-VI) are computed by dividing the number of infected erythrocytes forming rosettes with the total number of erythrocytes infected with mature trophozoites. In paper VI, rosetting was also scored at schizont stage. Binding to various endothelial receptors are of importance for assessing cytoadherent properties of infected erythrocytes. Parasites used in paper II and III were initially cloned based on the rosetting phenotype (3D7S8.4) and adhesion to CHO-CD36 transfectants (3D7AH1S2). For a more detailed adhesivity profile, infected erythrocytes of both clones were incubated on placental sections and endothelial receptors presented on cells (CHO cells transfected with CD36 and ICAM1), coated on plastic (CSA and TSP) and soluble and fluorochrome labeled receptors (heparin and CD31). The experimental procedures were in all cases very similar with an initial incubation and extensive washing followed by counting. Presented units are number of bound infected erythrocytes per 100 CHO cells, per mm^2 of placental sections, CSA and TSP, as well as percentage of infected erythrocytes showing surface fluorescence with heparin and CD31. The same clones were in paper III assessed for surface recognition with male and female immune sera and with sera raised in rabbits against different DBL-domains of *var2csa*. Recognition was in this case detected using flow cytometry upon incubation with sera and addition of fluorescing secondary antibodies.

3.4 ROSETTE DISRUPTION AND INVASION INHIBITION

Rosette disruption was performed on a panel of laboratory strains and clinical isolates at trophozoite and schizont stages using serial dilutions of antibodies towards DBL1α, human non-immune IgG and IgM as well as GAGs (heparan sulphate, heparin, the

depolymerized heparin Ba342 and chondroitin sulphate A). Parasite suspensions were incubated with the different reagents for 45 minutes prior to rosetting rate determination. The level of rosette disruption was computed using mock-treated (RPMI1640) cultures as controls. Invasion inhibition assays were performed for the same parasites and importantly during the same erythrocytic cycle as for which rosette disruptions were conducted. This was considered as a means of minimizing influence by potential oddities in parasite behavior between different erythrocytic cycles. Trophozoite stage cultures were mixed with the same reagents described above and allowed to rupture and re-invade new erythrocytes. Cultures were stained with acridine orange and parasitemias determined using flow cytometry. The level of invasion inhibition was subsequently computed, yet again with mock-treated (RPMI1640) cultures serving as controls. The chosen procedure, with rosette disruption and invasion inhibition performed on the same parasites, within the same erythrocytic cycle and with the same reagents, thereby presented an opportunity to correlate the results achieved for each specific reagent.

3.5 MICROARRAY

The DNA microarray technology provides a possibility to perform genotyping and transcriptional profiling at high resolution and high throughput. The *P. falciparum* whole genome microarray used in paper I-III contained 6850 70-mer oligonucleotide printed in quadruplicate. The great majority of these were designed in the DeRisi laboratory (299), where used for complete IDC transcription profiling (300, 301). 294 oligonucleotides were designed in-house, in order to complement this set (242 towards *var* genes, 24 towards stage-specific genes and 28 controls), before used for transcription profiling of isogenic 3D7 clones (paper II & III) and for comparative genomic hybridizations (CGH) of parasites from various geographical origins and with different phenotypic traits.

3.6 REAL-TIME PCR

Real-time PCR has been proven a very important and reliable method for accurate quantification of nucleic acids since introduced. This technique has in the studies presented in this thesis been used as a supplement to microarray experiments (paper I and III), but also for discrimination of duplicated gene transcripts containing slight sequence variability (paper IV). Gene specific primers and probes for all real-time PCR assays were designed using Primer Express 3.0 and risk of primer dimerization and secondary structure formation assessed using ΔG estimations in NetPrimer. Specificities of designed assays were confirmed through blasting towards all genomes used as design templates and experimentally from dissociation curves of amplified products using serially diluted DNA as template. Amplification efficiencies were evaluated using the same dilution series and were sometimes needed to be optimized through alterations in primer concentrations.

3.6.1 Relative gene copy numbers

Confirmation of already reported gene copy number variations (paper IV) and the ones observed using microarrays (paper I) was performed using specific primers to genes of interest and the endogenous control genes *β-tubulin* and *seryl-tRNA synthetase* (exist as single copies in all parasites genomes studied to date). Amplifications were performed in at least triplicate reactions containing SYBR Green mastermix, primers (100-900 nM) and approximately 2 ng of template. PCRs were carried out in an ABI 7500 real-time PCR system in 40-45 cycles (95°C for 15 sec, and 60°C for 1 min). Relative gene copy numbers were computed according to the ΔΔCt method with 3D7 or NF54 serving as calibrator samples and with *β-tubulin* as the choice of endogenous control gene for the analyses.

3.6.2 Relative gene transcription levels

In-depth analysis of *var* gene switching in paper III was achieved in a similar way, but with the use of reverse transcribed RNA as template and two endogenous controls known to have display stable transcription throughout the intraerythrocytic cycle (*seryl-tRNA synthetase* and *fructose biphosphate aldolase*). Levels of *var* gene transcripts were correlated to that of endogenous controls as ΔCt values.

3.6.3 Allelic discrimination

Real-time PCR discrimination of duplicated *var2csa* and *Pf332* genes in the HB3 parasite strain was achieved using primers and allele discriminative FAM or VIC labeled MGB-probes. Assays were designed based on the fully sequenced 3D7, FCR3, Dd2 and HB3 genome and validated with amplification of dilution series of HB3 gDNA, and mixtures of FCR3 and NF54 gDNA. Allelic discrimination (principle outlined in Figure 4) was performed on both gDNA and cDNA from whole parasite cultures and single cells, using primers (900 nM) and discriminative probes (200 nM). Amplifications were conducted as described above, but with an initial pre-read (for background fluorescence measurement before amplification) and a final post-read (for total fluorescence emission measurement after amplification). Relative allele frequencies were determined from ratios of fluorescence from the allele specific probes.

3.7 FLUORESCENT *IN SITU* HYBRIDIZATION

Fluorescent *in situ* hybridization (FISH), mainly used for the physical localization of genes in intact nuclei or chromosome spreads, was for the first time successfully performed in *P. falciparum* by Freitas-Junior et al (302). Unable to perform equally successful experiments using the published protocol, we developed a modified version in order to study duplicated genes (paper I) and the localization of transcriptionally active and non-active *var* genes in relation to telomeric repeat regions (paper III). Double stranded DNA probes were generated by PCR amplification of ~1 kb fragments of genes of interest. Fragments were subsequently either cloned into plasmids and propagated in competent cells before labeling or directly labeled. Fluorescein and biotin labeled probes were then column purified before ready to use. Parasites were isolated from their host erythrocytes using saponin and deposited as monolayers on either

regular or Poly-L-lysine coated microscope slides. Monolayers were air-dried, fixed with paraformaldehyde (PFA) and extensively washed before treated with RNase. At no time post fixation was preparations allowed to dry again, something that in our hands led to preserved morphology of parasite nuclei. Labeled and heat denatured probes were added to cell preparations, importantly without any prior drying step, and yet again denatured together with the cellular DNA on the slides before hybridized. After stringent washing and post-staining of bound biotin probes with avidine conjugated rhodamine were preparations visualized using a Leica DMRE microscope and imaged with a Hamamatsu C4880 cooled CCD camera. To asses the localization of active and non-active *var* genes (PFD0630c, PFF0845c and PFL0030c/*var2csa*) in respect to telomeric repeat regions (Rep20), numbers of co-localized (where fluorescent probes of *var* genes overlapped with probes of telomeric repeats) versus distantly localized were counted in at least 500 parasite nuclei for each probe combination.

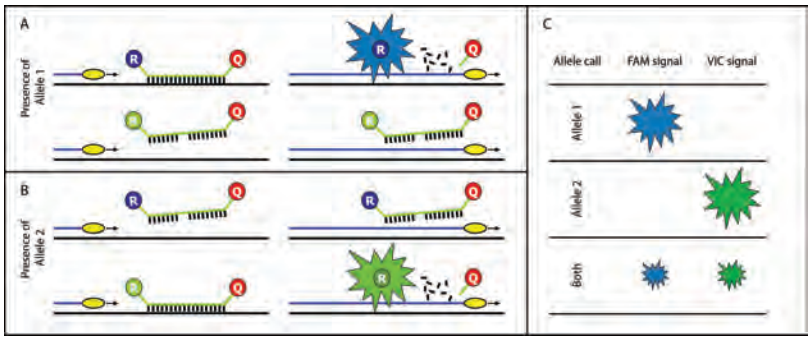

Figure 4. Principle of the real-time PCR allelic discrimination methodology. Two sequence-specific TaqMan MGB probes, labeled with different 5' reporter dyes (R), are used in each PCR amplification. Only probes with a perfect match hybridizes to the template sequence. Upon amplification, the Taq-polymerase cleaves the hybridized probe which separates the reporter dye and the quencher dye (Q), resulting in fluorescing reporter dye. **A)** In the presence of Allele 1 only blue light is emitted. **B)** In the presence of Allele 2, only green light is emitted. **C)** Allele calls (frequence of the two alleles) are deteremined from fluorescence detection of both dyes.

3.8 DBL1ALPHA RT-PCR AMPLIFICATION, SEQUENCING AND ANALYSIS

The degenerate PCR primer pair (α-AF/α-BR) previously published (303) was designed to amplify all possible DBL1α *var* gene sequences in a universal and unbiased manner. Using these primers we however experienced a bias towards a subset of sequences. Therefore complimentary primer sets were designed in order to elucidate dominantly transcribed *var* genes from heterogeneous mixes of transcripts in clinical isolates. The new oligonucleotides (nDBLf/nDBLr) primed different semi-conserved regions in the DBL1α than the primers designed by Taylor et al and led to an improved coverage in retrieved sequences when 3D7 gDNA was used as template of amplification. The newly designed primers were therefore used in amplifications of

reverse transcribed RNA from clinical isolates besides, and in combination with (α-AF/nDBLr), the α-AF/α-BR primers. PCR products from all three primer combinations were cloned and 48 inserts from each were cycle sequenced both in the forward and reverse direction using the MegaBace system. Several layers of quality filtering were applied to the sequence reads and corresponding translations before well defined consensus sequences were scored for within parasite dominance. The in-house designed MOTIFF *var* gene motif finder, designed to identify small motifs in pools of highly degenerate amino acid sequence sets, was thereafter used in order to identify potential DBL1α sequence tags over-represented in different malaria disease states.

4 ETHICAL CONSIDERATIONS

Ethical approvals for the human components used in the studies included in this thesis were obtained from research ethical committees in Uganda and Karolinska Institutet. Written informed consent was obtained from either patients themselves or parents / legal guardians for minor patients. Approvals for the generation of antisera in animals used in presented experiments were obtained from the ethical committee on animal research of Stockholm, section north.

5 RESULTS AND DISCUSSION

Results are detailed and discussed in each respective study included in this thesis (paper I-VI). The original results and interpretations of these are also summarized here, with additional discussions added in the light of more recent discoveries.

5.1 PAPER I

"Gene duplications and deletions are frequent in P. falciparum genomes"

Previous to this investigation, a few gene duplications had been shown to alter important traits of the parasite (304-306). We therefore wanted to understand how frequent copy number polymorphisms (CNPs) occur in the *P. falciparum* genome and also identify potential gene candidates for further explorations of CNPs and their impact on biology and virulence.

Seven laboratory strains (7G8, Dd2, F32, FCR3, R29, TM180 and TM284) and two clinical isolates (UAS31 and UAM25) of various geographic origins and with differences in several phenotypic traits were analyzed by CGH on 70-mer oligonucleotide microarrays using the 3D7 parasite as reference. Hierarchical clustering of the data revealed relatedness between parasites from the same geographical origin. Three parasites of Asian origin (Dd2, TM180 and TM284) clustered together and so did the two clinical isolates (UAS31 and UAM25) from Uganda. Three of the parasites (FCR3, F32 and R29) displayed near identical CGH results, suggesting a previous cross-contamination of these parasite strains. Relatively strict criteria were applied for detection of CNPs in the nuclear genomes of the different parasites in order to minimize the risk of sequence variation and experimental noise influencing the interpretation of the data. Using this strategy, 82 genes were identified as variable in copy numbers among all the strains and isolates relative to the 3D7 reference strain. Approximately half of these recurred among the investigated parasites, and some also to previously identified CNPs in other studies (307, 308), which implicates a presence of potential genomic hot spots where duplications and deletions are prone to occur. Identified genes clustered in 24 regions when superimposed on the genome build of 3D7, with sizes of these ranging from just under 1 kb to 110 kb and constituted of 1 to 22 genes. Validation of the microarray data was performed using real-time PCR, conventional PCR and FISH on a selected panel of variable and non-variable genes, and did in no case prove false positive or negative interpretations. Interestingly, besides confirming the amplification of the suggested erythrocyte invasion associated *surf4.1* and *PfRh1* genes (estimated to exist in 5-6 copies in the FCR3 and F32 genomes), the hybridization of FISH-probes towards the former also indicated a translocation of the duplicated genes to locations near the ends of different chromosomes. Apart from the genes suggested to encode proteins involved in invasion, a wide range of known or putative functions have been ascribed the proteins of identified copy number polymorphic genes. Even though the majority was of unknown function, other genes

were annotated as involved in cell cycle regulation, cell division, sexual differentiation and drug resistance.

In this study, relatively high numbers of CNPs were identified in a limited number of *P. falciparum* parasites. This finding, in conjunction with the acquired beneficial phenotypic traits observed, led us to propose that it is unlikely that these CNPs are selectively neutral. Increased amounts of genetic material to replicate will impose a cost for the parasite, a cost that must be balanced by increased chances of survival and adaptation to environmental changes. Supportive of this is the recent years surge in identification of various gene amplifications in *P. falciparum* genomes, mainly in response to antimalarials and resulting drug resistance traits (309-312). Besides the trendy approach of performing genome wide association studies in response to development of drug resistance, the same could be applied for associations of other parasite traits such as high multiplication potential or efficient sexual differentiation. This could generate a better understanding of the parasite biology and factors contributing to the virulence of the parasite.

5.2 PAPER II

"Complex transcriptional differences in isogenic clones with different adhesive phenotypes"

Both continuously grown laboratory strains and clinical isolates often contain several sub-populations with different phenotypes and sometimes also different genotypes. In order to study more homogenous parasite populations of interest as well as phenotypic traits of single parasites, the ability to clone single cells is a necessity. The possibility to study clonal populations of parasites is of major importance for studies of adhesive phenotypes and *var* gene transcription. This since the parasite rather quickly switches to transcribe and translate other *var* genes and PfEMP1 molecules respectively, often with a concomitant alteration in adhesive phenotype (280).

Single cell micromanipulation cloning was here used to generate a set of isogenic clones with specific and different adhesive phenotypes. 3D7S8.4 was selected based on the rosetting phenotype whereas the clones 3D7AH1S1-4 were selected from infected erythrocytes binding tightly to CHO-CD36 transfectants. The adhesive properties selected for were confirmed after short-term expansion of the clones. Receptor preferences for the 3D7S8.4 and 3D7AH1S2 clones were additionally evaluated in adhesion assays using a larger panel of soluble, cell-bound or immobilized receptors and competitive inhibition assays in case of observed binding. This revealed that 3D7S8.4, in addition to having a rosetting phenotype, also bound syncytiotrophoblasts on placental sections plus displayed a slight adhesiveness to CD36 (but not even close to levels seen for 3D7AH1S2). 3D7AH1S2 on the other hand was shown to bind TSP besides its strong CD36 binding capacity. In order to shed light on potential underlying transcriptional variations generating these differences in adhesive phenotypes we performed relative transcription profiling on microarrays. RNA was harvested at ring, trophozoite and schizont stages and used for comparative microarray hybridizations

between clones for each developmental stage. In total 262 genes were in this manner found differentially transcribed (≥ 2-fold change), out of which 100 genes were identified differentially expressed in rings, 113 in trophozoites and 49 in schizonts. Many of the encoded proteins from these genes were of unknown function, but for the ones with known or putative functions, the spectrum was wide. A particular focus was drawn to 15 genes that displayed the largest differences in relative transcription (≥ 5-fold change). Of these, three quarters were shown to harbor the host-cell targeting signal VTS/PEXEL, thus proposed to be transported towards the erythrocyte membrane and possibly involved in mediating the differences in adhesive phenotype.

The most striking transcriptional differences between the two clones were those involving *var* genes. One full-length *var* gene (PFF0845c) was found highly upregulated in 3D7AH1S2, whereas three full-length *var* genes were transcribed at higher levels in 3D7S8.4 (PFD0630c, PF08_0103 and PFL0030c/*var2csa*). The presence of PFF0845c and PFD0630c transcripts in respective clones was confirmed with northern blots, and western blots (using antibodies towards the conserved ATS domain) suggested these two to be the sole ones translated. The transcription of three full-length genes in 3D7S8.4 could either suggest a rapid transcriptional switch in the 3D7S8.4 clone or that single cells actually transcribe more than one *var* gene when they mature into trophozoites. A few reports by others have suggested the latter to be a possible scenario (313, 314) even though the literature arguing against is abundant (see chapter 1.4.2). Whatever the reason, the observation of only one translated protein suggests PFD0630c to be the only *var* gene in 3D7S8.4 participating in any of the binding observed with this parasite. Another intriguing finding in 3D7S8.4 was the placental binding, notably in the absence of translated VAR2CSA, no binding to CSA as well as no inhibition of this binding using HA or soluble CSA. Interestingly, one of the genes with unknown function, PFB0115w, which was found upregulated in 3D7S8.4 (≥ 5-fold change) has later also been identified by others as a potential mediator of placental sequestration. Substantially higher levels of transcripts and protein in parasites from pregnant women (315-317), with a trend of parity dependent immune recognition (316), argue for PFB0115w to mediate placental binding albeit via a receptor distinct from CSA. Taken together, this investigation thus presents important clues on PfEMP1-receptor specificities, novel sequestration mediating proteins as well as potential regulating factors in the maze of abundant and complex transcriptional differences.

5.3 PAPER III

"var gene switching and post-transcriptional regulation of var2csa"

Continuous switching of *var* gene transcription and corresponding PfEMP1 surface exposure allows the parasite to evade the host immune response and thus establish a chronic infection. To gain a better understanding of the succession of *var* gene switching and simultaneous effect on adhesive phenotypes we here studied two parasite clones with distinct adhesive and antigenic properties.

The same parasite clones (3D7S8.4 and 3D7AH1S2) that were generated and phenotypically assessed in paper II were continuously propagated *in vitro* for approximately 200 parasite generations without any enrichment or panning. Changes in gene transcription and adhesive phenotypes were monitored within clones during this continuous cultivation using real-time PCR, microarrays and various adhesion assays. The real-time PCR and microarray approaches revealed the initially transcribed *var* genes to be gradually down regulated over time, linked to a simultaneous increase of a single *var* gene transcript (*var2csa*) in both clones. Switch rates were computed and revealed differences between the two clones, with higher on-rate of *var2csa* (5.24% versus 1.35%) and higher off-rate of the initially transcribed *var* gene (10.15% versus 2.43%) in 3D7S8.4 than in 3D7AH1S2. A set of new sub-clones were generated from the 3D7S8.4 that had been propagated for 200 generations and 16 out of 17 of these showed an identical transcription pattern, with high levels of *var2csa* transcripts. That the transcripts were of full-length and correctly spliced was confirmed not only by successful real-time PCR amplifications using primers spanning the intron of the gene, but also by northern blots. Whether the switch of *var* gene transcription was associated with a physical perinuclear repositioning of the activated and repressed genes was evaluated using FISH. Probes towards the initially transcribed *var* genes (PFD0630c in 3D7S8.4 and PFF0845c in 3D7AH1S2) and *var2csa* were exclusively found hybridized to the rim of the nucleus irrespective of actively transcribed or not. The physical appearance of PFD0630c and PFF0845c did not change in relation to the telomeric repeats Rep20 upon altered transcriptional activity. *var2csa* however, revealed a clear difference in appearance in respect to Rep20. In the parasites with repressed *var2csa*, the gene was found almost exclusively co-localized with Rep20, whereas the opposite was observed in parasites with active transcription. Achieved results thus suggested perinuclear repositioning to be an activation mechanism for *var2csa* transcription. Upon switching of *var* gene transcription, a concomitant loss of original binding phenotypes (rosetting and CD36 biding) was also observed in both clones. Interestingly, despite abundant levels of full-length and correctly spliced *var2csa* transcripts, no binding to CSA or surface recognition of VAR2CSA (using antisera from *P. falciparum* exposed multigravidae and antibodies raised in animals towards specific domains of VAR2CSA) was observed with infected erythrocytes. In addition, immunoblots displayed relatively low levels of VAR2CSA in whole cell lysates. Taken together this led to the conclusion that translation and surface exposure of VAR2CSA, at least in part, is regulated on a post-transcriptional level.

The presented data of parasites switching to *var2csa* transcription with a concordant loss of PfEMP1 surface expression upon continuous *in vitro* growth is fascinating. Whether this suggests *var2csa* to represent an actor in an off-switch pathway employed by parasites not to exhaust the repertoire of expressed surface antigens, needs further proof. Parasites not expressing PfEMP1 on the surface *in vivo* would intuitively be considered undergo a fate of clearance by the spleen due to the inability to sequester. Supportive of this is the lack of mature trophozoite infected erythrocytes in the peripheral circulation of malaria patients. In splenectomized humans and primates, on the contrary, parasite infected erythrocytes have been found without PfEMP1 on the surface with a concordant abundant presence of mature trophozoites in the periphery (233, 318, 319). If indeed an off-switch pathway exists *in vivo*, *var2csa* may not be the only *var* gene participating, since other clonal parasites have been noticed to switch to

other *var* genes than *var2csa* upon continuous cultivation (278). It is however unclear if these parasites also stopped expressing corresponding PfEMP1s on the surface since the adhesive properties of infected erythrocytes were not investigated. Whether *var2csa* is truly an off-switch variant or not, the data presented here do shed considerable light on the possible regulatory mechanisms behind transcription and surface exposure of this maternal malaria associated virulence factor. The notion of *var2csa* being a target of post-transcriptional regulation, as suggested here, has indeed been subsequently supported by Amulic et al (320). The translational repression was here assigned the conserved upstream open reading frame (uORF) found intimately linked to the *var2csa* gene (244), a mechanism that has been observed in other species (321).

5.4 PAPER IV

"Simultaneous transcription of duplicated var2csa genes in individual parasites"

Sequence polymorphisms are often introduced in duplicated genes, either during the event of amplification or through subsequent mutations. Introduced alterations such as SNPs, insertions and deletions, can result in creation of pseudogenes (suggested to be abundant in *P. falciparum* (237)) or fully functional genes. If transcriptionally functional, the corresponding gene product can be of altered (loss, gain or antimorphic) or retained function as a result of these mutations. Hence, a linear correlation between gene dosage and transcription level cannot be automatically assumed. Even though close to perfect linearity has been observed in the case of a few duplicated genes in *P. falciparum* (301, 322, 323), the contrary has also been shown (301, 322). In addition, assumptions of linearity between gene dosage and biological effect cannot be drawn. To determine the impact on biology imposed by gene duplications, these issues therefore require either experimental testing or strong evidence of biological association.

The *var2csa* and *Pf332* genes were previously found duplicated in the genome of the HB3 parasite (241, 324). Sequence variations were here used as the base in the design of real-time PCR allelic discrimination assays intended to be used for elucidation of transcriptional activity of the amplified gene copies. *var2csa* and *Pf332* sequences of the fully sequenced HB3, FCR3, 3D7 and Dd2 parasite genomes were retrieved from database repositories. Primers towards conserved regions and FAM and VIC labeled TaqMan MGB probes towards variable regions were so designed to discriminate the different gene copies (alleles) in HB3, with the other parasites serving as controls for the individual alleles. This resulted in two assays for *var2csa* (towards DBL2x and DBL4ε) and one for *Pf332* (towards the S326P mutation). Relative copy number estimations were performed (using the primers of designed assays only) before any allelic discrimination experiment was conducted. In all of these, HB3 was shown to harbor two gene copies, thereby confirming the presence of duplicated genes but also suggesting three additional DBL4ε fragments in the genome sequence to be due to misassembly. The allele discriminative assays were subsequently and successfully used to distinguish all alleles on gDNA level of HB3, FCR3, NF54 (ancestor of 3D7) and

Dd2. *var2csa* transcripts in three of these parasite lines (HB3, FCR3 and NF54) and their CSA binding counterparts were analyzed using the same allele-discriminating approach. Transcriptional activity was confirmed for both *var2csa* genes in HB3 and HB3CSA as well as the respective single alleles in NF54/NF54CSA and FCR3/FCR3CSA. Transcripts of both *Pf332* copies were similarly shown to be present in the HB3 parasite, signifying transcriptional functionality off all duplicated alleles. Single HB3CSA parasites, collected using micromanipulation, were further analyzed with a nested PCR / real-time PCR approach using the assay towards the DBL2x region. Intriguingly, both allele types were shown transcribed in individual parasites collected at 24±4 hrs post invasion, independent of the use of reverse transcription priming procedure (random primers, oligo(dT), specific primers or combinations thereof).

With this study we demonstrate a real-time PCR allelic discrimination approach, which was successfully used to discriminate between highly similar duplicated gene copies in *P. falciparum* parasites. The results achieved from the single cell transcription experiments using this approach are intriguing since the transcription of *var* genes at mature trophozoite stage is presumed to be mutually exclusive ((274-277). Both *var2csa* copies of HB3CSA were here shown simultaneously active in individual cells, thereby challenging this dogma of mutual exclusive transcription. Yet, the case of simultaneous activity of the duplicated *var2csa* could be a special case and questions remain regarding how this is mechanistically achieved. The high sequence similarity (compared to sequence similarities in between *var* genes in general) possibly suggests the presence of a *var2csa* specific transcription factor with preserved DNA-binding regions associated with the duplicated gene copies. In addition, the two copies have been shown adjacently localized in the subtelomeric region of chromosome 12 in HB3 (241). Perhaps this allows the duplicated genes to reposition together to a transcriptionally active zone with beneficial chromatin environment in the nucleus, or by any other means (see chapter 1.4.2) circumventing the strict rules of mutual exclusive transcription. The interesting fact that transcripts of both alleles were detected using only oligo(dT) primers in the reverse transcription, suggests transcripts to be destined for translation. The presence of the 3´ poly(A) tail, added through polyadenylation in the maturation process of the mRNA, is however not enough to claim that both alleles are translated. Translational repression by the 5´uORF (highly conserved uORFs are indeed present upstream of both *var2csa* copies in the HB3 genome) could circumvent this, to only allow one of the alleles being expressed. All this taken together, discriminating transcriptional activity could generate a better understanding of the impact of gene duplications on the biology of the parasite, as well as of molecular aspects of the pathogenesis of malaria. This is here exemplified by the finding of yet another layer of complexity on the issue of antigenic variation.

5.5 PAPER V

"Degenerate PfEMP1-DBL1α motifs over-represented in parasites from patients with severe malaria"

Due to the fact that *var* genes are transcriptionally regulated (with the only known exceptions being *var1csa* and *var2csa*) and expressed in a mutual exclusive manner, *var* gene sequences retrieved from parasites at trophozoite stage should correspond to the antigen expressed on the infected erythrocyte (274-277). This concept constituted the base in the characterization and comparisons of *var* gene sequences transcribed by parasites from patients with different disease states.

P. falciparum infected blood samples were collected from children with severe or uncomplicated/mild disease at two different locations with distinct panorama of endemicity. The first group of patients was from Apac, Uganda, where the transmission is pan-seasonal and the rate of infective mosquito bites (1563 per person and year) is the highest recorded in the world to date (325). A number of patients were also recruited from Mulago hospital in Kampala, Uganda, where the transmission is instead holoendemic/mesoendemic. Concurring well with the endemicity, severely ill patients from Apac suffered mainly from respiratory distress syndrome whereas those from Kampala had an over-representation of cerebral malaria (see chapter 1.3.3.3). A total of 96 patients were thus sampled and of these suffered 52 from severe disease and 44 from mild malaria. Freshly collected parasites were cultured until the majority had propagated into mature trophozoites before rosetting rates and the presence of giant rosettes was noted. Both these traits were shown to be significantly higher in parasites from severe cases than from mild. Trophozoites were enriched and then extracted for total RNA that was reverse transcribed and PCR amplified using three sets of degenerate DBL1α primers. Amplified products were sequenced, retrieved sequences assembled, and corresponding contiguous sequences analyzed. Apparent from these was a high abundance of unique *var* transcripts in each isolate, ranging from a minimum of 9 to a maximum of 72. After translation of the sequences, the three most dominant contigs from each isolate were fed into the MOTIFF finder. Interestingly, six degenerate motifs were shown statistically over-represented in highly rosetting isolates and isolates from children with severe malaria (three in each group). When the severe cases were further sub-divided into groups based on the clinical manifestations observed in patients (cerebral malaria, malaria NUD and respiratory distress) 12 additional motifs were identified. Kullback-Liebler plot analysis coincided with the location of statistically over-represented motifs in majority of cases and thereby strengthened the results achieved using MOTIFF. To take the analysis of over-represented motifs further, we mapped them to three well-characterized DBL1α domains (from FCR3S1.2var1, R29var1 and VarO) structurally modeled using the EBA-172 F2 domains as template. We observed that motifs preferentially mapped to putative alpha-helical semi-variable sequences flanking hyper-conserved domains. These were here argued to be the parts of DBL1α mediating binding, since the hyper-conserved domains and also hyper-variable loops are unlikely to interact with various receptors due to a likely location in the interior scaffold for the former and too high redundancy to interact with receptors for the latter.

The data presented here, where DBL1α sequences were in-depth scrutinized down to the level of short motifs, does not only present an important methodological advance but also important clues on potential virulence associated ligand-receptor interactions. Apart from the motifs identified over-represented in different disease states, the mass of

sequences generated also supported previous findings of low numbers of cysteine-residues in the block III of DBL1α to correlate to rosetting (256, 258-260). Rosetting has by now been shown repeatedly to be a prominent phenotype among parasites causing severe disease (159, 197-202), and the data presented herein could represent important tools for combating this virulence trait. The motifs are potential candidates for the construction of adhesion-blocking antibodies, and such work is in progress. However, whether possible to generate cross-reactivity of antibodies raised towards these DBL1α motifs remains to be seen, but if so they could possibly act beneficially not only through release and clearance of sequestered parasite loads, but also through hampering invasion (discussed in paper VI).

5.6 PAPER VI

"Merozoite invasion is facilitated by PfEMP1 mediated rosetting"

The peripheral and sequestered biomass of *P. falciparum* parasites has been shown to be higher in patients suffering from severe malaria than in children with uncomplicated disease (130). Whether this difference is dependent on factors of the host, parasite or combinations thereof is not fully understood. Underlying variations in the ability of parasites to multiply could potentially be such a factor, and are therefore investigated here.

The problems involved in adaptation of clinical isolates to growth *in vitro* have historically hampered proper phenotypic characterizations of isolates recently collected from patients. For comparisons of multiplication potential and other traits between cryopreserved isolates, an optimization of suitable growth conditions was therefore essential. In paper VI, we show cultivation involving growth in suspension on an orbital shaker (326) and the use of fixed gas to maintain a stable microaerophilic environment were conditions of choice. All isolates could thereby be established in culture with good outgrowth, higher rates of multiplication, few numbers of multiple invaded erythrocytes and preserved adhesive capacity over time compared to other culture conditions tested. 76 clinical isolates, a large part the same as in paper V, were under these conditions assessed for their outgrowth, multiplication rates, timing and rates of rosetting as well as frequency of multiple invaded erythrocytes. An early observation with all clinical isolates, though at variable rates, was the formation of rosettes at the stage of schizogony with a concordant invasion of bound uninfected erythrocytes. This led us to also investigate various laboratory strains for their timing in rosetting. Parasites previously selected for the rosetting phenotype at trophozoite stage, known to express high levels of PfEMP1 on the infected erythrocyte surface, were also shown to rosette to high degrees during schizogony. Continuously grown, non-selected parasite clones and strains, with little or no PfEMP1 on the surface, did however also rosette but at much lower levels. All this argued for PfEMP1 to be the main mediator of rosetting also at the schizont stage, and that observed phenomenon diminishes in the absence of adhesion-selection or immunological stimuli. Of the 76 clinical isolates, 36 originate from children with severe malaria and 40 from children with uncomplicated disease. When compared, the severe group was found to multiply and rosette (both at

trophozoite and schizont stage) at significantly higher rates. Interestingly, the rate of multiplication of individual isolates was shown to positively correlate to the original parasitemia at the time of sampling as well as to the individual rosetting rates. From this it was hypothesized that rosetting is associated to the parasites ability to invade new erythrocytes. To test this, rosette disruption and invasion inhibition experiments were carried out on a panel of laboratory strains and clinical isolates using reagents targeting the three levels of interaction in rosettes. Antibodies towards the presumed parasite ligand PfEMP1 and bridging non-immune human IgM and IgG as well as different GAGs including the erythrocyte receptor HS, were all shown to affect the invasion, but only for parasites exhibiting sensitivity to rosette disruption with the same reagents. Taken together, the link unveiled between merozoite invasion, rosetting and the pathogenesis of severe malaria suggests that rosetting promotes the growth of *P. falciparum* by creating a favorable, close proximity to the uninfected red blood cell.

That there would be a direct link between merozoite invasion and rosetting has been hypothesized before (203), but all attempts to prove this have previously failed (327, 328). The reasons for observed discrepancies between these studies and the one presented here are probably due to the chosen experimental procedures. For example, in these studies invasion rates were monitored using rosetting and non-rosetting parasites from the same parental strains, with the rosetting and non-rosetting counterparts selected using either plasmagel flotation or centrifugation through percoll. Both these types of selection could select for unwanted parasite features, such as parasites harboring subtelomeric deletions, possibly confounding the results. Here, we instead performed all experiments on identical cultures and with rosette disruption achieved with specific reagents. The results presented herein could, apart from increasing the understanding of the pathology seen in severe malaria, have an impact on the development of malaria vaccines, as PfEMP1 may be the target of both growth and sequestration inhibitory antibodies. Rosetting-assisted invasion, leading to a minimal exposure of the merozoites, could also be a possible explanation to why subunit vaccines aimed at the merozoite surface have shown only marginal success in generating protection in human trials (329).

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