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REGULATION OF *PLASMODIUM FALCIPARUM* VIRULENCE GENES AND IMMUNE RESPONSE TO SURFACE ANTIGENS IN PLACENTAL MALARIA

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ABSTRACT

Malaria infection caused by the parasite *Plasmodium falciparum* is a deadly torment, especially for young children and pregnant women residing in sub-Saharan Africa. Much of the parasites virulence is due to its ability to constantly vary the adhesive molecules expressed on the surface of infected red blood cells (iRBCs). This antigenic variation permits the parasite to successfully sequester in various organs and tissues, thereby causing adverse effects and the clinical symptoms of malaria as well as enabling evasion of the host immune response. However, protective antibodies against surface exposed antigens are developed via exposure to infection, explaining the partial immunity seen in adults living in endemic areas. Another important aspect of the deadliness of *P. falciparum* is its astounding ability to successfully proliferate and multiply within the RBC. Numerous genes encode proteins that allow the daughter merozoites to effectively invade new RBC. While antigenic variation is a well-studied phenomenon in pathogens, very little is known concerning the regulation of invasion genes. In this thesis, we have explored both epigenetic regulation and immune recognition of *P. falciparim* virulence genes.

The *var* gene encoded *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) VAR2CSA is the main adhesin involved in placental malaria. We investigated the differential recognition of various VAR2CSA DBL-domains by immune sera from pregnant women and found DBL5ε to be widely recognized in a gender and parity-specific pattern. Further studies revealed that while the affinity of acquired antibodies to DBL5ε is similar between primigravidae and multigravidae, HIV co-infection impair the binding capability of these antibodies in women in their first pregnancy. Transcriptional regulation of *var2csa* as well as other *var* genes has been shown to be a complex and tightly regulated process. Our studies on duplicated *var2csa* paralogs in the *P. falciparum* strain HB3 revealed simultaneous transcription of both alleles. This suggests a less strict *var* gene regulation than previously thought and questions whether PfEMP1s are mutually exclusive expressed. Our findings support the presence of an active *var* gene expression site in the nuclear periphery but also suggest additional layers of gene regulation to be important, such as trans-factors and histone modifications. The five *P. falciparum* histone deacetylases are interesting therapeutic targets but have not been extensively characterized. By using reverse genetics techniques, we were able to create a conditional knockdown of the class II histone deacetylase PfHda1. The phenotypic change upon PfHda1 knockdown suggests this protein to be essential for cell cycle progression and successful proliferation but also for differential expression of invasion ligands. Moreover, dysregulation of *var* gene expression is seen in PfHda1 knockdown parasites, which provides insight into mechanisms behind virulence gene regulation in the context of histone modifications. To conclude, we here present a multi-faceted study of mechanisms behind multi-family gene expression important for parasite virulence and explore the complexity of antibody acquisition to VAR2CSA.

LIST OF PUBLICATIONS

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

I. **Brolin K.J**., Persson K.E., Wahlgren M., Rogerson S.J. & Chen Q. Differential recognition of *P. falciparum* VAR2CSA domains by naturally acquired antibodies in pregnant women from a malaria endemic area.
PLoS ONE 2010, 5(2):e9230

II. **Brolin K.J**., Ribacke U., Nilsson S., Ankarklev J., Moll K., Wahlgren M. & Chen Q. Simultaneous transcription of duplicated *var2csa* gene copies in individual *Plasmodium falciparum* parasites.
Genome Biology 2009, 10(10)

III. **Brolin K.J**., Ribacke U., Coleman B., Wirth D.F., Wahlgren M., Chen Q. & Duraisingh M. Characterization of a novel *Plasmodium falciparum* class II histone deacetylase important for cell cycle progress and virulence gene expression.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ANC	Antenatal care
ARDS	Acute respiratory distress syndrome
ATS	Acidic terminal segment
CIDR	Cysteine-rich interdomain segment
CM	Cerebral malaria
CNV	Copy number variation
CR1	Complement receptor 1
CSA	Chondroitin sulfate A
CSP	Circumsporozoite protein
DBL	Duffy binding-like
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding-like
FISH	Fluorescent *in situ* hybridization
HDAC	Histone deacetylase
ICAM1	Inter cellular adhesion molecule 1
IDC	Intraerythrocytic developmental cycle
IFA	Immunofluorescence Assay
IPTp	Intermittent preventetive treatment in pregnancy
iRBC	Infected red blood cell
KAHRP	Knob-associated histidine-rich protein
LBW	Low birth weight
MAHRP	Membrane-associated histidine-rich protein
MC	Maurer's cleft
MESA	Mature parasite-infected erythrocyte surface antigen
MTCT	Mother to child transfer
ORF	Open reading frame
p.i.	Post invasion
PAM	Pregnancy-associated malaria
PECAM1	Platelet endothelial cell adhesion molecule 1
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PTM	Posttranslational modification
PVM	Parasitophorous vacuole membrane
RESA	Ring parasite-infected erythrocyte surface antigen
RIFIN	Repetetive interspersed protein
RBL	Reticulocyte binding-like
SA	Sialic acid
SP	Sulphadoxine-pyrimethamine
SPR	Surface Plasmon resonance
STEVOR	Subtelomeric variable open reading frame protein
SURFIN	Surface associated interspersed protein
TNF	Tumor necrosis factor
TSP	Thrombospondin
TVN	Tubovesicular network
VCAM1	Vascular cell adhesion molecule 1
VSA	Variant surface antigens

1 INTRODUCTION

1.1 GLOBAL BURDEN OF MALARIA

Before Alphonse Laveran identified parasites in the blood of malaria patients in 1880, it was thought malaria was caused by bad air, something that also gave the disease its name; mal'aria, bad air in Italian. The disease however, goes further back than the 19th century and references to malaria occur in texts as old as 2700 BC [1]. Almost 20 years after the findings of Laveran, Ronald Ross discovered that a *Plasmodium* species is transmitted by the bite of an infected mosquito [1]. The discoveries of Laveran and Ross paved the way for extensive efforts to eradicate the disease for many years to follow. Nevertheless, today approximately 225 million clinical cases are reported world wide with almost 1 million deaths occur every year [2]. Half of the worlds population still live in malaria endemic areas [3,4] despite massive efforts to eradicate malaria from 1945 [5]. 90% of malaria morbidity and mortality occurs in sub-Saharan Africa [6,7] affecting a population already vulnerable due to other diseases, widespread poverty, lack of infrastructure and inadequate health care. There are several inherent reasons to why sub-Saharan Africa is so badly affected by malaria, such as a high base case reproduction rate of infection due to a favorable climate and the extraordinary capacity of parasite transmission by *Anopheles gambiae,* the main vector mosquito in Africa [8].

Young children and pregnant women are most severely affected by malaria, with children under five encompassing the majority of deaths [4]. Not only is malaria one of the worlds biggest killers among infectious diseases, it also hampers the economic development in affected regions. Sachs and Malaney have demonstrated a striking correlation between malaria and poverty with the probability that one is increasing the other [8]. Aside from the huge cost of wasted human lives, malaria is also expensive for the individual in terms of cost for prevention, diagnosis, treatment and loss of income due to illness. Furthermore, malaria is costly for the society as a whole, with high expenditure for vector control, health facilities and proper drugs [8]. In 2010, international funding invested 1.8 billion US$ in malaria treatment and research when WHO estimates that at least another 4 billion US$ is needed in order to effectively control malaria [2].

1.1.1 Controlling malaria

As of yet, there is no effective vaccine to malaria and the parasite is rapidly becoming resistant to existing drugs. The first control measures against malaria were introduced shortly after the discovery that the disease is mosquito-borne. After measurements such as window and door screens, control of mosquito breeding sites and the use of DDT, several countries managed to eradicate malaria by 1946 [4]. The Global Malaria Eradication program, initiated by WHO in 1955 added chloroquine to the line of measurements, which helped another 27 countries to get rid of malaria by 1969 [4], making the disease what it still is today – a plague for tropical and subtropical poorer regions. Chloroquine was long the drug of choice for malaria treatment, until resistant emerged in South East Asia and South America in 1960's [9], further spreading to Africa in the 1980's [10]. This led to the use of sulfadoxine-pyrimethamine (SP) treatment, which caused resistant parasites already a year after introduction [10]. The 1998 Roll Back Malaria effort included vector control such as long-lasting insecticide

treated bed nets and indoor residual insecticide spraying as well as improved diagnostic tools and treatment [2]. Artemisinin-based combination therapies (ACT's) are mostly used as a first line treatment today but new treatment variations are required due to the constantly emerging drug-resistance [11]. A highly effective vaccine is severely needed in order to properly combat malaria. However funding for malaria vaccine development is scarce [12] and the complexity of the parasite life cycle ([13,14], antigenic variation [15] and lack of knowledge concerning parasite interaction with the human immune system [16] represents further challenges. Currently, the RTS,S vaccine that target the pre-erythrocytic stage, is the most advance developed vaccine. Phase II clinical trials indicate this vaccine to have a rather short-lived 30-50% protection against clinical malaria in African children [17,18,19,20]. Since repeated exposure to malaria induce partial immunity, a vaccine targeting the asexual parasite stage seems reasonable. However, while several blood-stage antigens are in clinical development as vaccines [12], no efficacy has been seen so far [21,22]. Ideally, a vaccine would involve antigens from various stages in the parasites life cycle and be effective against establishment of infection, induce protective antibodies against the asexual stage as well as hinder further transmission. Unfortunately for all children, pregnant women and other people affected by malaria, there is still a very long way there.

1.2. PLASMODIUM SPECIES

There are five *Plasmodium* species known to infect humans, *P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi*. *P. falciparum* is causing the majority of mortality and is also the parasite most prevalent in sub-Saharan Africa where it accounts for 75% of all malaria infections [2]. The previously largely neglected *P. vivax* is getting increased attention as an important cause of morbidity and mortality and is also the most widespread of the human malaria parasites [23]. So far, efforts to grow *P. vivax* longterm *in vitro* have failed, making extensive studies difficult to perform. *P. vivax* causes endemic malaria throughout most of the tropics as well as in certain temperate regions in central Asia. An estimated 130 to 390 million people are infected every year [4] and 2.6 billion people are at risk for infection [24]. *P. ovale* and *P. malariae* are relatively rare and both cause a benign form of malaria. Whereas *P. vivax* and *P. ovale* can remain in the liver as hypnozoites for years before causing infection, the remaining three human parasites do not cause these kinds of relapses. Endemic *P. ovale* occur only in western Africa and at some isolated spots in Southeast Asia and Oceania. The geographical distribution of *P. malariae* is similar to that of *P. vivax,* however much less prevalent [25]. The fifth human *Plasmodium* species, *P. knowlesi* was recently found to not only infect macaque monkeys, but also being able to cause severe disease in humans residing in Malaysian Borneo [26]. It is likely however, that many earlier cases of *P. knowlesi* have gone surpassed due to its morphological similarity to *P. malariae* in blood smears [27]. *P. knowlesi* have repeatedly shown its capability to be a very fast killer despite the relatively low overall mortality rate, and is also restrained by its jungle-dwelling vector, *Anopheles hackeri* [28,29,30,31].

1.2.1 Plasmodium falciparum life cycle

The life cycle of the protozoan parasite *Plasmodium falciparum* is complex, and involves both a human host and a mosquito vector. Human infection is initiated when an infected female *Anopheles* mosquito injects 10-100 sporozoites into the human dermis, from where they continue to the blood stream before finally reaching the liver. Not every infectious mosquito bite results in infection as some sporozoites remain in the dermis and others enter the lymphatic circulation and are degraded in the lymph

nodes [32,33]. Once in the liver, the parasite invades hepatocytes and within the next 10-12 days replicates to form up to 30 000 merozoites. These are then released into the blood stream and subsequently invade red blood cells (RBCs), commencing the 48h asexual intraerythrocytic developmental cycle (IDC). The clinical manifestations of malaria take place during the IDC where parasites develop from young ring-stages to trophozoite stages before entering schizogony. The infected RBC (iRBC) is subsequently ruptured and 12-32 merozoites that can invade new RBCs are released. Upon various environmental cues [34] some parasites differentiate into sexual male and female gametocytes that, when ingested by a feeding mosquito, fuse in the mosquito midgut to form a zygote. The zygote then develops into a motile and invasive ookinete. After successfully traversing the midgut epithelium the ookinete develop into the oocyst stage that after multiple nuclear divisions render several thousands haploid sporozoites [35]. These subsequently migrate into the salivary glands and ducts of the mosquito, completing the life cycle of this deadly parasite [36].

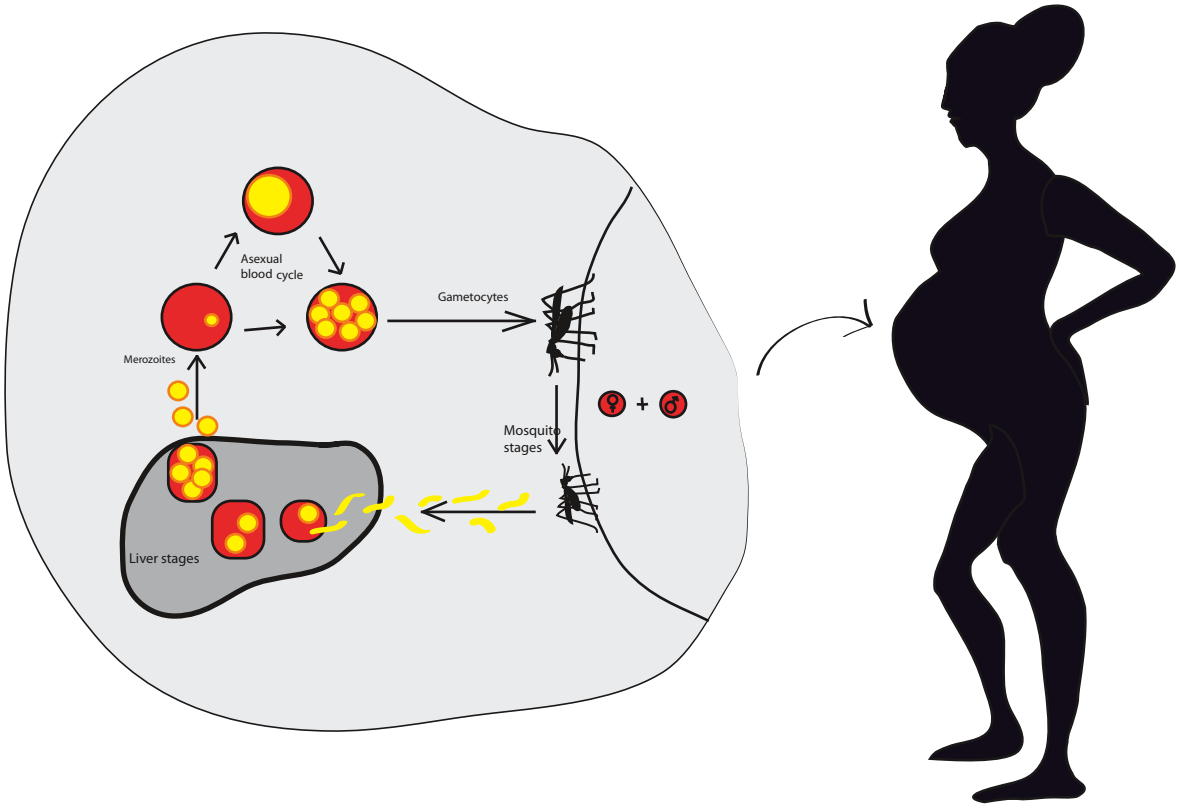

Figure 1. *P. falciparum* life cycle. Illustration by Jannike Simonsson.

1.3 TRANSCRIPTIONAL REGULATION IN *PLASMODIUM FALCIPARUM*

Transcriptional control in *P. falciparum* differs significantly of that from other eukaryotes despite retaining many of the canonical characteristics of eukaryotic transcription. The malaria parasites genome is extremely AT-rich, especially in the intergenic sequences [14], this perhaps being the reason for the believed paucity of specific transcription factors. Recently however, a family of DNA-binding putative transcriptional regulators was identified in apicomplexans [37]. These ApiAP2 proteins contain a version of the Apetala2/ethylene response factor (AP2/ERF) DNA binding domain, which is present in numerous plant transcription factors [38]. Currently, 27 members of the ApiAP2 family have been indentified in *P. falciparum* [14] and the various targets and functions are being unraveled. They are expressed not only throughout the asexual life stages but have also in been found to be important in gametocytogenesis [39], in the formation of ookinetes [40] and in the liver stages [41]. In *P. falciparum,* PfSip2 (PFF0200c) is implicated in *var* gene silencing via its role as a DNA tethering protein involved in heterochromatin formation [42].

Epigenetics is referring to inheritable changes in phenotype or gene expression caused by mechanisms other than changes in DNA sequence. DNA methylation, which generally plays a role in gene regulation in eukaryotes by addition or removal of methyl groups to or from bases in DNA, seems to be absent in *P. falciparum* despite the presence of a gene containing the DNA methyltransferase motif [43,44,45]. The RNA interference machinery is also lacking in the *Plasmodium* genome [14,46]. In *Plasmodium,* chromatin-mediated gene regulation is achieved through chromatin remodeling, posttranslational modifications (PTMs) of histones and replacement of core histones by histone variants. Change in nucleosome occupancy is common in eukaryotes where H2A is exchanged against H2A.Z in order to help promoter regions stay free from repressive nucleosomes [47]. This was recently shown to be the case also in *P. falciparum* and H2A.Z promote transcription by recruiting histone modifying/remodeling complexes and facilitating access for transcription factors [48].
PTMs include acetylation, methylation, phosphorylation, ubiquitination, poly-ADP-ribosylation and sumoylation [49]. The highly dynamic "histone code" is created by specific combinations of these, rendering the chromatin more or less accessible for downstream processes. In *P. falciparum,* the most plentiful marks are histone methylation and acetylation [50]. Histone acetylation is linked to active genes and lessens the attraction between the basic histone protein and acidic DNA by adding an acetate group to a basic amino acid on the histone tail. The reaction is catalyzed by histone acetyltransferases (HATs) of which several have been indentified in malaria parasites [51]. The *P. falciparum* genome contains five annotated histone deacetylases (HDACs) genes, encoding for enzymes that remove acetate groups from histone tails. HDACs in general can be divided into four different classes based on their primary structure. The I, II and IV enzymes share a zinc-dependent catalytic mechanism whereas the class III, sirtuins, utilize a NAD-dependent mechanism to catalyze the deacetylation reaction [52]. Class I HDACs are homologous to the yeast enzyme RPD3, exclusively found in the nucleus, acting on chromatin [53,54] whereas the class II HDACs have been shown to shuttle in and out of the nucleus and also deacetylase non-histone substrates [55,56]. These are generally larger proteins, sharing homology with Hda1 from yeast. Drug-target studies on PfHDAC1 shows it to be effectively inhibited by the human HDAC inhibitor Trichostatin A and SAHA [57,58] and the resulting hyperacetylation affects the global gene expression [59,60,61]. A recently published study showed that the drug Apicidin inhibit both class I and II HDACs in *P. falciparum*, which cause severe deregulation of the whole transcriptional cascade [60].

The class III sirtuins are related to the yeast Silent information regulator 2 protein and have been described among prokaryotes, eukaryotes and archaea [62]. *P. falciparum* Sir2a and 2b both belong to class III and have been extensively characterized. Among other things, they act important regulators of the *var* gene family [63,64,65]. Class IV enzymes, homologous to HsHDAC11 are less common among metazoans and also remain mostly uncharacterized. No class IV enzyme has been annotated in *P. falciparum* [14].

Histone lysine methylation is involved in both transcriptional activation and silencing. There are at least ten members of histone lysine methyltransferases (HKMTs) [66] in *Plasmodium,* as well as two families of lysine demethylases (LSD1 and JHDMs) [67]. The *P. falciparum* histones can also be modified via arginine methyltransferases (PRMTs) [68], ubiquitinating and de-ubiqutinating ezymes as well as by ATP-dependent chromatin remodeling proteins [69]. The PTMs creating the histone code are subsequently recognized by various effector molecules such as bromo- and chromodomains, Royal superfamily, plant homeodomain (PHD) fingers just to mention a few [70]. Despite the presence of several PTM-binding modules in malaria parasites, only one has so far been characterized ([69]. This PfHP1 is involved in H3K9me3 binding and dimerization, and has been shown to associate with both subtelomeric and intrachromosomal silent *var* genes [71].

These regulatory processes are all part of enabling successful proliferation and progression through the *P. falciparum* cell cycle. Gene expression in the malaria parasite is a complex continuous cascade where 60% of the genes are only expressed once during the life cycle, in close concordance with the function of the resulting protein [72]. This tightly synchronized but yet dynamic regulatory machinery ensures the establishment of successful infection by this deadly malaria parasite.

1.4 MALARIA PATHOGENESIS

In malaria endemic areas, non-sterile immunity against malaria is gradually developed. Older children and adults are less likely to develop severe disease but nevertheless remain vulnerable to infection and often sustain parasitemia without any clinical symptoms.

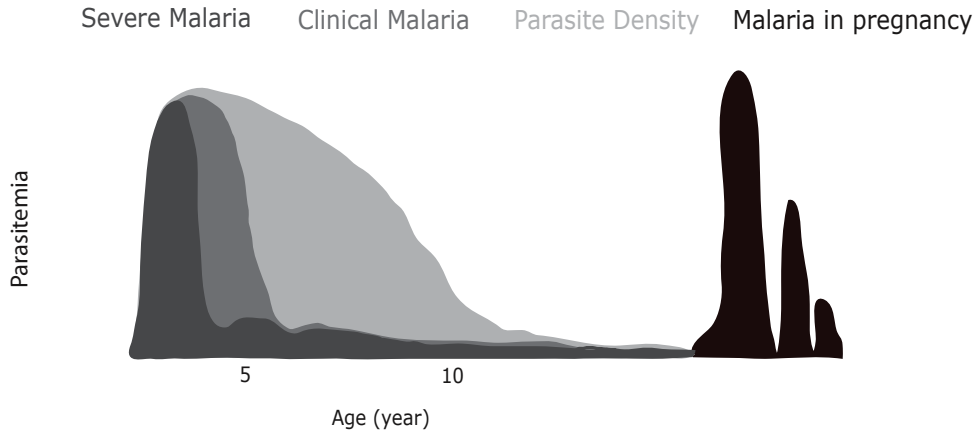

Figure 2. Acquisition of partial immunity to malaria infection

The situation is quite different in non-endemic areas where people of all ages are at high risk to develop complicated malaria. Clinical symptoms of malaria include fever, general malaise as well as other flu-like symptoms such as headache, body ache, vomiting, diarrhea, coughing and stomach ache. Hypoglycemia, hyperlactatemia, anemia and altered consciousness are other signs of malaria infection [73]. Non-treated, the infection can quickly develop into severe malaria since parasitemia can augment by several orders of magnitude within a few hours.

1.4.1 Immunological response to malaria infection

Mosquito-injected sporozoites are exposed to the human immune response for only a short period of time, and so far there is no evidence for naturally acquired protective immunity to this stage [74]. Despite this, volunteers inoculated with attenuated sporozoites produce antibodies that at least gradually protect from malaria infection [75]. The mechanisms behind this protection are unclear however, but it is possible that protection is due to the lower number of merozoites released from the liver when the amount of infective sporozoites is reduced. These sub-clinical levels of blood-stage malaria would then enable partial immunity to form and protect against severe infection [76]. The RTS,S vaccine consists of hepatitis B surface antigen (HBsAg) particles fused to the thrombospondin domain of the circumsporozoite protein (CSP) which is expressed on sporozoites and liver stage schizonts [77,78]. As mentioned above, this is the most advanced vaccine candidate to date.

Passive transfer of antibodies from the mother to the fetus protects the newborn for the first months of life [79]. After that, immunity to malaria is acquired via exposure to infection and the host immune response to this pathogen is very complex and poorly understood. Humoral immunity consists mostly of cytophilic IgG antibodies that are activating various functions of neutrophils and monocytes. Protective antibodies may target various stages of the *P. falciparum* life cycle, such as merozoite invasion and iRBC sequestration, and may also mediate phagocytosis of iRBC [80]. CD4+ T cells are an important part of the humoral immunity as helper cells for B-cells [81]. They are also part of the cell-mediated immunity to malaria by releasing inflammatory cytokines such as IFN-γ and IL-12 that activate macrophages and other cells to produce TNF, nitric oxide and reactive oxygen species [82]. Innate immunity cells such as dendritic cells, natural killer cells, Kuppfer cells and macrophages help stimulate and regulate the adaptive immune response via cytokine production [83]. There is a fine line however between protective adaptive immune response and excessive inflammation and severe pathology of the disease [84]. Despite the extensive knowledge of immune mechanisms to blood-stage infection, very little efficacy of vaccine candidates based on blood-stage antigens is obtained [21,22].

1.4.2 Severe malaria

Severe disease is most often characterized by high parasite density in a wide range of organs, tissues and blood vessels. Determinants of severe disease include host factors such as age, immune status, transmission rate and gender. Host genetic factors also play a part in disease severity. These include genetic variability in genes encoding host erythrocyte receptors as well as endothelial receptors, such as CD36 [85,86], ICAM1 (intercellular adhesion molecule 1) [87,88,89], PECAM1 (platelet endothelial cellular adhesion molecule 1) [90] and CR1 (complement receptor 1) [91,92] even though studies show non-uniform results. Malaria has also been suggested to be the selective force for various RBC disorders in human populations such as sickle cell disease and

α-thallasemia [93,94]. The manifestations of severe disease include unrousable coma, acute respiratory distress syndrome (ARDS), severe anemia, renal failure, splenomegaly and circulatory collapse. A subset of these, as well as the special case of pregnancy-associated malaria (PAM) will be described in more detail below.

1.4.2.1 Cerebral malaria

The mortality rate of cerebral malaria (CM) is close to 20% [95] and the pathogenesis is believed to be caused by massive parasite sequestration in brain microcapillaries via the receptor ICAM1 [96]. The immune response to malaria involves a significant increase of type 1 cytokines such as TNF-α and IFN-γ and these are causing an upregulation of surface expressed adhesion molecules such as ICAM1 and VCAM1 (vascular cell adhesion molecule 1). The heavy parasite load caused by sequestration via these receptors in the brain cause local hypoxia, vascular occlusion, inflammation and damage of the blood-brain barrier [97]. Unrousable coma is characteristic for CM and may arise either gradually or abruptly after severe seizures. The former is more common in adults whereas the latter is mostly seen in children [95]. In surviving CM patients, neurological sequelae and permanent brain injuries are widespread. This is most prevalent in children and includes epilepsy, speech and language difficulties, motor deficits and concentration disorders [98,99,100,101].

1.4.2.2 Respiratory distress

Acute respiratory distress syndrome (ARDS) in malaria is linked to high mortality and is one of the most severe manifestations of malaria caused by. ARDS is more common in adults than in children, and pregnant women and non-immune individuals are most vulnerable [102]. 20-30% of patients with complicated malaria develop ARDS [103,104] which via airflow obstruction, increased phagocytic activity and reduced lung function can lead to life-threatening hypoxia and respiratory failure [105]. ARDS is a common complication in PAM and can occur before, during or even after labor [103,104]. The pathogenesis of ARDS is not completely understood but studies have shown iRBC to sequester in lung capillaries [106,107]. This results in accumulation of monocytes and both pro-inflammatory and anti-inflammatory cytokines as well as endothelial cytoplasmic swelling and edema [108]. ARDS often co-exist with high parasitemia, acute renal failure, hypoglycemia, metabolic acidosis and bacterial sepsis, all which can worsen the prognosis for the patient [109,110].

1.4.2.3 Severe Anemia

Severe anemia is the major cause of malaria-related hospital admissions as well as morbidity and mortality in sub-Saharan Africa. It is defined as a hemoglobin level lower than 5g/dl or hematocrit beneath 15%. There are various mechanisms leading to anemia in malaria infection and the condition is further worsened by nutrition deficiencies, which are common in affected populations [111,112]. Rupture of iRBC, impaired erythropoiesis and loss of unifected RBC (uRBC) loss all contribute to amemia in malaria infection [113,114]. The spleen filters out altered RBCs, hence the need for the malaria parasite to sequester by binding to various endothelial receptors. Also, uRBC are often tagged by parasite molecules that are released during invasion [115,116,117], which leads to their destruction by the spleen. Phagocytosis of uRBC is also likely to contribute to anemia [118] as is hemozoin, released by the parasite during schizont rupture. The presence of heme-products alters the rigidity of surrounding RBC, thereby targeting them for splenic clearance [119]. Consequently, splenomegaly

is very common in malaria patients due to consequential splenic clogging by infected cells [120].

1.4.3 Placental Malaria

125 million pregnancies are at risk for malaria infection each year, and malaria during pregnancy cause 200 000 infant deaths annually in sub-Saharan Africa [121,122]. Despite having developed partial immunity to malaria through multiple infections since childhood, pregnant women are experiencing an increased susceptibility to infection. This is most pronounced in the first pregnancy and protective immunity is gradually built up in subsequent pregnancies [121,122,123,124,125]. The sex-specific, parity-dependent IgG recognition of variant surface antigens (VSA) seen with immune sera from pregnant women is characteristic for malaria in pregnancy and antibodies are protecting against adverse outcomes in later pregnancies [126,127,128]. The humoral immune response plays a huge role in the protection against placental malaria. Antibodies have been found to inhibit parasite binding to CSA [129] and opsonization of iRBC by macrophages is also an important protection mechanisms [58,130,131,132]. The majority of studies on malaria in pregnancy are from endemic areas. In low-transmission areas, women of all parities are equally susceptible to severe disease since less of a protective immune response has been produced [133]. The increased susceptibility to malaria in pregnant women is thought to be caused by pregnancy-associated immunological and hormonal changes [134,135] as well as the new niche for malaria parasites that the placenta constitutes.

Placental sequestration
P. falciparum parasites infecting pregnant women have the ability to sequester in the placenta and thereby avoiding both clearance by the spleen [136]. Mature iRBC are binding to chondroitin sulphate A (CSA) that is abundant on the syncytiotrophoblasts and in the intervillous space of the placenta. Hyaluronic acid (HA), also present in the placenta, [137,138] has been shown to be targeted for sequestration by parasites and it has also been suggested that non-immune IgG are acting like a bridge between adhesins on the iRBC surface and neonatal Fc receptors on the placenta [139]. Even though other proteins have been suggested to be involved in placental binding [140,141], VAR2CSA has repeatedly been shown to be the main culprit in mediating malaria in pregnancy [142,143,144,145]

Consequences for the mother, fetus and infant
Placental malaria is causing miscarriage, low birth weight, stillbirth and congenital malaria as well as maternal severe disease, anemia and increased morbidity [146]. Low birth weight (LBW) alone is causing half of the deaths attributed to malaria in pregnancy [147] and is defined as a birth weight less than 2.5 kg. Fetal growth restriction is the main cause for LBW and is probably caused by placental insufficiency due to the presence of parasites and substantial amounts of pigments in placental cells and fibers [148]. Acute infection however, particularly with high density of parasites is closely associated with pre-term delivery [149,150], which is also increasing risk of LBW. Maternal anemia is common in PAM and is further worsened by micronutrient (eg, iron and folic acid) deficiency [151]. Anemia might also be caused by the placental increase of pigmented monocytes, since these cells discharge inflammatory mediators that can hinder erythropoesis [152,153]. Increased amounts of cytokines are needed to eliminate parasites from the placenta but pro-inflammatory cytokines has also been shown to endanger the pregnancy, causing an immunological paradigm [154,155].

Thus, a precarious balance between the Th1 and Th2 response needs to be maintained in order to ensure a healthy pregnancy without parasites in the placenta.

Timing of infection
The severity of malaria infection in pregnancy depends on when in pregnancy it occurs. It is difficult to say however whether problems in late pregnancy might be caused by earlier infection. Malaria during the critical time of trophoblast invasion impairs remodeling of uterine spiral arteries and this is likely to hinder sufficient placental blood circulation later in pregnancy [156,157]. The mechanistic effects of large deposits of iRBC, monocytes and fibrins causing decreased blood flow as well as placental inflammation also hampers adequate nutrient transport to the fetus [158,159,160]. Placental malaria can also have severe effects on the newborn child. Congenital malaria is today recognized as a large problem and can cause both symptomatic and asymptomatic disease in neonates [161] with both fever and death being likely outcomes. Moreover, children are likely to be more susceptible to malaria later in life due to maternal malaria, but the mechanisms behind this is unknown. [162,163,164]. Also, transplacental transfer of maternal IgGs to other pathogens (such as measles, *S. pneumoniae* etc) is decreased when the mother is infected with malaria [165] whereas the effect on transfer of antimalarial antibodies remains unclear [146] as studies have shown ambiguous results.

Intermittent preventative treatment in pregnancy (IPTp)
The WHO guidelines for IPTp recommend at least two doses of SP given after quickening and with four weeks apart [166]. This strategy minimizes fetal toxicity of the drugs but leave women susceptible to malaria both during trophoblast invasion and placentation early in pregnancy as well as during the peak fetal growth later in pregnancy. As with all antimalarials, parasites resistant to SP is prevalent and alternative drugs are few due to unknown effects on the fetus. Artemisinin compounds are currently not recommended as treatment during the 1st trimester, but are considered safe for uncomplicated and severe malaria treatment later in pregnancy [167]. However, more studies on fetal toxicity are needed to complement both the IPTp and treatment drug collections. In addition, IPTp administration requires antenatal clinic (ANC) visits, something that not all women in malaria-endemic areas have access to. Therefore, the need is great for a functional vaccine against placental malaria.

1.4.4 Malaria co-infection with HIV

Malaria endemic areas overlap with areas where HIV is of high prevalence. In sub-Saharan Africa, 23 million people live with HIV [168] and nearly 250 million cases of malaria occur each year in the same area [2]. Malaria is the third largest cause of HIV-related morbidity, just after bacterial infections and drug-related events [169]. There is a higher prevalence of clinical malaria in HIV-infected children than in children without HIV and severe malaria is much more common in children over 1 year of age with HIV [170,171]. In non-pregnant adults, HIV infection is linked to increased cases of clinical malaria and higher prevalence and density of parasitemia [172,173,174] something that is especially severe in patients with extensive immunosupression [175]. This is signifying the importance of also considering HIV infection when discussing malaria treatment strategies and public health policies.

HIV and placental malaria
In pregnant women, HIV changes the pattern of acquisition of immunity where multigravidae generally are protected against malaria in pregnancy due to previous

pregnancies. When co-infected with HIV, this acquired protection disappears and renders women of all parities susceptible to severe disease [176]. HIV infection increases the risk of both clinical and placental malaria as well as the risk of maternal anemia, LBW and pre-term delivery [177]. Importantly, co-infection with HIV also undermines the efficacy of IPTp, indicating a need to evaluate current guidelines for both drug of choice and dosing regimen [178]. There are several mechanisms proposed to how HIV impairs the immune response to malaria in pregnant women. Studies are indicating that while there is no generalized suppression of immune response in pregnant women, the IL-12 mediated IFN-γ pathway is impaired by HIV infection, enabling the intracellular malaria parasite to proliferate without risk of being cleared by an active cellular response involving macrophages [179,180]. The humoral response towards several important *P. falciparum* antigens is also severely affected by HIV, which is most pronounced in women with advanced HIV infection [181]. Not only is HIV affecting the severity of malaria infection in pregnant women but malaria appears to also increase the HIV viral load [182,183,184]. Malaria infection causes up-regulation of pro-inflammatory cytokine production and increases the amount of macrophages and monocytes, both cell types targeted by the HIV virus [185,186]. The effect of malaria and HIV co-infection on mother-to-child-transfer (MTCT) is unknown. Studies have shown contradictory results, leading to the hypothesis that co-infection can be either protective or enhance MTCT, depending on the characteristics of placental infection and severity of HIV infection [187]. Important protective antibody functions such as phagocytosis are hampered by HIV infection in multigravidae [130,131,132] and a decrease in binding affinity of antibodies towards DBL5ε of VAR2CSA is shown in primigravidae [188]. Hence, changed antibody properties upon co-infection with HIV are important to consider within both treatment and preventative strategies.

1.5 PLASMODIUM FALCIPARUM VIRULENCE

The parasites ability to invade red blood cells and the cytoadherence of mature parasites to the host endothelium are both important virulence factors of *P. falciparum.* While the selective expression of the *var* gene family is central for sequestration, multigene families involved in invasion can also be variantly expressed. Further knowledge concerning these two processes is imperative in order to decipher regulatory mechanisms behind parasite virulence.

1.5.1 Invasion

RBC-invasion efficiency of *P. falciparum* is closely linked to the morbidity and mortality caused by this parasite. The invasion process starts with the egress of formed merozoites from its infected host cell, an intricate process that involves an increase in intracellular pressure and multiple biochemical changes [189]. In order for parasites to egress successfully, disruption of both the parasitophorous vacuole membrane (PVM) as well as the host-cell membrane is needed and these processes are in large mediated by various proteases [190,191,192,193] and kinases [194]. After egress, the merozoite needs to find, attach to and enter its new host cell, something that occurs in various steps. It is important that this extracellular stage is brief in order for merozoites to avoid recognition and clearance by the host immune response [195].

Initial attachment
P. falciparum merozoites have a plasma membrane and the basic cellular machinery of eukaryotic cells, as well as a plastid [196,197]. Additionally, it also contains several

invasion-specialized organelles located at the apical end, such as rhoptries, micronemes and dense granules [196,198,199,200,201]. The polar merozoite can attach to erythrocytes at any point of its surface and multiple merozoite surface proteins are implicated in this initial contact. The abundant merozoite surface proteins (MSPs) constitute a family of immunogenic proteins that are important in the initial contact of merozoite binding to erythrocytes [202,203,204]. MSP-1 has been most extensively studied and is a blood-stage vaccine candidate albeit rendering only low levels of protection [205,206]. The merozoite then reorients in order for the apical tip to face the erythrocyte membrane. An irreversible tight junction is formed and enables the parasite to enter the erythrocyte with the help of its actin-myosin motor [207] and simultaneous shedding of the merozoite protein coat [208]. The apical membrane antigen, AMA-1 is implicated in apical reorienteation of the merozoite [209] via interaction with rhoptry neck protein RON2 [210]. AMA-1 is essential for the invasion process, not only in *P. falciparum* but also in *Toxoplasma gondii* [210,211].

Secondary interaction

Several proteins located at the apical end of the merozoites play various secondary interaction roles in the invasion process, including the erythrocyte binding antigens (EBAs) and the reticulocyte binding-like homologue (PfRh) proteins [195,212]. The EBAs belong to the duffy binding like (DBL) family, which are orthologous of *P. vivax* DBL-proteins. PfRhs include PfRh1, 2a, 2b, 3, 4 and 5, and belong to the conserved multi-gene family reticulocyte binding-like (RBL) proteins. These proteins bind various receptors on the erythrocyte surface and mediate different invasion pathways, enabling the parasite to switch means of entering the host cell [213,214,215,216]. Invasion phenotypes are typically either dependent or independent on sialic acid (SA) residues of erythrocyte receptors. EBA-175, EBL1 and EBA-140 bind to glycophorin A, B and C respectively and mediate SA-dependent invasion [217,218,219]. EBA-165 is suggested to be a pseudogene and the receptor for EBA-181 is unknown albeit being SA-dependent [220]. The ligands for the PfRhs are unidentified except for the recent discovery that PfRh4 bind to CR1 and mediates a SA-independent invasion pathway [221,222]. While PfRh3 seems to be a pseudogene, studies show PfRh1 to bind erythrocytes in a SA-dependent manner [223,224]. Erythrocyte binding was recently demonstrated to be mediated by PfRh2a and 2b and both have been suggested to be important for merzoite invasion [215,225,226]. Interestingly, the native PfRh2a/b is processed near the N-terminus, yielding two different sized fragments that differ in their dependence on SA-residues on the RBC [227]. The atypical PfRh5 is smaller in size than the other PfRh's and lack a transmembrane domain, leading to the hypothesis that it is part of a larger protein complex [228,229]. Unlike the other PfRh's, PfRh5 disruption has been shown to be unachievable in all parasites tried so far, indicating essentiality for parasite invasion [228]. Both the EBA and PfRh gene families described above are highly polymorphic, which might affect both receptor affinity and specificity. This is important in order to overcome the host immune response but also the many host receptor polymorphisms that are present in various geographical areas. Several studies have shown invasion genes to be variantly expressed between parasite strains and that the various pathways they enable are redundant as individual EBAs and PfRhs can be knocked out with a resulting switch in pathway [212,215,220,224,230,231]. Copy number variation (CNV) in these genes has been observed in parasites, and been linked to various levels of expression [215,231]. Not only genetic differences but also epigenetic changes play important roles in invasion gene expression and enable the parasite to switch between sialic acid-independent and dependent growth [216,232,233]. The mechanisms behind these epigenetic changes are so far unknown but might involve chromatin modifications such as methylation and acetylation as well

as repositioning to active or silent expression zones in the nuclear periphery. Studies on EBAs and PfRhs indicate that they function not only similarly but also cooperatively, giving the notion of a combination vaccine based on members from both families [234].

Inside the RBC

Once the merozoites have successfully invaded a new red blood cell, several less studied molecular processes and modifications of the host cell take place in order for the parasite to successfully proliferate in this environment. A new PVM is formed as the parasite enters the host cell and keeps the parasite apart from the host cell cytoplasm. The dense granules are believed to release various proteins and chemicals that help creating a favorable environment for proliferation [200,201,235] but as of yet, only a few of these have been indentified [236,237,238,239,240]. The invasion process appears highly structured and complicated and of an obvious interest from a therapeutic point of view. Several invasion proteins are currently considered potential vaccine candidates. Much is still unknown however concerning regulatory mechanisms behind expression of invasion genes.

1.5.2 Sequestration

All *P. falciparum* isolates studied to date sequester and even though sequestration is known to affect pathogenesis only a fraction of malaria infections leads to severe disease. Countless studies have investigated what specific binding types are causing life-threatening disease such as cerebral malaria. The malaria parasite drastically modifies its RBC host, altering both the rigidity and adhesive properties of the iRBC. A myriad of parasite proteins are exported to the iRBC surface and these are enabling various types of sequestration. Foremost, mature iRBC can cytoadhere to endothelial receptors in various organs and tissues. However, iRBC also adhere to both uninfected (rosetting) and infected red blood cells (autoagglutination). While cytoadhesion enable the parasite to proliferate successfully without being cleared by the spleen, rosetting obstruct the blood flow and is speculated to protect the parasite against immune cells and alleviate erythrocyte invasion by merozoites by keeping uninfected cells near [241].

1.5.2.1 Erythrocyte membrane modifications

The parasite modifies the host cell immediately after invasion. Internal modifications such as an extensive tubovesicular network (TVN) that extends from the parasite vacuole helps to guarantee adequate nutrient transport into the parasite as well as waste transport out [242,243,244]. The permeability of the RBC membrane also changes, allowing for easier transport of various molecules in and out of the infected cell. Other dramatic changes to the RBC membranes take place, with the primary purpose of aiding the parasite to evade the host immune system. The various proteins exported to the RBC membrane constitute important virulence factors and contributes to the pathology of *P. falciparum.* Instead of using its endogenous trafficking system, the parasite assembles novel membrane structures in the RBC cytoplasm, such as the mentioned TVN and Maurer's Clefts (MCs) [245,246,247,248,249]. MCs are disc-shaped structures, tethered to the RBC membrane and are involved in delivering virulence proteins to the RBC membrane [250,251]. While normal RBCs are remarkably deformable in order to move through tiny capillaries, the rigidity is rapidly altered upon infection by *P. falciparum.* Various membrane modifications by parasite proteins are contributing to this increased rigidity [252,253]. The ring-infected

erythrocyte surface antigen (RESA) stabilizes the membrane skeleton via its associations with spectrin, which is present in the RBC membrane [236] and thereby curb further invasion of other merozoites [254]. Proteins such as the skeleton binding protein 1 (SBP1), membrane-associated His-rich protein 1 (MAHRP1), mature-stage erythrocyte surface antigen (MESA), *P. falciparum* erythrocyte membrane protein 3 (PfEMP3) and Pf332 are all involved in the formation and morphology of Maurers clefts, RBC membrane rigidity and/or trafficking of PfEMP1 [244]. The knob-associated His-rich protein (KAHRP) is expressed in mature RBCs and self-associate to form electron dense structures that interact with spectrin and actin in the RBC membrane [255,256]. While not essential, these so called knobs are important for surface presentation of PfEMP1 [257,258]. The amount of proteins exported by *P. falciparum* widely exceeds that of other *Plasmodium* species, and this is mostly due to expansion of various gene families such as the *var*, *stevor*, and *rif* genes [259].

1.5.2.2 Cytoadherence

An important factor of *P. falciparum* parasites is their ability to adhere to vascular endothelium in organs such as brain, intestine, liver, lung, skin and to syncytiotrophoblasts in the placenta. However, it is still not clear how the parasites use of different human receptors is connected to disease severity. CD36 is perhaps the most described receptor for adhesive iRBC and is expressed ubiquitously on the endothelium, platelets, monocytes and dendritic cells [260,261]. Most clinical isolates bind CD36 [262,263,264] but despite the obvious importance of this receptor, no association to severe disease has been shown as of yet [265,266]. ICAM1 has been associated with severe disease as was found to be heavily expressed in the brain of deceased CM patients [96,267]. Expression of ICAM1 can be upregulated by proinflammatory cytokines, which are common in severe disease as a natural response to infection [96,268]. *P. falciparum* parasites also bind other endothelial receptors such as thrombospondins (TSP), PECAM1 and VCAM1 among many others [269,270,271,272,273]. *P. falciparum* receptors present in the placenta are also of interest since they are so clearly linked to the severe syndrome placental malaria. The sulfated glycosaminoglycan CSA is the best described receptor for infected erythrocyte binding in the placenta [274] and it normally functions as a reversible immobilizer for cytokines, hormones and other molecules [146]. While *P. falciparum* isolates binding CSA rarely bind other common iRBC receptors such as CD36 [275], HA is another receptor proposed to mediate placental binding [276,277].

1.5.2.3 Rosetting

The adhesion of a *P. falciparum* infected red blood cell to uRBCs is termed rosetting and was discovered in the late 1980s [278,279]. Later studies showed this phenomenon to be present in both clinical and laboratory isolates and that rosetting is linked to severe malaria in African children [270,280,281]. Interestingly, rosetting is a phenotype that greatly varies between isolates and studies have shown that it is associated with cerebral malaria, severe malarial anemia and respiratory distress [282,283,284]. Malaria isolates infecting pregnant women and bind to syncytiotrophoblast cells in the placenta do not form rosettes however [285] despite being able to cause severe disease. The main parasite rosetting ligand is the protein PfEMP1 [286,287] that will be discussed in greater detail below. Multiple erythrocyte receptors can mediate rosetting. CR1 is a glycoprotein expressed at various levels on the surface of erythrocytes and is an important ligand to PfEMP1 [286]. It has been found that CR1 density polymorphism [286,288] and *cr1* gene alterations [286] both are important

determinants for severe disease. Human populations with low levels of CR1 as well as populations completely lacking CR1 are to a high extent protected against severe malaria [92,289,290]. Heparin sulphate (HS) is present on RBCs and heparin as well as other sulphated glycans both inhibit rosette formation and disrupt already formed rosettes [291,292]. Blood group ABO antigens are important for rosetting levels and size of rosettes. Blood group A is particularly linked to more frequent and larger rosettes among both laboratory strains and clinical isolates whereas blood group O results in lower levels and smaller rosettes [281,291,293,294,295]. Immunoglobulins, especially IgM, also appear to play important parts in rosetting, acting like bridges between the parasite ligand and RBC receptor [296,297,298,299].

1.5.2.4 *P. falciparum* surface exposed antigens

PfEMP1 in general and VAR2CSA in particular

The main parasite adhesin PfEMP1s is encoded by approximately 60 hyper-variable *var* genes per parasite genome [300] and are considered to be the most important virulence factor in *P. falciparum*. The multi-domain protein varies in size from 200-350 kDa and undergoes highly controlled regulation leading to antigenic variation, which constitutes an important parasite defense against the human immune response [15]. PfEMP1s mediate sequestration via a multitude of receptors in the endothelium and on red blood cells. All members contain two exons where the polymorphic extracellular domain (exon 1) comprise the N-terminal segment (NTS), a variable number of Duffy-binding-like (DBL) adhesive domains and cysteine rich interdomain region (CIDR). There are four types of CIDR domains (α, α_1, β and γ) and seven types of DBL domains (α, α_1, β, γ, δ, ε and x) and these different types are mediating the various binding specificities of PfEMP1s.

var genes are mainly located in the polymorphic subtelomeric regions [14,300], and can be divided into various groups based on their 5' upstream open reading frame, chromosomal location and transcriptional orientation [301,302]. The three main groups (ups A, B and C), two intermediate (B/A and B/C) as well as the unusual single *var* gene containing ups E are conserved in *P. falciparum*, indicating strict patterns of recombination of *var* genes [303]. Recombination occurs both in the mosquito abdomen as well as during human infection, rendering the *var* gene repertoire hyper-variable with very low levels of conservation between isolates [304,305,306].

The unusually conserved ups E var gene is located in the subtelomeric region of chromosome 12 and encodes VAR2CSA. It is by far the best-characterized *var* gene, mainly due to its important role in the pathogenesis of placental malaria. Single *P.falciparum* parasites may have several copies of slightly variable *var2csa* [301,310,311,312], which has not been seen for other *var* genes. Identification of multiple *var2csa* alleles in field isolates indicate that multiple alleles are more common in pregnant women than in other individuals and that these isolates accumulate during the course of pregnancy [311,312]. The level of antibodies towards VAR2CSA also correlates with the *var2csa* copy number [312], indicating that host immunity is driving the selection of parasites containing several *var2csa*. The domain architecture of VAR2CSA is different from other PfEMP1s, does not contain CIDR domains and instead consists of DBL1-3x and DBL4-6ε. Due to this unusual PfEMP1 structure, VAR2CSA has distinctive binding properties compared to other PfEMP1s, which bind a variety of receptors present in organs and tissues such as CD36, ICAM1, PECAM1, and VCAM1, all extensively discussed above. While the various domains have been thoroughly examined in terms of elicited protective antibody response and binding

capacities [188,313,314,315,316,317,318], later studies indicate the whole length protein to be important for high affinity binding to placental receptors [319,320]. However, studies on the various individual DBL-domains of VAR2CSA have shown several of these to bring forth a protective immune response against pregnancy-associated malaria. VAR2CSA is an important vaccine candidate that could possibly save the lives of both pregnant women and their unborn children.

Others surface exposed antigens

Other hypervariable gene families are proposed to encode proteins that are exposed on the iRBC surface. These families share chromosomal localization features with the *var* genes. The multigene family *stevor* encode STEVOR (subtelomeric open reading frame) proteins [321], that were recently shown to be expressed on the surface of schizont infected RBC and on the merozoite surface [322]. Variation in STEVOR expression appears important for the immunogenic properties of the parasite and might have a role in mediating immune evasion [323]. Often adjancent to *stevor* are the 150-200 gene copies of the *rif* (repetitive interspersed family) [14]. RIFINS are expressed on the surface of iRBC and protective antibodies are acquired with exposure [324,325]. Even though the function of RIFINS is still not understood, a member of the *rif* gene family have been found to dominate transcription in both sporozoites and gametozytes [326]. Also the function of the surface associated interspersed protein (SURFIN) family remains unknown. SURFINs have been localized to the surface of iRBCs and on merozoites [327]. The family of 13 *pfmc-2tm* genes is encoding proteins found in the PV, PVM and MC in late stage parasites [328] and possibly participates in iRBC adhesion [329].

1.5.3 Antigenic variation

Antigenic variation is employed by a multitude of human pathogens. By altering molecules exposed to the host, species like African trypanosomes [330] and *Giardia lamblia* [331,332,333] as well as *Plasmodium* are able to pertain a long-lasting infection and increase chances for transmission. While the above-described adhesins are essential for successful proliferation and escape from splenic clearance, they are also targets for the host immune response. Hence, the parasite needs to constantly change the surface exposed antigens, and is doing so by means of highly controlled antigenic variation. This has been best described for PfEMP1 but also other surface exposed antigens are suggested to undergo antigenic variation in *P. falciparum,* however. Antigenic variation is regulated epigenetically in the sense that activation and silencing of individual genes are inherited without any changes in the DNA sequence.

1.5.3.1 Antigenic variation of PfEMP

The ~ 60 *var* genes that a single *P. falciparum* genome contains is considerably less than the hundreds of variants surface *vsg* genes encoded by African trypanosomes. In order to not exhaust this relatively small repertoire, the family is constantly evolving by ectopic recombination [334]. Individual *P. falciparum* parasites supposedly express only a single *var* gene at a time, while remaining family members are in a silent state [335,336]. Recent data support a strict regulation of *var* gene expression, albeit not as strict mutually exclusive [310,337,338]. In order for the parasite to optimally use the *var* gene repertoire, there seems to be a highly structural pattern of transcriptional change [339], at least *in vitro*. Switching rate could be individual to each *var* gene, where *var* genes located internally on the chromosomes experience significantly slower off rates than subtelomeric *var* genes [340]. A more recent study however suggest the

switching pattern to be dependent on the *var* gene repertoire as a whole [339] and that switches never occur between closely related *var* genes.

Transcriptional regulation of *var* genes occurs at various levels. All *var* genes contain two transcriptionally active promoters, where the first is producing mRNA and is located upstream of exon 1. The second promoter constituted by the *var* gene intron leads to expression of non-coding RNA [300,341] and is an important regulatory element as a silencer and in recognizing other *var* genes [342,343,344]. While only one or a few upstream promoters may be active at a time, the intron promoter has no such regulation and is consequently active in all *var* genes simultaneously [345]. The unique gene *var2csa* contains a small upstream open reading frame (uORF) that functions as a translational repressor [346]. This is hypothesized to aid in repressing *var2csa* when infecting a non-pregnant individual and thereby only establish a placental infection when in a pregnant host.

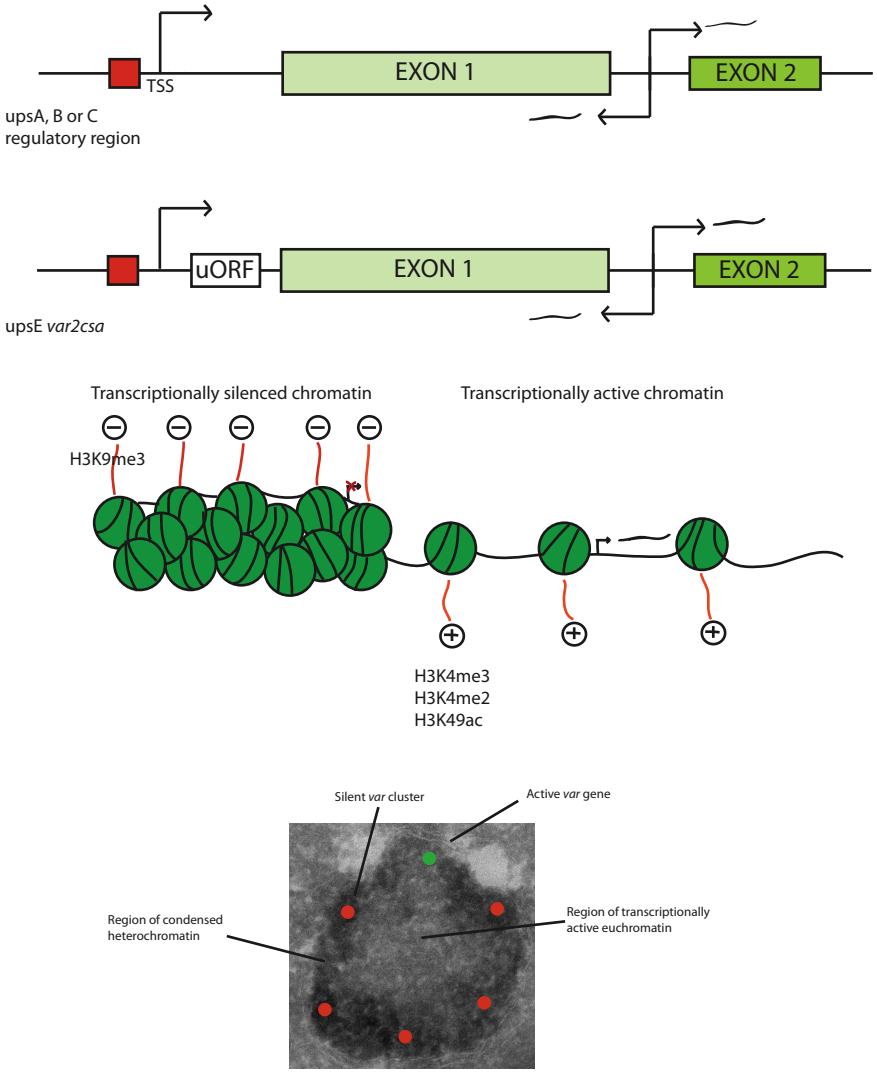

Figure 3. Overview of the various layers of regulatory processes controlling antigenic variation. (Inspired by Dzikowski and Deitsch, 2009, illustration by Jannike Simonsson and EM-picture by Ulf Ribacke).

Chromatin modifications are another important aspect of *var* gene regulation by rendering chromatin more or less accessible for transcription [69]. Active *var* genes are associated with H3K9ac and di- and tri-methylation of H3K4 in the ring stage and H3K4me2 in schizonts whereas H3K9me3 is enriched around silent genes [347,348,349]. The enrichment of di- and tri-methylation of H3K4 in trophozite and schizont stage is intriguing since var genes are no longer actively expressed that late in the IDC. It is possible that H3K4me2 / me3 therefore constitute an epigenetic memory by priming a certain *var* gene for the following cell cycle. The class III histone deacetylase PfSir2a has been suggested important for epigenetic control of *var* genes due to its association with transcriptionally inactive promoters [64,65,350,351,352]. Non-coding RNA could also play a role in chromatin assembly and thereby possibly in the transcriptional regulation of *var* genes [345].

The subnuclear organization represents another possible level of transcriptional regulation in antigenic variation. The *P. falciparum* nucleus is divided into two distinct compartments where the nuclear periphery consists of mostly electron-dense transcriptionally silent heterochromatin. Loose euchromatin that promotes DNA accessibility, which allows transcription factors to activate their target genes is located in the internal part of the nucleus [353]. However, the nuclear periphery contains active zones devoid of heterochromatin as well as the more prevalent inactive zones [354] and multiple studies have shown that *var* genes reposition to a specific transcription site in the nuclear periphery when active [63,310,343,355,356]. Whether or not this site co-localizes with telomeric end clusters is under debate [63,310,357] however.

1.6 GENETIC MODIFICATIONS OF *P. FALCIPARUM*; FORWARD AND REVERSE GENETICS

The first transfection of intracellular *P. falciparum* occurred in 1995 [358] and this started a new era in malaria molecular biology. The full-genome sequencing of *P. falciparum* in 2002 [14] then paved the way for extensive genetic manipulations that promise to facilitate vaccine and drug design by exploring parasite biology. Over 50% of the approximately 5300 *Plasmodium* genes encode hypothetical proteins of unknown functions that lack orthologues in other eukaryotes [14]. While transient transfection has given great insight into regulation of gene expression [359], stable transfectants allow for more extensive functional studies. Various selective marker genes that each encode a protein that confers drug resistance enable a positive selection process that ultimately lead to parasites with a disrupted wild type locus.

Despite the recent development of transgenic tools, there are several challenges with genetic modifications of *P. falciparum* iRBCs. First, the high AT-content of *Plasmodium spp.* DNA renders it highly unstable in *E.Coli* [360], which results in difficulties to prepare transfection constructs. Also, the targeting DNA needs to cross four membranes in order to reach the parasite nucleus [361], something that contributes to the low transfection efficiency seen in *P. falciparum.* The transient creation of micro-sized holes in the plasma membrane by electroporation is most commonly used in order to insert vector DNA into the parasite nucleus and this has been shown to work best on ring-stage parasites. The *piggyBac* transposon mutagenesis system allows for large-scale forward genetic screens [364]. While gene disruptions are not specific, the benefits of this system is its high efficiency and relatively short period of time it take to generate stable clones of insertional mutants. The *piggyBac* insertion approach have

been shown to work very well in *P. falciparum* [365] and adds to the increasing number of methods that enable the proteome of this deadly parasite to be unraveled. The function of malaria proteins can be elucidated by disruption of target genes via homologous recombination [362] but this approach is inadequate for essential genes. Hence, there is a great need for regulatable and inducible transfection systems in order to elucidate the function of these. Epp *et al* reports a regulatable expression system where a bidirectional promoter drive the expression of both the transgene and the selectable marker, allowing for significantly smaller constructs that therefore are more stable [363]. By changing concentrations of the selection drug, the copy number of concatameric episomes varies and thereby regulates the transgenes level of expression. In paper III, we successfully use the mutant version of the human rapamycin-binding protein FKPB12, called 'destabilization domain', or DD [366,367], fused to the C terminus of our target gene. By adding the small molecule Shield 1 (Shld1) that function as a DD ligand, the fusion protein is protected from the degradation that would occur with no addition of Shld1. While different *P. falciparum* proteins inherently will be knocked down at varying levels, this system is a useful tool for investigating functions of essential genes. By varying the concentration of added Shld1, protein degradation can be effectively tuned and controlled.

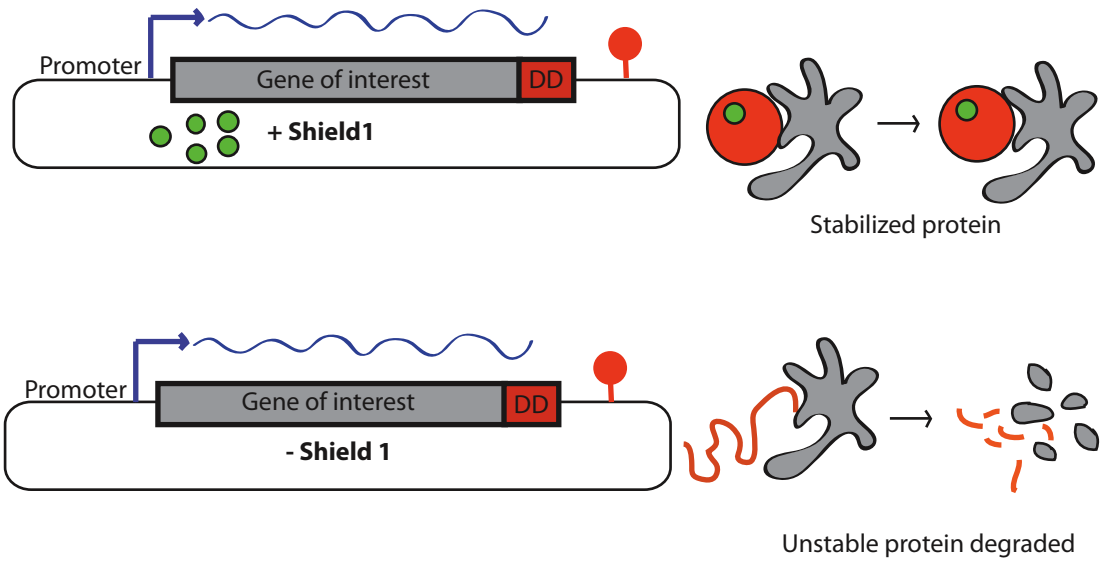

Figure 4. Inducible knockdown system using the DD-domain. (Illustration by Jannike Simonsson).

2 SCOPE OF THE THESIS

The general objective of the studies presented in this thesis was to increase the understanding of acquired immune response to VAR2CSA as well as investigate gene regulatory mechanisms behind expression of *P. falciparum* virulence genes, with a focus on antigens involved in placental malaria.

Specific aims:

The specific aims of the presented papers were as follows:

I. To explore the differential acquisition of immune IgGs towards VAR2CSA DBL-domains and investigate the impact of HIV co-infection on antibody affinity.

II. To gain insights into gene regulatory mechanisms behind antigenic variation and the rationale of gene dosage by scrutinizing active transcription of duplicated *var2csa* alleles in individual parasites.

III. To elucidate essentiality and gene regulatory functions of the *P. falciparum* histone deacetylase PfHda1 with the use of reverse parasite genetics.

3 EXPERIMENTAL PROCEDURES

While material and methods are thoroughly explained in each respective study included in this thesis (paper I-III), experimental procedures considered of extra importance are systematically described here.

3.1 *Plasmodium falciparum in vitro* culture conditions

Trager and Jensen published their success in cultivating *P. falciparum* parasites in 1976 [368] and this was obviously a big landmark for malaria research. The candle-jar technique they describe was used for parasite cultivation for paper II. In brief, iRBC in complete media were grown at 37°C in open flasks in an airtight desiccator where lit candles consume excess oxygen and thereby creates a beneficial environment for the parasite to proliferate. Parasites used for paper I and III were cultured using similar complete media, however grown in petri dishes within airtight containers individually gassed with 5% O_2 and 5% CO_2 in N_2. No significant difference between these similar methods in terms of parasite growth was seen and the reason for using the various methods was purely logistical.

3.2 VAR2CSA DBL-domain recognition by immune sera from pregnant women

The transfectant CHO-745 cells we used in paper I are comprehensively described in [369] and was further characterized in [277]. The serum samples used in this study were part of a large study that took place in Malawi and investigated interactions between HIV and malaria in pregnancy [181]. Using these sera, we measured VAR2CSA DBL-domain recognition by flow cytometry. Compared to an earlier study measuring non-immune IgG and IgM recognition of the various domains expressed on the same CHO-745 cells [277], experimental procedures were slightly modified. Fetal calf serum (FCS) was added to blocking steps and antibody incubations, something that significantly increased the amount of viable cells appropriate for analysis. While we originally used cells expressing all VAR2CSA DBL-domains (DBL1x, DBL2x, DBL3x, DBL4ε, DBL5ε and DBL6ε), we decided to continue to work with domains DBL3x, DBL5ε and DBL6ε. Due to the impracticality of testing large number of patient samples using all DBL-domain expressing CHO-745 cells, the three most interesting domains were chosen for a more thorough analysis. These three domains were all recognized in a gender specific pattern to a much higher extent than the other VAR2CSA DBL-domains. Further analysis showed especially DBL5ε to be interesting due to also being recognized in a parity dependent manner, something that is characteristic for immune acquisition within malaria in pregnancy. Also, the antibody levels to DBL5ε positively correlated to those against total variant surface antigens expressed by VAR2CSA expressing parasites (VSA-PAM). Hence, we chose to further analyze the properties of VAR2CSA DBL5ε-specific antibodies.

3.3 Antibody affinity measurements

In order to investigate antibody affinity to recombinant VAR2CSA DBL5ε, we used the surface Plasmon resonance (SPR) technique, measured with the biosensor analytical system Biacore. SPR occurs when polarized light strikes the gold-coated sensorchip and generates electron charge density waves called Plasmons. The reflected light is detected and measured using the Biacore system. There are multiple uses of the Biacore

system and in addition to determinate affinity between two molecules, it can also measure kinetics for interaction, binding specificity and molecule concentration. While the affinity of an interaction is evaluated from the level of binding at equilibrium as a function of sample concentration, it can also be determined from kinetic measurements where the equilibrium constant K_D equals k_d/k_a (dissociation rate constant and association rate constant respectively). In our experiments however, there are several uncertainties. First, we do not know which molecules in the patient sera that bind to the VAR2CSA domain DBL5ε. Hence, we cannot know the concentration of molecules in the interaction. Therefore, we determined k_d as a measurement of binding affinity and compared these from our various patient groups (primi- or multigravidae with or without HIV infection).

After the target protein was coupled to the CM5 sensor chip, the amount of samples we could test before the protein either lost its conformation or was downgraded by exposure to the 10 mM glycine, pH 1.5 varied. To make sure the recombinant DBL5ε remained intact, our control sera from hyperimmune Malawian multigravid women was tested regularly. In order to measure only binding by immune antibodies specific to VAR2CSA DBL5ε, sera from malaria-naïve Melbourne donors and sera depleted from IgG as well as non-immune IgG and IgM was used as controls, none of these showing significant binding to our test protein. We also used the control protein 3D7 DBL6γ in order to adjust for the rare event of non-specific binding. The k_d of individual samples was subsequently evaluated using the software BIAevaluation 3.0 (Biacore AB).

3.4 Single cell cloning of parasites

We used two different methods of cloning single cell parasites within the studies that this thesis comprises. In paper II, single infected RBC was picked by a micromanipulation technique using sterile glass capillaries (~3-5 μm internal diameter) and a micromanipulator MN-188 in conjunction with an inverted Diaphot 300 microscope. The single cells were directly deposited into RNA harvesting buffer and snap frozen on dry ice. For the downstream assay, the use of actual single cells were imperative and the precision of micromanipulation ensured that this was indeed what we were working with.

In paper III, we instead used limiting dilution in order to obtain clonal parasites. Each transfected bulk population was dilution into three different dilutions and plated into 96-well microtiter plates. Media containing drug was changed every 2-3 days and presence of parasites were monitored after 14-16 days of culturing using a parasite-specific lactate dehydrogenase assay (pLDH) [370,371]. The parasite LDH is able to utilize 3-acetylpyridine NAD (APAD) as an NAD analogue whereas RBC cannot. Hence, by adding nitroblue tetrozolium (NBT) and phenylethyl sulfate as well as Malstat reagent to 20 μl of well contents and incubating at room temperature for ~2 h, positive wells can be spotted without the need for a plate reader. The pLDH assay is very specific and sensitive as well as significantly faster than giemsa staining followed by microscopy. Six clones from each plate was subsequently transferred to 5 ml dishes and cultured for southern blot analysis and downstream assays. If parasite growth was only observed in wells with highest concentrations, plates were discarded and cloning experiment repeated, in order to ensure clonal parasite cultures.

3.5 Real-time PCR

Real-time PCR was extensively used in both paper II and III. In paper II, we used this method in order to quantify relative gene copy number of *var2csa* and to discriminate between highly similar gene paralogs. In paper III, we investigated relative gene transcription levels in PfHda1 knockdowns in the sense of both invasion related genes and the *var* gene family. All used gene specific primers and probes were designed using Primer Express 3.0 and further assessed using ΔG estimations in NetPrimer. However important and useful, the real-time PCR technique is limited to the quality of material used as well as of proper assay design. Therefore, extensive efforts to ensure specificity of designed assays were taken by blasting towards all genomes used as design template as well as performing dissociation curves of amplified products. By using serially diluted DNA and modifying primer concentrations, amplification efficiencies were optimized. In the rare cases when amplification efficiencies differed more than insignificantly, this was taken into consideration when analyzing the data.

Relative gene copy numbers was performed using specific primers for *var2csa, Pf332* and the single copy gene *β-tubulin*. After amplification reactions were performed in at least triplicates, relative gene copy number was calculated using the ΔΔCt method.
By using primers and allele discriminative FAM or VIC labeled MGB-probes, we were able to distinguish between the highly similar duplicated *var2csa* and *Pf332* genes in the parasite HB3. After extensive validation of assays, relative allele frequencies were determined from ratios of fluorescence from the allele specific probes via an initial pre-read followed by amplification and a final post-read.

For the relative gene quantification we performed in paper III, several endogenous controls were used. Due to the stage delay observed upon PfHda1 knockdown, parasites grown on and off Shld1 were harvested at slightly different time points. Also, since inhibition of histone deacetylases are likely to affect a large variety of genes, we made sure that our control genes did not change between the parasites we compared. Initially, primers targeting *seryl-tRNA synthetase*, *adenylosuccinate lyase, arginyl-tRNA synthetase* and *glutaminyl-tRNA synthetase* were all used but since values were identical in balanced samples, we chose to use only *seryl-tRNA synthetase* for our final analysis.

3.6 Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) has been extensively used in *P. falciparum* [338,345] and considerable improvements to the method have been developed by the Wahlgren group [355,372]. FISH is effective for visualization of the physical location of genes, either within intact nuclei or on whole chromosomes and is used in paper II to investigate *var2csa* localization. By using double stranded probes targeting both *var2csa* alleles in HB3CSA and the telomeric repeat regions (Rep20), we were able to assess gene localization. Five different scenarios were seen and clearly distinguishable; co-localization of duplicated *var2csa* and co-localization of these with Rep20, co-localization of *var2csa* but no co-localization with Rep20, non co-localized *var2csa* where both co-localize with Rep20, non co-localized *var2csa* where none co-localize with Rep20 and non co-localized *var2csa* where one paralog co-localizes with Rep20 and the other not.

In order to visualize active transcription of the duplicated *var2csa* alleles in paper II, we developed a RNA-FISH assay. CSA-selected parasites were picked at 16 ± 4 h p.i. in order to cover peak *var* gene transcription. 100 bp single stranded antisense and sense

RNA probes with Sp6 promoter tails were designed to being able to distinguish between the highly similar *var2csa* paralogues in HB3CSA as well as incorporating also FCR3CSA and NF54CSA as controls. The various probes were generated by *in vitro* transcription with either fluorescein or biotin and Sp6 RNA polymerase. RNA is inherently more difficult to work with than DNA, and is easily degraded. Hence, great care was taken to store RNA probes at -70°C until use and to keep all reagents and bench spaces free from RNases. The methodology for RNA-FISH is thoroughly described in paper II and our results clearly shows the usefulness of this discriminative assay.

3.7 Inducible knockdown using the DD-domain system

The use of the human FKBP12-domain in transfection techniques [367] was rapidly adapted for use in *P. falciparum* [366] and has since then been used successfully in several studies (paper III; [194,373]. A thorough description of the generation of the DD-HA plasmid is found in [194]. In brief, the DD-domain was amplified and cloned into a single crossover vector with the *Pfhsp86* 3'UTR as an XhoI/Acc65I fragment. Triple HA-tags was then inserted into the DD vector, resulting in plasmid pJDD41 (here callded DD3HA). A 1159 bp fraction of PF14_0690 was then amplified using primers F: 5'-AAGCTGCGGCCGCTGTTGTCCTTTTA and R: 5'-AGGGTACTCGAGATTCCA GAAATTTA. Resulting PfHda1 fragment was then cleaved using NotI/Xho1 and ligated to the DD3HA vector. We transformed XL10-Gold cells (Agilent Technologies) with this plasmid and checked for positive colonies that were then further expanded in order to test cleave and sequence the vector. Large amounts of the DD3HA_Hda1 plasmid was then generated using the same procedures and the resulting DNA was precipitated and used for transfection. Parasites were sorbitol-synchronized and used at ring-stage parasites at a 5-10% parasitemia. For transfection, 200 µl packed iRBC were transfected with ~100 µg plasmid DNA in cytomix (120nM KCl, 0.15mM $CaCl_2$, 2mM EGTA, 5mM $MgCl_2$, 10mM K_2HPO_4 / KH_2PO_4, pH 7.6, 25mM HEPES, pH 7.6) by electroporation. Transfected parasites were then moved into pre-warmed media containing 10% human serum. After 6 hours of recovery, media was changed and drug added (0.5µM Shld1 and 2.5nM WR99210 (Jacobus Pharmaceutical Company)). The following day, fresh red blood cells were added for a hematocrit of 4%. Transfected parasites were maintained with 0.5µM Shld1 and stable single crossover parasites were selected by cycling on and off WR99210. When off drug pressure, parasites carrying the plasmid episomally will dispose of it, which is seen as massive cell death when drug is added anew. The cell death will gradually decline after several cycles of 2 weeks on drug and 2 weeks off drug until single crossovers are obtained. Stable transfectants were then cloned by limiting dilution and correct integration subsequently confirmed using gel electrophoresis and southern blots.

3.8 Assays for proliferation, invasion, cell cycle progression

In order to carefully monitor the effect PfHda1 knockdown has on the growth phenotype of the parasite, several assays were performed. First, we wanted to look at parasite proliferation over several generations. To do this, we sorbitol-synchronized ring-stage NF54DD3HA_Hda1 and FCR3DD3HA_Hda1 parasites and washed them extensively in RPMI, in order to remove all traces of Shld1. Parasites were then plated in triplicates in a 12-well plate, at 0.05% parasitemia and 0.25% hematocrit. A low starting parasitemia as well as low hematocrit ensures to successful growth of parasites

for up to 3 generations without adding anything but fresh media. This minimizes any variation in our results that could be due to different treatment of cultures. For each strain, we either grew them completely off Shld1 or on 0.5μM (+), 0.125μM (+M), 0.0625μM (+L) Shld1 as well as 2.5nM WR. Non-infected RBCs and wildtype parasites were used in parallel as controls. Parasitemia was subsequently monitored by SYBR stain and flow cytometry every second day until day 6 (third round of invasion).

In order to specifically look at invasion efficiency upon PfHda1 knockdown, parasites were treated in a similar fashion as above. However, parasites were grown in 10 ml dishes either in the presence or absence of 0.5μM Shld1 (+) and (-) respectively. Initial parasitemia at ring stage was measured as described above and subsequently measured after one round of invasion.

During these experiments, a clear delay in parasite growth was observed, which led us to study the parasite cell cycle in more detail. To do so, cultures were set up as described above for the invasion assay, and smeared every 4-8 hours for the next 80 hours. Parasite morphology was then monitored with microscopy and cells determined as either ring, trophozoites or schizonts. We also carefully checked for abnormal cells, such as exoerythrocytic parasites and RBC ghosts but found most parasites grown without Shld1 to normally develop albeit at a slower pace than wildtype parasites.

4 ETHICAL CONSIDERATIONS

Ethical approvals for the human components used in Paper I in this thesis were obtained from research ethical committees in Malawi and Melbourne University. Written informed consent was obtained from patients themselves.

5 RESULTS AND DISCUSSION

5.1 Paper I

"Antibody responses to VAR2CSA DBL-domains suggests DBL5ε as a potential vaccine candidate and indicate HIV infection to impair antibody affinity in primigravidae"

Parasites infecting pregnant women have distinct binding phenotypes that enable them to sequester in the placenta, where they contribute to the pathogenesis of PAM. Multiple studies have shown that women in their first pregnancy are at highest risk to develop malaria but that protective antibodies are acquired throughout the pregnancy. These antibodies are gender-specific and levels increase with parity. The PfEMP1 VAR2CSA is the main binding ligand for placental sequestration and is also a potential vaccine candidate. However, its large size calls for increased knowledge of elicited antibody response against parts of the protein, such as the different DBL- domains. The aim of this study was to study the dynamics of the acquired immune response involved in protection to PAM and investigate how HIV affects both the acquisition and function of protective antibodies.

First, we investigated antibody levels to variant surface antigens (VSA) expressed by the VAR2CSA expressing parasite CS2 by flow cytometry. Not surprisingly, multigravid women were shown to have significantly higher levels of VSA-PAM antibodies than primigravidae from the same area. We then went on to a more detailed analysis of the immune response to the different DBL-domains of VAR2CSA (DBL1x, DBL2x, DBL3x, DBL4ε, DBL5ε and DBL6ε. Using CHO-745 cells, that each express one of the VAR2CSA DBL-domains on its surface [369], we found DLB5ε to be particularly well recognized in a gender and parity dependent manner. DBL5ε is highly conserved among both laboratory strains and clinical isolates [374] and contain numerous regions that are targeted by protective antibodies [375]. Importantly, these antibodies are highly cross-reactive between isolates, further indicating DBL5ε as an interesting vaccine candidate [315]. Also, levels of DBL5ε antibodies correlate well with levels of adhesion-inhibitory antibodies [376] as well as with levels of IgG to total VSA-PAM as seen in our study.

Further, we characterized the affinity of acquired DBL5ε antibodies. Affinity has recently been recognized as highly important for the understanding of receptor-ligand interactions for VAR2CSA [314,319]. Our results suggest that primigravidae and multigravidae acquire antibodies that bind DBL5ε with similar affinity but that HIV infection significantly impairs binding affinity in primigravidae. It is well known that HIV co-infection with malaria hampers the parity-dependent acquisition of protective antibodies, but less is known about the mechanisms behind this [181]. Our study suggests that HIV co-infection further endangers already highly malaria-susceptible women in their first pregnancy by reducing the potency of antibody binding. This signifies that not only antibody levels but also their functionality are important markers of protection to PAM, and adds another layer to the complexity of the acquired immune response that protect pregnant women from malaria.

While several of the VAR2CSA DBL-domains have been implicated to bind to the placental receptor, CSA, very little is known about the molecular interactions in this

binding [369,377,378]. It has also been implicated that this interaction is not specific for VAR2CSA DBL-domains and that DBL-domains belonging to other PfEMP1s bind CSA to a similar extent [379]. Two recent important studies expressing recombinant full-length extracellular VAR2CSA has shown that its binding affinity and specificity to CSA is significantly higher than that of single DBL-domains [319,320]. This questions the previous model of VAR2CSA structure as "beads on a string" and instead indicates a higher-order structure of the VAR2CSA extracellular region that would lead to a native CSA-specific binding site. While the full-length VAR2CSA exhibits high-affinity binding to CSA, it is clear that certain DBL-domains are indeed generating protective and highly functional antibodies. A functional vaccine should include DBL-domains with as many highly conserved residues involved in receptor-ligand interactions as possible, and future research should focus on finding these residues.

5.2 Paper II

"Simultaneously transcribed var2csa alleles in individual P. falciparum parasites questions mutually exclusive var gene expression and explores the potential of var2csa duplications for placental malaria"

The aim of paper II was to investigate transcriptional functionality of duplicated genes, as a means to further understand mechanisms behind antigenic variation and to explore the relevance of multiple *var2csa* alleles for placental malaria pathogenesis. While earlier studies had shown various *P. falciparum* genomes to contain multiple *var2csa* copies [301,311], this study was the first to prove that the gene paralogs can be simultaneously transcribed in individual parasites. To do this, we designed a highly specific allelic discriminative real-time PCR assay that was able to distinguish between the *var2csa* alleles in the parasite strain HB3, as well as single alleles from other parasite lines. This assay could then be used both for bulk population as well as on single iRBC. In order to investigate *var2csa* transcripts in individual parasites, we developed a method for successful extraction of RNA from individual iRBC that were selected by micromanipulation and a nested PCR/ real-time PCR approach.

Our findings that both *var2csa* alleles indeed can be transcribed simultaneously in single cells were confirmed by a discriminative RNA fluorescent *in situ* hybridization assay (RNA-FISH), which concurred with the PCR results. Moreover, the RNA-FISH revealed the two transcripts to co-localize to a high extent in the nuclear periphery. The presence of the 3' poly(A) tail as well as the cytoplasmic localization seen for *var2csa* paralogs indicate them to be destined for translation. We also performed DNA-FISH in order to further elucidate the nuclear positioning of active *var2csa* genes, which supported co-localization of the two paralogs, this despite being located on different chromosomes. In the majority of cases, the signals also overlap with those of the telomere-end representative Rep20. One suggested layer of *var* gene regulation is nuclear repositioning and a *var*-specific active transcription site has been proposed to exist in the nuclear periphery [353]. Whether or not actively transcribed genes co-localize with telomeric clusters has been debated [343,355,356,380]. Our study suggests that a specific active site appear to exist but that *var* genes can be actively transcribed regardless of whether they are located distant or adjacent to telomeric clusters, at least as seen with the resolution that is achieved by Rep20 labelling.

The simultaneous transcription of more than one *var* gene indicates *var* gene regulation to be less strict as previously thought. It is likely that histone modifications play an important role in determination of transcriptional status of the *var* gene family and it is possible that nuclear co-localization of *var2csa* alleles results in their synchronized transcription by the state of chromatin accessibility.

While our study did not investigate surface expression of the *var2csa* alleles, another study has shown more than one PfEMP1 on the surface of individual iRBC, mediating two distinguished binding phenotypes [338]. Whether or not this is true also for VAR2CSA paralogs remains to be proven. The *var2csa* specific 5' uORF functions as a translational repressor and adds an extra layer of complexity to the possibility of surface co-expressed VAR2CSA paralogs. In high-transmission areas, parasites with multiple copies of *var2csa* seem to have a selective advantage and are more often found in pregnant women than in non-pregnant hosts (Sander Plos One 2009). Pregnant women infected with multi-copy parasites tend to have a higher parasite load and more

IgG to the VAR2CSA domain DBL4ε (Sander JID, 2011). While the mechanism behind this selection is unclear, it is possible that parasites with several VAR2CSA alleles benefit from either the ability to switch between which to use or use paralogs simultaneously and therefore are able to better establish and maintain prolonged infection in the placenta.

5.3 Paper III

"PfHda1 functions as an important regulator of gene expression and cell cycle progression in P. falciparum"

In this study, we used an inducible knockdown system to characterize the class II HDAC PfHda1. The fusion of a destabilization domain (DD) to the target protein marks the product for degradation. However, in the presence of the DD ligand Shld1, the fusion protein is protected from destruction. This system is attractive in particular for functional characterization of essential proteins where traditional knockout approaches are ineffective. Using this approach, we found that PfHda1 localizes in both the cytoplasm and the nucleus, something that is common for class II HDACs. Upon the removal of Shld1 degradation of PfHda1 commence early and is even more pronounced later in the cell cycle.

A striking growth defect could be observed for parasites with less PfHda1, and this defect comprises both a cell cycle delay and significantly lower increase in parasitemia after one round of invasion. A more detailed study of the cell cycle progression revealed parasites grown off Shld1 to experience a longer cell cycle with the majority of parasites stalling before transition into S-phase.

We also investigated differential gene expression and decided to focus on invasion related gene families due to the phenotype seen upon PfHda1 knockdown. The class III HDACs PfSir2a and 2b are known regulators of *var* gene expression, which led us to also investigate *var* gene transcription. Our findings show that decreased levels of PfHda1 leads to downregulation of PfRhs and EBAs, both families encoding important invasion ligands, possibly explaining the defective proliferation phenotype. However, since HDACs generally are considered to be silencing proteins, our results could indicate that the decrease in gene expression is not a direct result of PfHda1 loss but rather a downstream effect. *var* genes are clearly dysregulated in the absence of PfHda1 with varying results in NF54 and FCR3 clones. It is clear however that a more in-depth analysis is needed to further elucidate how PfHda1 is connected to *var* gene regulation. Both *var* and invasion genes are encoding proteins that are important for parasite virulence. Our results that a chromatin-binding protein such as PfHda1 can affect transcription of both these groups are exciting, especially as very little is known about transcriptional regulation of invasion genes.

Interestingly, PfHda1 has previously been found differentially transcribed between different parasites. In a study by Mok et al, two isogenic clones with specific and different binding phenotypes were shown to differentially express over 250 genes [381]. In one of the clones, 3D7AH1S.2, PfHda1 is clearly upregulated. Fewer other genes were upregulated in this clone compared to clone 3D7S8.4. It is tempting to link this general downregulation to increased levels of PfHda1 but it is likely that significantly more factors than that weighs in. Genes coding for transcription factors, RNA methylase and cell cycle genes were among those genes downregulated in 3D7AH1S.2 and interestingly only one *var* gene were transcribed compared to the three *vars* in 3D7S8.4. Therefore, it would be useful to also investigate how increased levels of PfHda1 affect the regulation of gene expression in *P. falciparum*. The present study highlights the benefits of genetic tools such as inducible gene knockdown and establishes PfHda1 as an important protein in both cell cycle progression and as a transcriptional regulator.

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