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TOWARDS UNDERSTANDING THE SURFIN PROTEIN FAMILY OF *PLASMODIUM FALCIPARUM*

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To my loving parents and family

But those who hope in the Lord
will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint
Isaiah 40 vs 31 (The Holy Bible)

ABSTRACT

Plasmodium falciparum, the parasite responsible for severe malaria, has been shown to use different protein families for its survival and proliferation within the human host. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), a protein responsible for the cytoadherence of parasitized red blood cells (pRBCs) has been implicated in severe disease. PfEMP1 is transported to the pRBCs surface together with a recently identified surface membrane protein, $SURFIN_{4.2}$, a member of the SURFIN protein family. SURFINs are encoded by *surf* genes, which in the parasite clone 3D7, ten paralogs were identified two of which are most likely nonfunctional pseudogenes. However, little understanding about the function of SURFINs in the parasite is known.

This thesis aimed to further describe the SURFIN family and to study *var* gene expression in organs of fatal malaria patients:

- The gene expression and localization of another member of the family, the $surf_{4.1}$ gene was analyzed. Previously annotated as a pseudogene, the analysis revealed the $surf_{4.1}$ gene is a functionally complete gene transcribed from approximately 32hrs post invasion through to the infectious developmental stage, the merozoites.

- Experiments were conducted to identify a potential receptor for the $SURFIN_{4.2}$, previously shown to be expressed on the surface of pRBC. The analysis involved a wild type CS2 parasite line (CS2WT) and a knockout line where $surf_{4.2}$ gene was disrupted (CS2Δ$surf_{4.2}$). Initial data suggests that the receptor, on the RBC surface, is resistant to chymotrypsin and is sensitive to heparin.

- Analysis of the SURFIN family using various bioinformatics tools identified two major groups of SURFINs, GroupA (3 members) and GroupB (4 members), and an intermediate group (2 members). An additional SURFIN did not fit in the above mentioned categories. With the exception of two pseudogenes, the SURFINs were observed to have conserved domains (SCDs) which were predicted to be duplicated and inserted in different locations within the sequence.

- The expression of *var* genes, that encode for PfEMP1 protein, was studied in various organs of fatal malaria patients. A clear dominance of certain *var* types in the brain was observed and the *var* types varied between organs.

LIST OF PUBLICATIONS

I. Fingani Annie Mphande, Ulf Ribacke, Gehard Winter, Fred Kironde, Mats Wahlgren

SURFIN$_{4.1}$ a schizont-merozoite associated protein in SURFIN family of *Plasmodium falciparum*

Malaria journal,2008; **7**: 116 online open access

II. Fingani Annie Mphande, Kristina Persson, Alex G. Maier, Alan F. Cowman, Mats Wahlgren

Investigating a Receptor for *Plasmodium falciparum* SURFIN$_{4.2}$ using CS2-wild type and CS2-*surf*$_{4.2}$ knock out lines

Manuscript

III. Fingani Annie Mphande, Kevin Brick, Mats Wahlgren

Dissecting the SURFIN family of *Plasmodium falciparum*

Manuscript

IV. Jacqui Montgomery, Fingani Annie Mphande, Matthew Berriman, Arnab Pain, Stephen J. Rogerson, Terrie E. Taylor, Malcolm E Molyneux, Alister Craig

Differential *var* gene expression in the organs of patients dying of *falciparum* malaria

Molecular Microbiology, 2007. online open access

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ABBREVIATIONS

ALP	Alkaline phosphatase
AM	Amodiaquine
AMA1	Apical membrane antigen 1
ATS	Acidic terminal segment
cDNA	Copy DNA
CIDR	Cysteine rich inter domain region
CM	Cerebral malaria
CQ	Chloroquine
CR1	Compliment receptor 1
CRD	Cysteine rich domain
CSA	Chondroitin sulphate A
DABP	Duffy antigen binding protein
DBL	Duffy binding like
DC	Dendritic cells
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EM	Erythrocyte membrane
G6PD	Glucose-6-phosphate dehydrogenase
GroupC	SURFIN group C
GroupA	SURFIN group A
GroupB	SURFIN group B
GYPA	Glycophorin A
GYPB	Gylcophorin B
GYPC	Glycophorin C
HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molecule 1
LMV	Large multimeric vesicles
MACS	Magnetic cell sorter
NO	Nitric oxide
ORF	Open reading frame
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
pRBC	Parasitized red blood cell
PVM	Parasitophorous vacuole membrane
PvSTP1	*Plasmodium vivax* subtelomeric protein 1
QN	Quinine
RBC	Red blood cell
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
Rt-QPCR	Real-time quantitative PCR
SA	Severe anemia
SCDs	SURFIN conserved domains
SDS	Sodium dodecly sulphate
SM	Severe malaria
SMA	Severe malarial anemia
SP	Sulphadoxine pyremithamine

SSV	Single small vesicles
Surf	Corresponding gene to the SURFIN antigen
SURFIN	SURFace associated INterspersed antigen
var	Variable genes encoding PfEMP1
vir	Family of gene encoding variable antigens
WRD	Tryptophane rich domain

1 INTRODUCTION

Malaria is one of the oldest diseases known to man. Malaria was first linked to "bad air" from bad smells from swamps hence termed malaria (from the Italian *mala* "bad" *aria* "air"). The name was then shortened to what we know today, malaria, in the 20th century.

Malaria is an infectious disease which is spread by a mosquito vector. The parasite that causes malaria, *Plasmodium*, survives both in the mosquito vector and in the animal and or human host. Malaria is a big public health problem and one of the biggest killer diseases in sub Saharan Africa and South East Asia (SE Asia).

Malaria exerts a heavy social and economic burden on the affected countries and the outcome of the disease has been further complicated by the complex host-parasite interactions.

Malaria parasites have several gene families that encode for various proteins that are used by the parasite for survival and dispersal within the human host. These proteins can interact with the host directly through different host specific receptors or indirectly by triggering changes within the host that allow for parasite proliferation.

To address these problems, studies are being conducted to further understand different protein families in the malaria parasite so as to identify their function. Understanding of protein function and their possible receptors on the human host is a key to identifying drug and vaccine targets to combat the parasite.

1.1 THEME AND OVERVIEW OF THESIS

This thesis is based on a PhD study investigating a family of proteins in *Plasmodium falciparum* called SURFINs and distribution of *var* genes in organs of fatal pediatric malaria cases. SURFIN stands for "SURFace associated INterspersed protein family". These proteins are encoded by the *surf* multi-gene family.

The SURFINs were studied in both laboratory adapted and in wild (patient) *P. falciparum* isolates. Two members of the GroupA SURFIN family, SURFIN$_{4.1}$, SURFIN$_{4.2,}$ and three members of the GroupB SURFINs were studied in detail. The

expression of *var* genes, encoded by PfEMP1, was also studied in organs of children who died from malaria in Blantyre, Malawi.
The findings of these studies are outlined in the chapters that follow.

1.2 INTRODUCTION TO MALARIA

1.2.1 Global Malaria Situation

During the past decade, there has been considerable increase in the burden of malaria mostly in Africa. Global estimates from 2007 from 81 countries most affected by malaria showed that there were 660 million people infected in malaria endemic areas in Africa and 1.24 billion in Asia and America. It was estimated that the annual incidence of severe malaria was 10.7million in Africa and 3.3 million in Asia and the Americas [1]. Episodes of clinical *Plasmodium falciparum* malaria occur every year with 70% of the episodes in tropical Africa, and 25% in SE Asia [2] (Figure 1). Malaria has been ranked as one of the biggest killer diseases in Africa apart from HIV/AIDS with over 3000 deaths per day most of which are children under five years.
Clinical manifestations of *P. falciparum* infection have been found to be dependent on a combination of various parasite, host, geographical and social factors [3]. Local differences in the parasites have evolved and these have led to emergence of clearly identifiable geographical variants [4].

The distribution of malaria in endemic parts of the world has defined according to the degrees of endemicity. Malaria is said to be endemic in areas where there is constant transmission for successive years. Malaria is said to be hypoendemic in areas where the infection prevalence is less than 10%, and mesoendemic in areas where the infection prevalence is between 11%-50%. In regions where the infection prevalence is above 50%, the areas are defined as holoendemic and hyperendemic.
Figure 1 shows the distribution of *P.falciparum* malaria, sub-Saharan Africa contains areas which exhibit all forms of malaria endemicity.

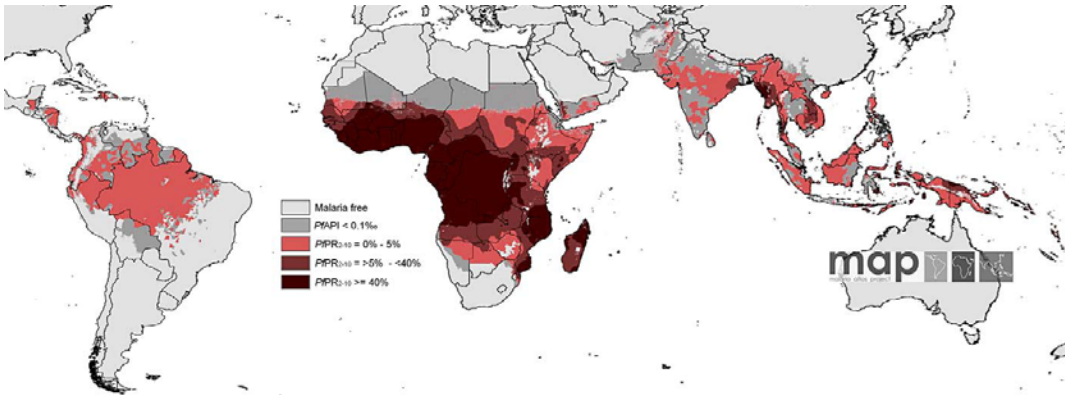

Figure 1: ***P.falciparum* malaria distribution**. Map showing *P. falciparum* malaria distribution according to endemicity as of 2007.The light red colour denotes hypoendemic areas, the reddish brown denote mesoendemic areas and the dark red hyper and holoendemic areas [5].

1.2.2 Malaria History and Pathology

The malaria parasite, *P. falciparum,* was first described by Dr. Alfonse Laveran on 20th October 1880 from the blood of a patient with intermittent fevers at a military hospital in Consantine, Algeria. Since then there has been considerable research to find ways of eliminating and or controlling the disease.

Malaria is caused by protozoa of genus *Plasmodium* family Plasmodiidae, sub order Haemospondiidae order Coccidia. There are over 120 species of *Plasmodium* found in blood of mammals, reptiles and birds.

Malaria parasites can infect birds, rodents, monkeys and humans. Malaria is transmitted by Anophiline mosquitoes carrying parasites during a blood meal. Anophiline mosquitoes transmit the human parasites but not all *Anopheles* mosquitoes transmit malaria. Malaria can only be transmitted by a female Anophilline mosquito when it is taking a blood meal; male Anophilline mosquitoes feed on nectar from plants.

The human parasites are of two sub genera *Laverania* and *Plasmodium*. Three malaria parasites have been found exclusively in humans, these are *P. falciparum* (Welch., 1897), *P. vivax* [6], and *P. ovale* (Stephens, 1922). *P. malariae* is found in both humans and African apes [6]. Recently *P. knowlesi* has been described to infect humans in Malaysia Borneo [7]. *P. falciparum* causes the most severe form of malaria.

2 THE DISEASE

2.1 *PLASMODIUM FALCIPARUM* MALARIA

Malaria burden poses socioeconomic challenges in most of the developing countries in the world. In Africa, both urban and rural settings are affected by *falciparum* malaria. Due to the large number of malaria episodes per year, especially in sub-Saharan Africa, the cost of treatment is an economic challenge both at household and national level. In adults, the labour force, there are many days of work lost due to malaria illness, through sickness and mortality thereby reducing the total productivity. On the other hand in children malaria illness results in malnutrition, loss of parents and absence from school which may result in poor education and reduction in skilled labour force. With many countries in Africa still having struggling economies, the impact of malaria is a big challenge to their economy.

Several studies have been carried out in several African countries which shed a light on the impact of malaria in these populations. In Ghana, intra-city variations in the disease were observed with the urban poor being the most vulnerable group [8, 9]. In Malawi, children suffer an average of 9.7 malaria episodes per year and malaria is responsible for over 40% of deaths in children under two years of age [10]. The disease is not only restricted to children, mortalities in adults have also been observed both in Africa and SE Asia [2, 8] this has a large impact on the socio-economic growth of these populations.

The disease challenge in malaria endemic areas, has led to adults from these areas of stable transmission, to develop immunity that protects against severe forms of the disease. Among the adults, pregnant women in malaria endemic areas, especially those in their first pregnancy (primigravidae), are vulnerable to severe malaria due to parasites that sequester (attach) in the placenta resulting in what is known as placental malaria (PM) [11-13]. During the first pregnancy, women from endemic areas lack antibodies to placental binding parasites suggesting a new set of antigens that these women have not been exposed to [14-18]. As such primigravid women are at very high risk of PM and even severe forms of the disease. PM affects both the mother and the developing foetus resulting in severe anaemia in the mother and low birth weight in the newborns. There are estimated 100 000-200 000 infant deaths that are associated with

PM [19, 20]. Conversely, multigravid women with prior PM have a lower risk of disease.

2.2 CLINICAL FEATURES OF MALARIA

2.2.1 Clinical features

The first symptoms of malaria can be observed 8-10 days after the parasite was introduced into the human host. The release of parasite toxins during erythrocytic rupture is what brings about malaria symptoms. Malaria presents different fevers to the host depending on the form of the malaria. The bouts of fevers are due to synchronous lysis of pRBCs. Some malaria forms present everyday fevers (semi-tertian malaria) while others present fevers every second day (tertian malaria) and there still other forms which present fevers every third day (quartan malaria). *P.falciparum* presents both a tertian and semi-tertian fever forms, while *P.vivax* in addition to *P.ovale* present a tertian malaria form and *P.malariae* presents a quartan malaria form. In *P.falciparum* malaria, after a few erythrocytic cycles, the haemolysis loses synchrony and with irregular haemolysis patients tend to have continuous fevers. Therefore *falciparum* malaria starts off as tertian malaria and presents semi-tertian forms as well. It is with this reason that *falciparum* malaria is termed malignant tertian malaria (MTN) while *P.vivax* and *P.ovale* malaria are termed benign tertian malaria.

Focussing on *P.falciparum* malaria, initial malaria symptoms include, headaches, high temperature followed with chills and sweating, muscular pains, dizziness, vomiting, diarrhoea, and in severe cases convulsions and impaired consciousness. Malaria caused by *P. falciparum* can be broadly classified as mild (uncomplicated) malaria and severe (complicated) [21]. Mild malaria is characterized by fevers and rigours associated with parasite toxins released during erythrocytic rupture [22].

When a patient presents with malaria, a blood smear is prepared in order to detect presence of early stages (rings) which are in circulation. Trophozoites and schizonts are not observed from blood smears as they sequester in vessels hence not be observed in circulation.

The definition for severe malaria (SM) is complicated but severe disease is often associated with a phenomenon known as cerebral malaria and several other cytokine responses which can trigger complicated disease. The cause of severe malaria is not

fully understood. Clinically severe malaria is composed of three often overlapping clinical syndromes; impaired consciousness, respiratory distress and severe anaemia [23]. CM has been attributed to sequestration of infected erythrocytes (IE) to brain endothelial cells in the microvasculature which results in clogging and finally rupture of the blood vessels. Another mechanism in pathogenesis of cerebral malaria involves cytokine induction of secondary mediators such as nitric oxide (NO) which may cause intracranial hypotension due to excessive expansion of the blood vessels (vasodilation) and aberrant neurotransmission [24]. Tumour Necrosis factor α (TNFα) may also be linked to both mechanism outlined above as it causes up regulation of adhesion receptors as well as modulating the effects of NO [25].

To further understand the phenomenon of severe malaria, several studies have looked at the interrelationship and association between the factors associated with severe disease. Studies in Kenya and Gambia have revealed that the peak of CM and SMA does not occur at the same time in the human host. It has been observed that CM incidence peaks at older age compared to SMA which led to the hypothesis that CM occurs only after some developmental changes in the host brain or that there are certain strains of *P. falciparum* which can cause CM while all strains of *P. falciparum* may cause SMA [26].

Severity of malaria has also been associated with occurrence of parasite genotypes described in MSP1 and MSP2 surface proteins [27]. These two proteins have allelic protein families unique to each protein. MSP1 has three allelic families, K1, MAD20 and RO33 [27], while MSP2 has two allelic families, 3D7 and FC27 [28, 29].The distribution of these alleles in asymptomatic, mild and severe malaria has been shown to vary in different regions. RO33 was observed in malaria cases in Brazil and Senegal [30] while in Gabon RO33 was observed in asymptomatic cases [31]. On the other hand K1 was predominant in asymptomatic cases in East Africa [32] but was observed in severe cases in Gabon [31]. A study conducted in a hyper endemic region in India showed that severe malaria was associated with higher parasite density and increased multiplicity of infection using *P. falciparum* genotypes 3D7 and FC27 [33].

Studies in malaria epidemiology are being carried out in different parts of the world in order to better understand the disease incidence, distribution and how the disease can be controlled.

2.2.2 Disease Epidemiology

Malaria epidemiology varies in different regions of the world. Malaria is endemic in sub-Saharan Africa, and SE Asia with diverse degrees of endemicity.

In malaria epidemiology there is interplay of different factors which add complexity to understanding the disease mechanisms. These factors could be host specific, environmental and or geographic and they play an important role in the disease outcome.

Age-specific patterns have been observed in malaria endemic areas [26, 34]. Children under five years of age are prone to CM while adults mostly suffer from mild malaria. The risk of death due to malaria is very high among children compared to adults living in endemic areas. Interestingly, when adults have high parasitaemia there is a higher risk of disease as compared to children with the same parasite density [35]. This phenomenon has been explained by proposing that immunity that protects against disease develops during the first 5 to 6 years of exposure to malaria while non-sterilizing immunity that regulates infection occurs after 15 years of residence in a malaria endemic area [36]. In Papua New Guinea, age dependent level and prevalence of antibodies against the merozoite protein apical membrane protein 1 (AMA1) which paralleled the development of protective immune responses were observed [37]. On the other hand in Guinea Bissau and Cameroon it was observed that there was a small age related change to antibody responses while in Senegal this was not observed. [38, 39].

Presence or absence of certain receptors between ethnic groups have shown striking differences in susceptibility to various *Plasmodium* species. It has been observed that human red blood cells (RBC) that are deficient of GYPA or B (GYPA and GYPB) show resistance to invasion by *P. falciparum* [23]. EBA175, a micronemal protein, present on the merozoite, has Duffy Binding-Like (DBL) domains that recognise specific sialic acid residues on GYPA molecule, therefore the absence of GYPA and GYPB affects the protein's recognition of the RBC. Deletion of GYPC exon 3 is responsible for the Gerbich blood group negativity which is found in 50% of humans living on the coastal areas of Papua New Guinea. In Gerbich negative individuals EBA140 does not bind and *P. falciparum* invasion is blocked [40]. No epidemiological evidence has been found yet that GYPC deletion affects malaria infection.

Erythrocyte polymorphisms such as Haemoglobin S, α-thalassemia and a polymorphism where there is deficiency of the complement receptor (CR) 1 in individuals have been associated with providing protection against severe forms of malaria.

Individuals with heterozygote sickle cell disease (HbS) generally have 10-fold protection from life threatening forms of malaria and lower levels of protection against milder forms [41-43]. Mutations to haemoglobin C (HbC) is common in West Africa but less than HbS. Sickle cell haemoglobin and glucose -6-phosphate dehydrogenase (G6PD) A- deficient heterozygous females are protected against severe malaria [44]. A recent study in Kampala, Uganda has shown that G6PD A- heterozygous females and hemizygous males had a higher incidence of uncomplicated malaria and high parasite densities compared to children without the mutation concluding that G6PD deficient individuals have an increased incidence of malaria [45-47]. Similar outcomes have been observed in Gabon [45, 46].

Polymorphisms in the ABO blood groups also play a role in malaria disease outcome. The ABO blood group polymorphisms were discovered in the early 20th century by Karl Landsteiner and the polymorphisms were shown to be medically important by Johnson and Hopkins [48]. Strain specific preferences were observed in rosetting parasites when cultured in blood group A, B, A/B and O RBCs with reduced rosetting observed with the blood group O compared to the A and B blood groups [49, 50]. It has also been shown that individuals with the blood group O suffer from less severe forms of malaria as compared to those with A, B and A/B blood groups, this outcome can be correlated to the reduced rosetting observed with parasites cultured in blood group O [49].

The other factor that has contributed to malaria complications is the development of parasite resistance to antimalarial drugs. Parasite drug resistance has led to treatment failures of many first drug regimens in different parts of the world.

3.1. DRUG RESISTANCE

Antimalarial resistance is the ability of a parasite strain to survive and multiply despite the administration of a drug at therapeutic dosages making it difficult to clear the

parasites. *P. falciparum* drug resistance was first reported in the early 1950's in Colombia and Thailand and in the next 20 years resistance spread to Africa and SE Asia. Chloroquine, which had been used as a first line drug against malaria in several countries, was replaced due to resistance that was observed in most parts of Africa, South America and SE Asia. This led to the discontinuation in the use of the drug in these parts.

The issue of drug resistance became more complicated with the development of multi-drug resistant strains. Multi-drug resistance is defined as resistance to three or more antimalarial drugs; this has been observed in SE Asia. Varying levels of antimalarial drug resistance in different regions of the world have been reported. Parasite drug resistance has been attributed to mutations in certain parasite genes which are targets by the antimalarial drugs. Mutations in particular genes in parasite strains have been associated with resistance to different drug combinations both in Africa and SE Asia. Multiple mutations in *dhfr* a gene that encodes the enzyme *dihydrofolate reductase* (DHFR) and *dhps* which encodes the enzyme *dihydroptroate synthase* (DHPS) which are involved in the folate pathway contribute to Sulfodoxine pyrimethamine (SP) resistance [51-53].

Resistance of the malaria parasite to first line malaria drugs has led to treatment failures in most of the malaria endemic areas. Treatment failures have been observed in most parts of Africa and SE not only to Chloroquine (CQ) [54, 55], but also Sulfodoxine pyrimethamine (SP) [51, 56], and in some rare cases resistance to Quinine (QN) have been reported in Thailand and SE Asia. Mefloquine resistance was reported in the early 90's in SE Asia [57]. In Thailand, mefloquine resistance was associated with increase in copy numbers of the *Plasmodium* multi resistance gene, *pfmdr* [57]. True stable resistance has not yet been reported to artemisinin compounds.

Continued research on multi-drug resistant parasites and the genes that are involved is one way to address the understanding of this phenomenon. The release of the genome sequence of *Plasmodium* [58-60] and availability of new methods to genetically manipulate genes through gene disruption, transgene expression and allelic exchange has allowed for profound research on different genes and their functions [61]. As such there is hope that apart from the already known multi-drug resistant genes in parasites

more studies will identify ways to disrupt these genes and their pathways thereby help to develop drugs that can overcome this problem.

3 THE PARASITE

3.1 PARASITE LIFE CYCLE

All malaria parasites require two hosts in their life cycle: the definitive host where sexual development occurs (Anophiline mosquito) and the intermediate host (e.g. man) where asexual development occurs (Figure 2). The haploid parasite adopts three strategies during its life cycle in order to proliferate.

The first strategy is the ability to grow and replicate extensively, which is achieved through three different stages. The first stage is the oocyst which occurs in the mosquito (in a process called sporogony), and second is schizogony stage (also called erythrocytic schizony or pre-erythrocytic schizogony) and the third stage is erythrocytic schizogony.

The second strategy adopted by the parasite is dispersal and invasion of host cells. This includes the merozoite, sporozoite, and ookinite stages of the parasite.

The third strategy is sexual reproduction which involves the formation of gametocytes in the peripheral circulation of the vertebrate host which is completed upon formation of the ookinite in the mosquito after a blood meal.

The three parasite strategies are summarized in the life cycle both in the mosquito and parasite host (Figure 2). An infective bite from an Anophiline mosquito introduces malaria sporozoites through its saliva into the human host during a blood meal. The sporozoites then migrate to the liver cells (hepatocytes) where they mature into merozoites (about 5-15 days) and then they are released into the circulation starting what is known as the erythrocytic cycle. In *P. falciparum*, the erythrocytic cycle takes 48 hours depending on the parasite strain. The merozoites invade RBC and they develop into the ring stages (0-24hrs) then to trophozoite stage (24-35hrs) and finally schizont stage (35-48hrs) where the merozoites are produced and mature. The merozoites are released into the bloodstream after schizont rupture to start another cycle. Some parasites differentiate into gametocytes, sexual forms, which are then taken up by an Anophiline mosquito during a blood meal. The gametocytes undergo fertilization and produce ookinite in the mosquito midgut. The ookinite then matures into oocysts which cross the midgut epithelia and migrate to the salivary glands where they mature into sporozoites which are then transferred to a human host during a blood meal and the erythrocytic cycle begins again (Figure 2).

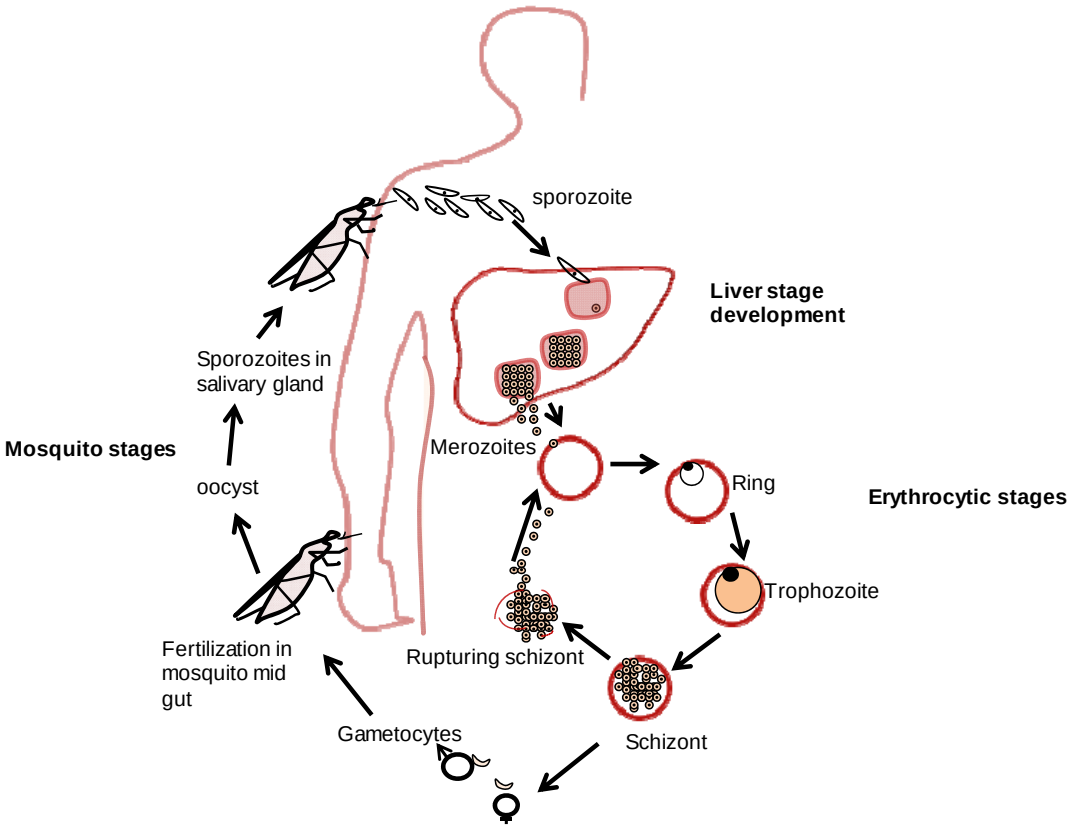

Figure 2: **Life cycle of *Plasmodium falciparum***.

3.2 PATHOGENESIS

As shown in the life cycle the malaria parasite survives in the mosquito where sexual reproduction of the parasite occurs and the mosquito vector transmits the parasites to the human host. In the human host the parasite needs to proliferate and survive without being destroyed by the host immune response. To achieve this *P. falciparum* uses several techniques to avoid the host immune system and to cause severe disease. Some of the well known techniques include cytoadherence and sequestration, rosetting and antigenic variation.

3.2.1 Sequestration and rosetting

3.2.1.1 *Sequestration*

The sequestration of pRBC in the microvasculature of vital organs is a prominent feature of *P. falciparum* malaria and is likely to be one of the most important factors in the pathogenesis of the disease [62]. Sequestration is defined as the removal of pRBC from the peripheral circulation by binding of the pRBC to vascular endothelium,

predominantly in post-capillary venules of the deep tissues. Impaired oxygen delivery due to occlusion of blood flow in the vessels may result in organ dysfunction. Massive sequestration in the brain is believed to be the cause of coma in cerebral malaria [62]. In the human host the spleen gets rid of all of all abnormal RBCs from circulation. pRBCs are deformed due to parasite development as such they are targeted by the spleen and are removed in a process known as splenic clearance (pitting). *P. falciparum* parasites sequester in blood vessels to avoid splenic clearance.

Mature stages of the malaria parasite, the trophozoites and schizonts accumulate a pigment known as heamozoin. Immunohistochemical studies have shown that this malaria pigment is not only present in trophozoites and intact schizonts but also seemingly free lying within vessels and within intravascular white blood cells [63]. Free lying pigments are indicative of ruptured schizonts and trophozoites which were sequestered in the vessels.

3.2.1.2 *Rosetting*

Parasite sequestration is an integral part in adhesion of pRBC to endothelium, and extended by clustering of cells through either rosetting (binding of pRBC to uninfected RBC) or clumping (pRBC binding to other pRBC). Both clumping and rosetting have been independently associated with severe malaria and anaemia [64-66]. Complement receptor 1 (CR1) has been identified as an important receptor for PfEMP1-mediated rosetting [67].

3.2.2 Antigenic variation

Antigenic variation was first discovered in primate malaria *Plasmodium knowlesi* (Brown and Brown, 1965 [68]). Proteins such as PfEMP1 have to display a form of variability. With approximately 60 *var* genes encoding PfEMP1 and only one dominant *var* gene being expressed at the mature stage of the parasite, PfEMP1 has achieved a form of variability, which in turn has allowed the parasite to cope with the host immune system. PfEMP1 is responsible for sequestration which is a mechanism used by parasites to escape splenic clearance [69]. Frequent gene recombination and shuffling during genomic fusion and division processes in both mosquitoes and human erythrocyte can result in extensive genetic and antigenic diversity [70]. The affinity of an expressed PfEMP1 to certain host receptors is suggested to determine parasite

virulence [71]. Supporting this hypothesis is the case of placental malaria where DBLγ or β are shown to mediate adhesion to placental tissue through CSA or non-immune immunoglobulins. Another example supporting the hypothesis is that of the 3D7 parasite where the CIDRα domains encoded in the parasite have affinity to CD36 receptors [72, 73]. This evidence postulates that certain *var* genes may be more virulent than others and parasite strains such as 3D7 which have more conserved *var* genes in their centromere may display more virulent phenotypes [70].

3.3 *P.FALCIPARUM* VARIABLE SURFACE ANTIGENS AND SURFINS

In order to survive in the human RBCs parasites employ different proteins that help to achieve different functions during various developmental stages.

P. falciparum has several multi gene families encoding different proteins. Of these three major variable surface antigens (VSAs) have been described. These include *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), RIFINS and STEVORS (subtelomeric variable open reading frame proteins).[3].

3.3.1 PfEMP1

PfEMP1 belongs to a large family of polymorphic proteins of 220 – 350 kDa and is encoded by the *var* multigene family, found at 55-60 copies per haploid genome [69, 74-76]. During the ring stage several v*ar* genes are expressed but during the trophozoite stage, only one PfEMP1 is dominant. In multiclonal infections, multiple *var* genes are transcribed in a population of mature stage parasites although a limited number of transcripts appear to dominate [77, 78]

3.3.2 RIFIN

The RIFIN proteins, 30-40kDa in size, are another group of variable surface proteins encoded by the150-200 *rif* (repetitive interspersed family) multi gene family [79, 80] . No definitive function of the RIFINS has been established. RIFIN have been grouped into two major groups A and B proteins [81]. It has been suggested that these proteins may play a role in rosetting using CD31 as a receptor [79].

3.3.3 STEVOR

The 40 member STEVOR (subtelomeric variable open reading frame proteins) encoded by the *stevor* multi gene family is transcribed in the gametocytes [82]. Clonal variants

of *stevor* have been observed on the surface of the schizonts [88].

The protein has been shown to be expressed in the asexual stages of *P.falciparum*. The proteins localize to Maurer's clefts in mature schizonts and have been shown to be exported to the erythrocyte surface [83-85] [86, 87].

3.3.4 SURFIN

Recently, a novel group of high molecular weight proteins called SURFINs encoded by the *surf* multi gene family has been identified. The protein is related to a *P. vivax* transmembrane protein, PvSTP1 (*Plasmodium vivax* subtelomeric transmembrane protein 1), the VIR proteins of the *P. vivax* external cysteine rich domain and the ATS (acidic terminal sequence) of PfEMP1.

One member of the SURFIN family, $SURFIN_{4.2}$ was observed both on the merozoites and infected erythrocyte $SURFIN_{4.2}$ is a polypeptide of 286kDa expressed on the surface of pRBCs and as merozoite associated material (MAM) at the apical end of the merozoite [89].

The SURFIN family is encoded by a family of 10 *surf* genes located within or close to the sub telomeres of five chromosomes of the 3D7 parasite strain. SURFINs are found on chromosome 1, with three genes, $surf_{1.1}$, $surf_{1.2}$ and $surf_{1.3}$. Chromosome 4 with $surf_{4.1}$ and $surf_{4.2}$, chromosome 8 with $surf_{8.1}$, $surf_{8.2}$ and $surf_{8.3}$ are located on and chromosome 13 and 14 each have one *surf* gene viz $surf_{13.1}$ and $surf_{14.1}$ respectively [89] [90].

3.3.5 SURFIN Structure

The SURFINs have conserved features which are unique to the SURFIN family. In the N-terminus, the SURFINs a cysteine rich domain (CRD) followed by a variable region, a transmenbrane domain. A tryptophane rich domain (WRD) separated by semi-variable regions is located towards the C-terminus of the proteins [89].

The *surf* genes have 2-5 exons between the members. The SURFIN family has been divided into groups according to similarity between the family members. The SURFINs have been divided into two major groups, GroupA and GroupB according

to sequence structure and similarity. There is also an intermediate group that is related to both GroupA and GroupB but not enough to fit in either of the groups. The intermediate group consists of two SURFIN members present on chromosome 1, PFA0625w and PFA0650w (Figure 3).

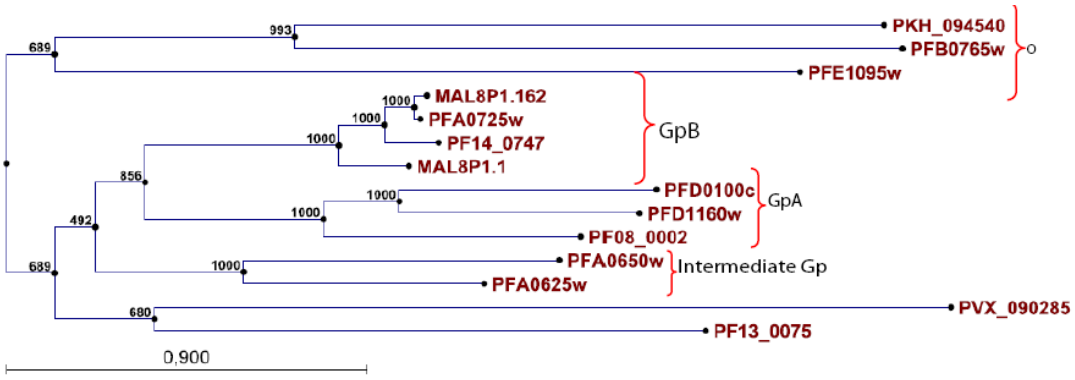

Figure 3: **SURFIN family tree**. Showing SURFIN family groups, GroupA (GpA), GroupB (GpB), the intermediate group (intermediate Gp) and the outgroups (o).

3.3.6 Cellular localization and trafficking SURFIN$_{4.2}$

SURFIN$_{4.2}$ is presumed to co-localize with PfEMP1 in the small transport vesicles (SSV) and accumulate in the PV in the 3D78 parasite line. In the FCR3S1.2 parasite line, which has LMV in over 90% of the trophozoite stage, SURFIN$_{4.2}$ co-localized with PfEMP1 in SSV and also prominently in LMV from where surface proteins appear to be delivered to the exterior. The SURFINs have also been found in an amorphous cap at the apex of extracellular merozoites.

In the chapters that follow, localization of other SURFIN members, SURFIN$_{4.1}$, and GroupB SURFIN members will be discussed in detail.

4 THE PRESENT INVESTIGATION

4.1 THE SCOPE OF THE THESIS

The purpose of the study was to explore the SURFIN family of proteins in *P. falciparum*, looking at gene transcription, localization and characteristics (Paper I, II, and III) and studying parasite distribution in organs of patients who died from malaria (Paper IV).

The specific objectives of the study were:

- To study $surf_{4.1}$ gene expression, transcription and localization.
- Explore a possible receptor for $SURFIN_{4.2}$.
- Describe the SURFIN family using different bioinformatics tools.
- Study *var* gene expression and distribution in parasites lodged in organs of fatal pediatric malaria cases in Malawi.

4.2 MATERIALS AND METHODS

The materials and methods that were used for each specific paper are presented in detail in Papers I –IV. In this section some of the methods that were used routinely in the experiments are presented.

4.2.1 SURFIN sequence retrieval and analysis

Protein, DNA and RNA sequences from 3D7, DD2 and HB3 parasite lines, for the SURFIN family were downloaded from the *Plasmodium* database, plasmoDB (www.plasmodb.org), genedb (www.genedb.org) and the Broad institute genome databases (http://www.broad.mit.edu/annotation/genome/plasmodium_falciparum_spp/Blast.html).

Allocation of the SURFIN family members into groups was done by comparing protein SURFIN sequences from 3D7 parasite line. Protein sequences from both SURFINs and out-group sequences from SURFIN orthologous proteins were retrieved from the *Plasmodium* database, plasmodb through BLAST using one SURFIN sequence as a seed sequence. The BLAST search was conducted using a maximum expectation value of 0.05, and a maximum description of 20 without a low complexity filter. The SURFIN and out-group sequences were aligned using Clustal W, Clustal X [91, 92] and K-align, with a gap penalty of 11.0, gap extension penalty of 0.85, a terminal gap penalty of 0.45 and a bonus score of 0. The alignments were viewed using BioEdit (BioEdit version 7.0.4.1, 1997-2005) and CLCsequence viewer, sequence editing programs. From the alignments obtained a neighbour joining amino acid tree was constructed from1000 replicates and bootstrap values were plotted. Sequence conservation between the two SURFIN members was determined by calculating percentage identities between the SURFIN groups. Protein identities and conservation between the SURFINs were calculated using Clustal X.

4.2.2 Comparison of GroupB protein sequences among 3D7, HB3 and DD2 parasites

GroupB protein sequences from 3D7, HB3 and DD2 parasites were aligned using Clustal W, Clustal X and K-align as outlined above. The sequences were viewed using BioEdit (BioEdit version 7.0.4.1, 1997-2005) and CLCsequence viewer, sequence editing programs. Percent sequence identities between the GpB members were calculated using clustal X. Thereafter to identify presence of similarity blocks within the sequences, the alignment was imported into Gblock_0.91b

(http://molevol.cmima.csic.es/castresana/Gblocks.html), an algorithm for identifying similarity blocks within a given alignment.

4.2.3 Identification of SURFIN features

SURFIN protein sequences from 3D7 parasite strain were each analyzed using simple modular architecture tool (SMART) (http://smart.embl-heidelberg.de/), using the genomic option. SMART analysis tool incorporates several algorithms which include Pfam, ARIADNE and Prospero. Presence of functional domains was analyzed using Pfam (http://pfam.sanger.ac.uk/) which uses Hidden Markov models (HMM) to predict functional domains in the protein. Presence of signal peptides was analyzed using Sigcleave an algorithm which uses the von Heijne method [93] to predict signal peptide sequences. SURFIN conserved domains (SCDs) were identified through ARIADNE programs, prospero and ariadne, which compare protein sequences and profiles using the Smith-Waterman algorithm [94]. Using Clustal W and K-align the SCDs were aligned using the parameters described earlier (4.2.1) and conserved protein motifs within the SCDs were identified in the GroupB SURFINs. Occurrence of the conserved sequences in different *Plasmodium* species was carried out using plasmid similarity/pattern tool which identifies genes based on protein motifs (http://plasmodb.org). Presence of transmembrane domains (TM) was determined using Phobius (http://phobius.cbr.su.se/).

4.2.4 Parasites used in the study

The following parasites were used on a regular basis during the study:

FCR3: A parasite isolated from Gambia, in vitro culture established by Jim Jensen and William Trager at Rockerfeller University.

FCR3S1.2: A multi-adhesive parasite which expresses one PfEMP1 and multiple RIFINs.

3D7AH1: Used for crosses in mosquitoes with HB3 (Tom Wellems) and DD2 (David Walliker).

3D7S8: Cloned from 3D7 by micromanipulation on the rosetting phenotype in Stockholm.

CS2WT: A clone of the It isolate, adheres to chondroitin sulfate (CSA) and hyaluronic acid (HA) in vitro.

CS2Δ*surf*$_{4.2}$: A PFD1160w (*surf*$_{4.2}$) knock out line of the CS2WT.

DD2: A laboratory adapted parasite isolate from Indochina.

HB3: A laboratory adapted parasite isolate from Honduras.

4.2.5 Parasite culture

Parasites were cultured according to standard protocols at 37°C using the candle jar method [95]. *P. falciparum* parasites were cultured in complete medium containing (RPMI-1640 with 25mM HEPES and the supplements, sodium bicarbonate (25mM), L-glutamine (2mM), gentamicin (0.5%) and heat inactivated B+ human sera (10%).
The parasites were sub-cultured twice per week and medium was changed on a daily basis in the culture. Synchronization of the culture was performed by adding 5% sorbitol to the culture and incubating at room temperature for 10min followed by 3 washes in RPMI (SIGMA).

CS2WT and CS2Δ$surf_{4.2}$ were cultured using the same medium as outlined above but instead 5% serum and 5% albumax were used in place of 10% serum. The parasite nucleus was stained using acridine orange and parasites were visualized under Nikon Optiphot 2 UV microscope.

4.2.6 Genomic DNA and RNA transcript analysis for *surf* genes

To study the distribution of *surf* genes in different laboratory and wild isolates, PCR was performed on parasite genomic DNA (gDNA) using two separate primer sets designed from the 3D7 genome (www.plasmodb.org v4.3) specific to each *surf* gene (Table 1, appendix 1).

To obtain the gDNA, parasites were harvested at required stages, rings, trophozoite and schizont stages depending on the experiment. Genomic DNA was extracted mostly from trophozoite stages using DNEasy mini blood extraction kit (QIAGEN, USA). All PCR amplifications were carried out at the following conditions 35 cycles of 94°C for 30sec, 55°C for 30sec, and 68°C 60sec.

For RNA analysis, highly synchronized parasites were harvested at different stages and RNA was extracted immediately using RNEasy mini kit (QIAGEN, USA). The RNA (100ng) was reverse transcribed (RT+) using MuLV reverse transcriptase enzyme and random hexamers (Applied Biosystems, USA) according to the manufacturer's

instructions. For each RT+ experiment, one RT- reaction (with reverse transcriptase omitted) was performed to ensure that there was no DNA contamination in the RNA.

For the analysis of the *surf*$_{4.1}$ gene, primers (*surf*C$_{4.1}$F/R and Sintra$_{4.1}$ F/R) were consequently designed on opposite sides of the intergenic region separating the two previously annotated genes PFD0100c and PFD0105c (plasmodb v.3). cDNA from rings, trophozoite and schizont stages was amplified using *surf*C$_{4.1}$F and *surf*C$_{4.1}$R primer set. The RT-PCR cycling conditions were as follows: 35 cycles of 94°C for 30sec, 55°C for 30sec, and 68°C 60sec. The cDNA from the different parasite stages was also amplified using primers specific to the *surf*$_{4.1}$ gene sections, these included 5CS-1F/R, 5CS-2F/R, *surf*C$_{4.1}$F/R, S$_{4.1}$F/R, 1C-S1F/R, 1C-S2F/R and 1C-S3F/R (Table 2, appendix 1).

4.2.7 SURFIN Protein expression in *E.coli*

For the analysis of SURFIN$_{4.1}$, the protein encoded by the *surf*$_{4.1}$ gene, a *surf*$_{4.1}$ construct was designed from the variable region of the first exon of PFD0100c. A protein BLAST was conducted against *P. falciparum* non-redundant proteins to check if the selected fragment occurred in other *P. falciparum* proteins. The construct was designed with a His–tag located in the C-terminal end. The construct was screened for presence of restriction sites so as to determine which restriction enzymes to use during the cloning process. PCR using specific primers flanking the construct was performed and the product was purified and cloned into TOPO II vector. The purified plasmid DNA was then sequenced to confirm the integrity of the sequence. Thereafter the plasmid was cloned into PQE70 expression vector (QIAGEN, USA) where the protein was expressed at room temperature and at 37°C in *Escherichia coli*. The expressed protein was then purified using a Nickel column and was dialyzed with PBS twice, for 3hrs and overnight respectively. To check the status of the expressed recombinant protein, a 10% SDS protein gel was run and a Coomasie stain was performed on the gel to confirm the size and purity of the purified recombinant protein. A Western blot was also performed using anti-His antibody to confirm integrity of the recombinant protein. The purified protein was used to immunize rats (100µg/ml) and rabbits (500µg/ml) to develop antibodies against SURFIN$_{4.1}$. The antibody against SURFIN$_{4.1}$ was called anti-SURFIN$_{4.1}$-C1 antibody.

For the GroupB SURFIN analysis, a peptide antibody was designed to three members of the group. Multiple alignments of SURFIN$_{1.3}$ (PFA0725w), SURFIN$_{8.3}$ (MAL8P1.162) and SURFIN$_{14.1}$ (PF14_0747) were performed using Clustal W.

Peptides were then designed from regions of similarity (100% identity). BLAST was run against *P. falciparum* non-redundant proteins from the *Plasmodium* database (plasmodb) to check for specificity of the peptide sequence. Two peptide sequences were designed initially and antibodies were developed in rabbits to both peptides. One of the peptide antibodies showed weak reactivity hence it was not used for analysis. The other peptide antibody showed better reactivity and this was the peptide sequence N–CQLKVFDEIEKRSIYLSSKRVLKWK-C to which the antibody was developed. The GroupB antibody, rabbit anti- $SURFIN_{GroupB}$ antibody was raised by a commercial supplier (INNOVAGEN AB) in rabbits and was KLH-conjugated. The $SURFIN_{GroupB}$ antibody was affinity purified by the same commercial supplier.

4.2.8 Western blot Analysis

Parasites were processed either as whole cell culture mix or were enriched for late stages, trophozoite and schizonts using a magnetic cell sorter, MACS system (Miltenyi biotech, Bergisch Gladbach, Germany) [96]. The cells were lysed in reducing SDS-PAGE sample buffer at 95°C for 5 minutes. Extracts ($5x10^7$cells/lane) were separated on a 4-15% gradient SDS- PAGE (BIORAD, USA) or 6% and 10% SDS poly acrylamide gels. Proteins were transferred onto nitrocellulose membranes (0.45µm) (BIORAD, USA) and transiently stained with 0.1% Ponceau S in acetic acid. The membranes were blocked with 5% milk-PBS-0.05%Tween overnight (O/N) at 4°C or 1hr at RT depending on the source of the protein. Immunostaining was carried out with either ALP conjugated secondary antibodies (SIGMA) or HRP conjugated secondary antibodies as described (New England Biolabs) [97]

4.2.9 Immunofluorescence Assay (IFA)

P. falciparum isolates were harvested at various stages of interest and the pellet (50µl packed RBC) was washed three times in PBS. For indirect immunofluorescence assays (IFA), monolayers of pRBCs were prepared as previously described [98]. Monolayers were incubated with primary antibodies for 30min and subsequently with a secondary antibody for 30min. The antibodies were diluted either in blocking buffer (5%milk–PBS) or in PBS. All incubations were performed at room temperature (RT) in a humid chamber. Cover slips were mounted with an anti-fading solution (20% DABCO (SIGMA) in glycerol) or with VECTASHIELD mounting medium with DAPI (VECTOR Labs, USA).Slides were analyzed using a Nikon Optiphot 2 UV microscope with x100 oil immersion lens.

4.2.10 Invasion Inhibition Assays

Red blood cells were treated with enzymes neuraminidase (1x), chymotrypsin (1mg/ml) and trypsin (2mg/ml) as single enzyme treatment or in combinations of two enzymes, chymotrypsin-Neuraminidase, and chymotrypsin-Trypsin. All enzyme treatments were done at 37°C. Invasion inhibition assays were carried out using enzyme treated RBCs and mock treated RBCs (control cells). Parasites were synchronized using MACS (Miltenyi biotech, Bergisch Gladbach, Germany) to enrich for late stages. Parasitized red blood cells were cultured in a 96 well plate in a total volume of 50µl (1% heamatocrit) with a starting parasitaemia of 1% trophozoites (28-32hrs). Enzyme treated RBCs were the only uninfected cells added to the culture mix. The culture was then incubated for one parasite cycle (48hrs) or two cycles and analyzed using flow cytometry. The percentage invasion into enzyme treated RBCs was calculated against parasites incubated with mock treated RBCs. Invasion inhibition assays using sera were conducted as outlined [99].

4.2.11 Recombinant protein binding assay

RBCs were washed 3x in PBS and 5µl of the washed RBCs was incubated with 100pmol and 200pmol of $SURFIN_{4.1}$ recombinant protein diluted in PBS in a 100µl total volume. The mixture was incubated for 2hrs at 4°C. Thereafter the RBCs were washed 5x with PBS. The washed RBCs were boiled at 95°C in 20µl SDS loading buffer. The mixture was run on a 10% SDS-PAGE and transferred to membrane. A Western blot was run as previously described (4.2.8) using anti His antibody as the recombinant $SURFIN_{4.1}$ protein was His tagged.

4.2.12 RNA extraction from organs

Organs were collected during autopsy and snap frozen using liquid nitrogen in frozen tissue matrix (OCT compound, tissue Tek) and stored at -80°C. 0.5g of the tissue was ground in liquid nitrogen and resuspended in 10x volume pre-warmed Trizol (invitrogen). Insoluble material was removed by centrifugation at 12000g for 10minutes. The mixture was incubated for 5min at RT and extraction was carried out according to manufacturer's instructions. DNAse treatment using DNA-free RNA kit (Genetex) was carried out to remove residual DNA. PCR using DBL1a sequence primers was carried out to confirm total removal of DNA by the treatment.

4.2.13 cDNA synthesis from tissue RNA and cloning

cDNA was synthesized from 2µl RNA using retroscript kit (Ambion). The cDNA quality was checked by agarose gel electrphoresis. A primary PCR using the cDNA was conducted using DBL-fo and BBL-ro primers as described by Duffy et al. [100] with 1 mM final concentration and 1 mM dNTPs, 4 mM Mg2+ and 0.025 U *Taq* DNA polymerase (QIAGEN). A nested PCR was carried out using 1µl of primary product with the same reaction components, oligonucleotides DBL-fi and DBL-ri and reaction conditions as described [100]. The PCR products were purified using a QIAquick PCR purification or gel extraction kit (QIAGEN) as required. PCR products were ligated into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5a bacteria. Colonies were grown in liquid media and frozen in 96 well plates. Plasmid purification and DNA sequencing were performed at the Wellcome Trust Sanger Institute.

4.2.14 Sequence analysis of tissue transcripts

Sequences obtained from the cDNA were aligned using CLUSTALW (http://align.genome.jp). Sequence identities were compared between the tissue clones and *P. falciparum* 3D7 genome using the *Plasmodium* database, *PlasmoDB* (http://www.plasmodb.org). DBL motifs were analyzed using a database kindly provided by Peter Bull. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers DQ519104–DQ519354, EF640985 and EF640986.

4.3 RESULTS AND DISCUSSION

SURFIN family of proteins encoded by the *surf* multi-gene family, and the distribution and expression of *var* genes in organs from fatal malaria cases were the focus of this study.

4.3.1 *surf*$_{4.1}$ gene analysis (Paper I)

The SURFINs are a ten member family, three of which were previously annotated as truncated (pseudogenes). *surf*$_{4.1}$ gene was previously annotated as a

truncated gene with two separate open reading frames, PFD0105c and PFD0100c each with two exons but was proved otherwise in this study.

A revised new gene structure of *surf*$_{4.1}$, which combines two previously identified open reading frames (PFD0100c and PFD0105c) (plasmodb.org v4.3) was the main outcome of this study. PCR across the intergenic area which separated the two open reading frames, showed that *surf*$_{4.1}$ is one complete gene with three exons separated by two introns. *surf*$_{4.1}$ transcript was shown to be a single open reading frame in 3D7S8, FCR3, FCR3S1.2. A detailed analysis of the gene revealed that *surf*$_{4.1}$ is one complete gene comprised of three exons with one continuous open reading frame (ORF) (Figure 1A, Paper I) [89]. Differences in length of the first intron were observed between FCR3 and 3D7S8 parasite strains with FCR3 having a longer intron. Single nucleotide polymorphisms (SNPs) were observed in laboratory strains (HB3, DD2, D10, 7G8, IT, RO33, K1, FCR3, and FCC-2), some clinical isolates from Uganda (UAM25 (from Apac, a malaria endemic rural area in the northern part of Uganda), UKS03 and UKS05 (from Kampala, an endemic urban area in Uganda) and GHANA 1 from Ghana in the *surf*$_{4.1}$ gene.

The *surf*$_{4.1}$ gene encodes a 258kDa protein, SURFIN$_{4.1}$, which is of a slightly smaller molecular weight than the previously characterized SURFIN$_{4.2}$ (286.4kDa) (Figure 1D, Paper I).

PCR analysis using *surf*$_{4.1}$ specific primers revealed that *surf*$_{4.1}$ gene was amplified in 11 out of 12 parasite lines.

With the knowledge that *surf*$_{4.1}$ exists as one complete gene, copy number polymorphisms were analyzed in different parasite isolates to determine how many copies of the gene exists in the genome. FCR3 and its daughter clone FCR3S1.2 (cloned by micromanipulation) were found to have six copies of the gene, in concordance with copy number estimates performed previously using microarrays [101]. The increase in *surf*$_{4.1}$ gene copy numbers correlated with a five-fold increase in RNA transcription observed in FCR3 compared to the 3D7S8 parasite strain. However, the increase in copy number of the gene was not reflected on level of protein, rather that the protein was present a similar levels in the two parasite lines. This might be due to sequence differences in the protein in FCR3, resulting in poorer recognition by the antibodies raised using a protein construct from 3D7S8. Another possibility may be that

there is a certain level of translational repression as has been previously described in *P. falciparum* [102].

The temporal transcriptional profile of $surf_{4.1}$ was analyzed in the 3D7S8 and FCR3 parasite strains. $surf_{4.1}$ is transcribed during the late stages from ≈32h post invasion peaking at the late schizont stages (44-48 hrs). The difference in transcription patterns between $surf_{4.1}$ (transcribed from late trophozoites and schizonts) and $surf_{4.2}$ (transcribed from early rings to the schizont stages) may suggest differences in function of the proteins encoded by the respective genes. The transcription profile of $surf_{4.1}$ correlated well with the protein expression, observed in both FCR3 and 3D7S8 parasite lysates (Figure 3, Paper I). No detectable protein for $SURFIN_{4.1}$ was observed in early parasite stages.

4.3.1.1 *Presence of surf genes in different parasite lines and Differential transcription of the genes*

In order to investigate the presence of *surf* genes in both laboratory strains and clinical isolates of *P. falciparum*, PCR was carried out on all the 10 *surf* genes using two different primer sets for each gene. Two independent primer sets were used in order to reduce bias in gene amplification, which may arise with the use of only one primer set due to sequence variation. The outcome showed that all the genes were amplified in either laboratory adapted or clinical isolates, but not every gene was amplified in every parasite (Additional File 3, Paper I).

To understand which *surf* genes are transcribed and at what times the genes are transcribed in 3D7S8 parasite clone, RT-PCR was performed with primers specifically targeting individual sequences of all 10 *surf* genes (Additional File 1, Paper I). Differential gene expression was observed in the SURFIN family. Some *surf* genes were found expressed throughout the erythrocytic cycle such as $surf_{1.3}$, $surf_{4.2}$ and $surf_{8.3}$ which were expressed from early rings to the schizonts while others were restricted to later trophozoites and/or schizont stages ($surf_{1.1}$, $surf_{4.1}$ and $surf_{14.1}$) (Figure 4). Most of the *surf* genes were expressed in the later stages of the parasite cycle from 30hrs onwards (Figure 4).

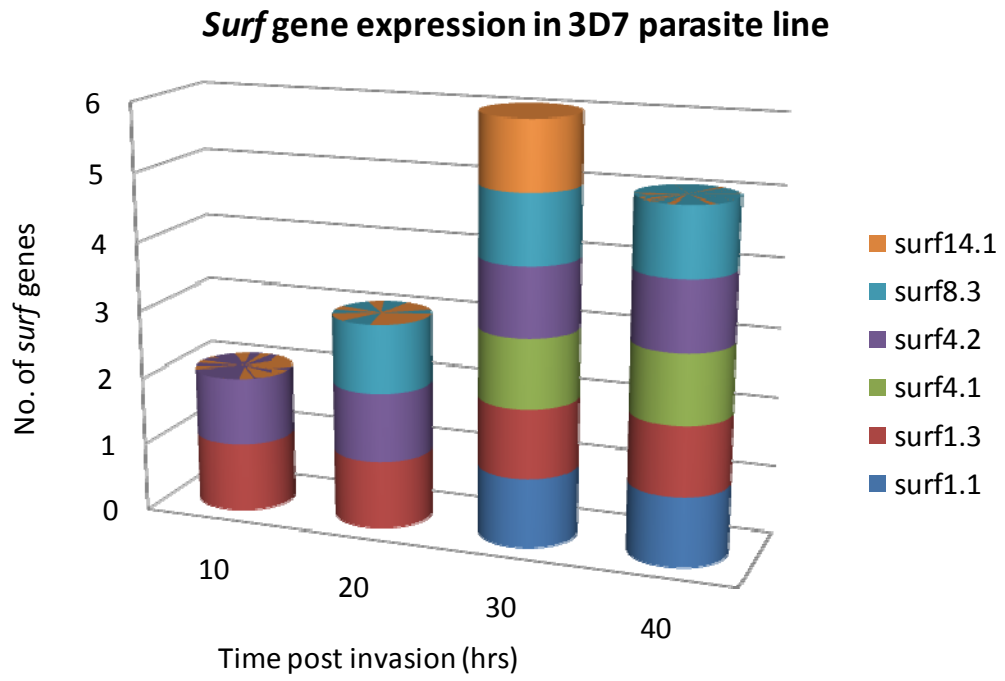

Figure 4: *surf* gene transcription. Graph showing *surf* gene transcription in 3D7 parasite line at different time points in the parasite cycle. Most of the *surf* genes are expressed during the late stages in the parasite life cycle.

4.3.1.2 *SURFIN$_{4.1}$ localization*

SURFIN$_{4.1}$ localized close to the food vacuole (FV) in the PV as a distinct spot, which later spread out within the PV in a dotty pattern in both 3D7S8 and FCR3 parasite lines. SURFIN$_{4.1}$ was also present on the merozoite as MAM. The protein was also observed around the newly formed merozoites in intact schizonts. The presence of SURFIN$_{4.1}$ on the merozoite suggests that the protein could have a function on the merozoite, whether or not the protein could be involved in merozoite invasion process is yet to be established.

Co-localization experiments were carried out between SURFIN$_{4.1}$ and the micronemal protein EBA175, and SURFIN$_{4.2}$ to determine whether SURFIN$_{4.1}$ was present at the apical end of the merozoite or not. In the intact schizont, SURFIN$_{4.1}$ appears to co-localize in part with EBA175, but the proteins differentiate in the ruptured (segmented) schizont. SURFIN$_{4.1}$ is spread around the pre-released merozoites, while EBA175 localizes at the apical end of the merozoite (Figure 4A, Paper I). SURFIN$_{4.1}$ is also co-localized with SURFIN$_{4.2}$ in the intact schizont but again the two proteins differentiate in the ruptured schizont as SURFIN$_{4.1}$ is spread around the pre-released

merozoites while SURFIN$_{4.2}$ is also seen on the merozoites but with apical staining (Paper I, Figure 4A-B).

The colocalization results suggest that SURFIN$_{4.1}$ is not at the apical end of the merozoite.

4.3.1.3 *SURFIN$_{4.1}$ and exposure on the infected erythrocyte surface*

3D7S8 and FCR3 late stage parasite cultures were examined for their ability to agglutinate in the presence of rabbit-anti-SURFIN$_{4.1}$-C1 antibody from whole serum. The serum showed no agglutinates in either 3D7S8 or FCR3 (30hr and 40hr) parasite cultures.

Failure of anti-SURFIN$_{4.1}$-C1 serum to agglutinate pRBC in FCR3 and 3D7S8, suggests that the protein is not exposed on the infected erythrocyte surface. This outcome correlates with the results from IFA that SURFIN$_{4.1}$ is contained within the PV. Even though SURFIN$_{4.1}$ is not transported to the erythrocyte surface it has a PEXEL-like motif RKIFE in the N-terminus of the protein.

4.3.2 *surf$_{4.2}$* gene analysis (Paper II)

The protein SURFIN$_{4.2}$ was the main focus of this paper. SURFIN$_{4.2}$ has been shown to be transported to the erythrocyte surface together with PfEMP1 [89]. The protein was also found to localize at the apical end of the merozoite as MAM. The receptor for the protein and the function are not yet known [89].

In this study *surf*$_{4.2}$ a gene encoding SURFIN$_{4.2}$ protein, was disrupted in CS2 parasite line. Analysis of both the CS2WT and the CS2Δ*surf*$_{4.2}$ were carried out. *surf*$_{4.2}$ was found to be a single copy gene in both 3D7 and CS2 parasite lines. The integrity of the knockout was confirmed by Southern blot and PCR.

4.3.2.1 *Growth and morphology of CS2WT and CS2Δsurf$_{4.2}$*

Growth and parasite morphology as well as pRBC morphology between CS2WT and CS2Δ*surf*$_{4.2}$ was studied during continuous culture of the two parasite lines. During the continuous culturing of the parasites some RBCs were found clumped to each other both in the knockout and CS2WT strains. The clumps were made of 4-8 RBC and sometimes pRBC were part of the clumps. The clumps were observed at different

stages of the parasites. During the trophozoite stage, the pRBC involved in clumps looked almost like rosettes. No classical rosettes were observed in either of the parasite lines.

Both CS2WT and CS2Δ*surf*$_{4.2}$ parasite lines showed no significant differences in multiplication rates during culture. The parasite lines showed a normal growth rate maintaining a 48hr growth cycle. No differences in morphology were observed between CS2WT and CS2Δ*surf*$_{4.2}$ both in the parasite and the pRBC when observed under a light microscope (x100 magnification).

These outcomes suggest that disruption of the *surf*$_{4.2}$ gene in the CS2 parasite does not have any visible effects on the parasite morphology and does not affect the 48hr growth cycle.

4.3.2.2 *Possible receptor for SURFIN$_{4.2}$*

In order to study the characteristics of the receptor of *surf*$_{4.2}$ gene, invasion assays using enzyme treated RBC and invasion in the presence of heparin, a glycosaminoglycan (GAG) were carried out.

The RBCs were treated with neuraminidase, which removes sialic acid residues from the erythrocyte surface, chymotrypsin, which removes GYPB on the erythrocyte surface and trypsin which cleaves amide and ester bonds of arginine and lysine of proteins on the RBC surface. It was observed that CS2WT and CS2Δ*surf*$_{4.2}$ were able to invade neuraminidase and trypsin treated RBCs at the same rate. A significant difference in invasion between CS2WT and CS2Δ*surf*$_{4.2}$ was observed in the chymotrypsin treated RBCs. CS2WT was able to invade into chymotrypsin treated RBCs at a higher rate compared to CS2Δ*surf*$_{4.2}$.

Invasion of CS2WT into neuraminidase treated RBCs was not significantly changed as compared to invasion into normal RBC. The same phenomena were also observed for CS2Δ*surf*$_{4.2}$ parasites. Neuraminidase removes sialic acid residues from the erythrocyte surface and makes pathways that are dependent on sialic acid inaccessible. Sialic acid is present in Gylcophorin A, B and C but also in other receptors.
Both CS2WT and CS2Δ*surf*$_{4.2}$ parasite lines invaded trypsin treated RBCs at a similar rate. However, CS2WT and CS2Δ*surf*$_{4.2}$ showed a significant difference in invasion

rate into chymotrypsin treated RBCs, clearly indicating that SURFIN$_{4.2}$ is functional in the CS2 parasite. A 50% reduction in invasion observed in the CS2Δ*surf*$_{4.2}$ indicates that disruption of *surf*$_{4.2}$ renders the parasite sensitive to chymotrypsin treatment, meaning that the receptor for the SURFIN$_{4.2}$ is resistant to chymotrypsin treatment. Chymotrypsin removes GYPB on the RBC surface, but also other unknown receptors such as receptor "E" and "Z". GYPA and C, and the unknown receptors X and Y are chymotrypsin resistant and might therefore be possible candidate receptors for SURFIN$_{4.2.}$

The ability of both the CS2WT and CS2Δ*surf*$_{4.2}$ to invade neuraminidase treated RBC suggests that disruption of the *surf*$_{4.2}$ gene still allows the parasite to invade through the sialic acid independent pathway [103]. General reduction in invasion rate by both parasite lines into trypsin treated RBCs, shows that CS2 parasite uses receptors on the RBC that are sensitive to trypsin treatment and this affects the merozoite invasion process regardless of the disruption of the *surf*$_{4.2}$ gene.
It is possible to speculate that disruption of the *surf*$_{4.2}$ gene does allow the parasite to compensate with other ligands, possibly other *surf* genes; therefore knocking out one SURFIN may not be enough [40].

Invasion inhibition of CS2WT and CS2Δ*surf*$_{4.2}$ by different concentrations of heparin showed that at lower concentrations of heparin, both the CS2WT and CS2Δ*surf*$_{4.2}$ were able to invade at a similar rate but as the concentrations were increased CS2Δ*surf*$_{4.2}$ invaded better than CS2WT.

The results of this study suggest that SURFIN$_{4.2}$ uses a receptor that is resistant to chymotrypsin. The possible candidates could be GYPA and C and the unknown receptor X and Y that are resistant to chymotrypsin. The significant difference in invasion at high concentrations of heparin suggests that SURFIN$_{4.2}$ uses a receptor that is sensitive to heparin.

More research is needed to determine the exact receptor for SURFIN$_{4.2}$.

4.3.3 Features of the SURFIN family of *Plasmodium falciparum* (Paper III)

The SURFIN family of ten proteins was previously divided into three groups, group A (GroupA), group B (GroupB) and group C (GroupC) depending on their sequence similarity and structure [89]. Structurally the SURFINs have two major domains, the CRD in the N-terminus and WRD in the C-terminus which are separated by variable or semi-conserved regions. The CRD and WRD domains are well conserved among the SURFIN family members. GroupC constituted all the truncated SURFINs (pseudogenes). The re-annotation of the *Plasmodium* genome led to updates in both PlasmoDB (www.plasmodb.org) and GeneDB (www.genedb.org/) as such the SURFINs were re/analyzed with regard to the recent updates.

SURFIN members were regrouped into two major groups, GroupA and GroupB and an intermediate group that was partially similar to GroupA and partially similar to GroupB but the similarity was not enough to include these members into either of the major groups.

GroupA contains three members, PFD0100c, PFD1160w and PF08_0002. GroupB has four members, PFA0725w, MAL8P1.1, MAL8P1.162 and PF14_0747. Sequence alignments revealed that the GroupB SURFIN members were highly conserved among each other compared to the GroupA members. Three members of the GroupB SURFINs, PFA0725w, MAL8P1.162 and PF14_0747, have between 85-96% sequence conservation between each other.

4.3.3.1 *Sequence conservation in the SURFIN family*

To further study the two SURFIN groups, sequence alignments were performed between members of GroupA and GroupB SURFINs to study the conservation between the group members in 3D7 parasite line. Percentage identities were calculated between the GroupA and GroupB SURFINs. A neighbor joining tree with bootstraps was constructed to illustrate the conserved nature of the members of each group. GroupB SURFIN members were found to be more conserved between each other compared to the GroupA members. Examination of protein topologies in the three GroupB closely related members ($SURFIN_{1.3}$, $SURFIN_{8.1}$ and $SURFIN_{14.1}$) confirmed the structural and sequence conservation between these proteins. These three proteins contained the same number of TM in similar locations in the protein. Percentage identities between the

GroupB members showed that there is high sequence conservation between the members. Using MAL8P1.162 as a reference sequence, 96%, 70% and 82% sequence conservation were observed in PFA0725w, MAL8P1.1 and PF14_0747 respectively (Table 1B, Paper IV). When percentage identities were calculated in GroupA SURFINs using PFD1160w as reference sequence, 13% and 37% identities were observed in PFD0100c and PF08_0002 respectively (Table 1A paper IV).

When conservation of GroupB SURFINS was compared between 3D7, HB3 and DD2 parasite lines, GroupB SURFINs were conserved in all parasite lines with percentage identities ranging from 70-99% (Table 2B, Paper IV). On the other hand, GroupA SURFINs were not so well conserved and had percentage identities between 6-98% (Table 2A, Paper IV).

This study showed strong sequence conservation between the GroupB SURFIN members. The sequence conservation between the GroupB SURFIN members in 3D7, HB3 and DD2 parasite strains suggests that these SURFIN members could possibly have similar and or related functions. The sequence identities between GroupA and GroupB SURFINs are of interest as it shows that GroupB SURFINs are closely related to each other compared to GroupA SURFINs. The conservation of GroupB SURFINs in 3D7, HB3, and DD2 parasite lines could suggest that these conserved regions in the proteins are of great importance to the parasite.

4.3.3.2 *GroupB SURFINs have duplicated domains*

When the SURFIN members were analyzed using prospero which applies the Smith-Waterman algorithm, to identify genetically mobile domains, it was discovered that SURFINs have domains which are conserved but not exactly similar within each gene except in PF13_0075 (Figure 5). These conserved domains; are hereby referred to as SURFIN conserved domains (SCDs). Each SURFIN had two SCDs, SCD1 (original) and SCD2 (duplicate spread within the sequence) (Figure 5). The two SCDs were separated by 182-579 amino acid stretch in between. When the two SCDs from GroupB SURFIN members were aligned against each other, four conserved sequences were identified. BLAST of the GroupB SCD retrieved all the SURFINs with the GroupB SURFINs being the highest on the list. Uniquely conserved regions in GroupB SCD were found in positions; 59-64, 119-124, 164-177 and 241-246. Four sequences with 99-100% conservation were identified in the GroupB SCD. The four sequences,

NKGDFL, QRQKIIWRKWIAK, WIKIYM, and MLEKWK contained 6-14 aminoacids in a row. When a BLAST was run on these sequences against other *P. falciparum* non-redundant proteins, it was revealed that the sequences were conserved in the GroupB SURFINs. When a gene search was carried out to check if these occur in other *Plasmodium* species other than *P. falciparum* it was observed that the 14 amino acid motif, QRQKIIWRKWIAK, NKGDFL, WIKIYM and MLEKWK motifs were conserved in *P. falciparum*. When each of the four conserved amino acid sequences in the GroupB members were run through plasmodb similarity/pattern tool, the sequences were found to occur twice or three times within the GroupB SURFINs. In GroupA SURFINs on the other hand, there was less sequence conservation in the SCDs when compared to the GroupB SCDs.

When percent occurrence of each amino acid in the SCDs was calculated, the SCDs were rich in glutamic acid (E) and lysine (K). The SCDs were located either in the N-terminus or C-terminus of the protein. The SCDs in the N-terminus of the SURFINs occurred after the cysteine CRD [89] and some of the SCDs overlapped with previously described WRD [89].

The presence of conserved sequences in the SCD1 and SCD2 segments in GroupB SURFINs is an indication that this region of the protein was duplicated and inserted at different positions within the same protein. Whether these motifs could contribute to localization pattern and or denote a function of the GroupB SURFINs is yet to be investigated. SURFIN conserved domains are different from the previously described WRD [89] even though there is partial overlapping of the domains in certain members.

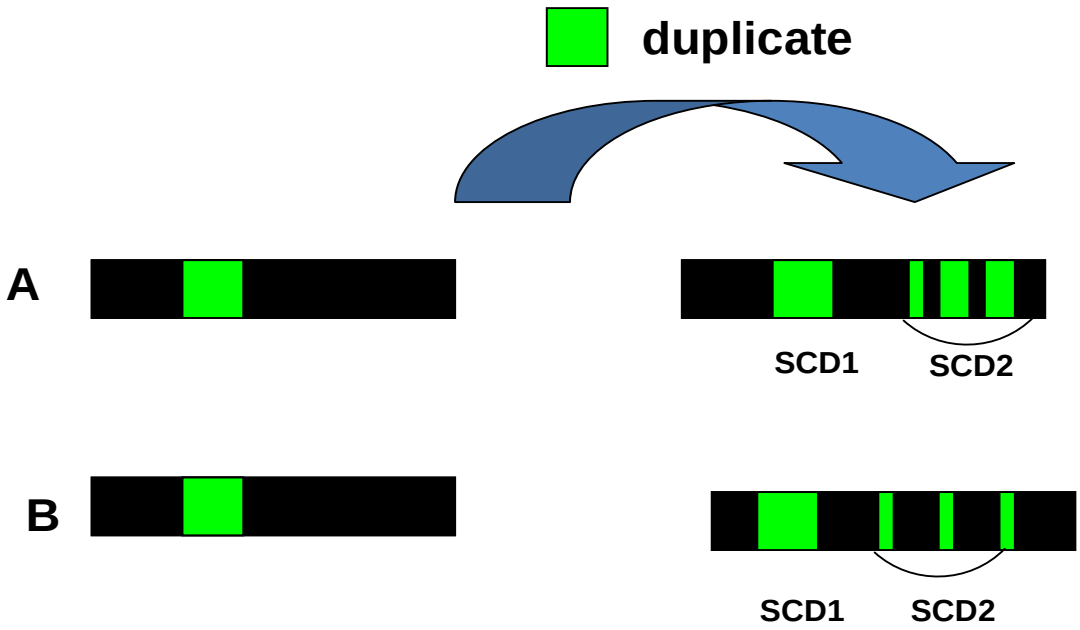

Figure 5: SURFIN conserved domains (SCDs). An illustration showing a duplication process in a protein sequence (black) where a segment was duplicated (green) and was split and inserted either close to each other (A) or spread all over the sequence (B). The duplicated segments are indicated as SCD1 (original sequence) and SCD2 (differentiated sequence).

4.3.3.3 *Localization of three GroupB SURFIN members*

The localization of $SURFIN_{1.3}$, $SURFIN_{8.3}$ and $SURFIN_{14.1}$ in pRBCs was determined on air dried monolayers of 3D7S8 and FCR3. Localization studies showed that GroupB SURFINs are expressed in the rings, trophozoites, schizont and merozoite stages. GroupB SURFINs were observed as three distinct dots on the merozoite, more research is needed to clarify whether these dots were on the apical end of the merozoite or not. Comparing localization of GroupB SURFINs to the previously studied GroupA SURFIN members, $SURFIN_{4.1}$ and $SURFIN_{4.2}$, GroupB SURFINs showed a unique localization pattern different from the GroupA localization pattern. All the SURFINs from the two separate groups localized on the merozoite but to different locations. The localization pattern of the GroupB SURFINs is in agreement with the transcription pattern reported previously [90].

$SURFIN_{4.1}$ was observed as MAM around the merozoite, while $SURFIN_{4.2}$ localized as MAM at the apical end of the merozoite. The three dot pattern observed with the three conserved GroupB SURFINs on the merozoite could suggest that each of the three SURFINs localize in three separate regions and or the proteins localize in separate organelles in the merozoite. The presence of the SURFINs on the merozoite could suggest involvement in the merozoite invasion process.

Western blots on enriched 3D7S8 parasites revealed three bands around 230-240KDa and there was an extra band which was also observed at 130KDa. The lower band observed from GroupB SURFIN IgG in Western blots (130KDa) calls for preparation of more GpB peptide sera to confirm specificity and localization of the GroupB SURFIN members.

4.3.4 Complex *var* Expression in the Organs of Fatal Malaria Patients (Paper IV)

Sequestration of parasitized erythrocytes in the microvasculature of tissues is thought to be one of the important factors in the pathogenesis of the severe form of malaria caused by *P.falcparum.*

Three patients, PM30 diagnosed with severe malarial anaemia (SMA), PM32 diagnosed with cerebral malaria (CM) and SMA and PM55 diagnosed with CM only were used in this study. The expression of *var* genes by *P. falciparum* parasites in the brain, lung, heart and spleen of these fatal pediatric malaria patients was carried out. The brain, heart and lung were chosen for analysis because these organs are major sites of *P. falciparum* sequestration. The spleen was chosen because that is where the pRBC are lodged after being removed from circulation (pitting) thereby representing the circulating population of parasites [104]. Primarily 96 clones, where "clone" stands for each cloned PCR product, were cloned, sequenced and aligned. A median of 26 *var* types (where "*var* types means each different DBL1α sequence identified) were amplified from each organ of the three patients. The range of the *var* types amplified was 11-49. In this study, it was found that 6% of the *var* types were cloned more than once from a single organ or were detected from multiple organs and or cases. The homogeneous distribution of genetic types throughout the organs of the CM patients is in contrast to the current finding discussed in this study where up to 102 *var* types were expressed in *P. falciparum* parasites in a single patient. Despite this many *var* types being expressed, only one or two types were expressed at high levels in the brain microvasculature.

In all the patients the infections consisted of both rings and mature stages and it was shown that immature asexual parasites transcribed the same dominant *var* transcript as

the mature stages. Mature pRBCs were not observed in the lungs, heart and spleen of PM55 though present at high numbers in the brain. Pigment accumulation in the organs provided evidence for sequestered parasites. PM55 had high circulating parasitaemia at the time of death and *var* expression observed is assumed to be from immature stages transiently present in organs other than the brain.

4.3.4.1 *High expression of var types in the heart and brain*

When the expression of *var* types was compared between different organs, there was a variation in *var* types amplified in each organ with the heart and brain tissues having less diversity. In all three brain samples one or two *var* types were detected at a far higher frequency than other types in the same organ. These findings suggest organ specific sequestration of particular *var* types and support the hypothesis that PfEMP1 type determines the site of cytoadherence. A major overlap in expression of *var* types between the lung and spleen was common in all three patients. Despite overlaps, the dominance in expression of particular *var* types in the brain and other organs mediate sequestration in these tissues.

4.3.4.2 *Patients from a single malaria season exhibit organ-specific var expression*

This study also investigated if any *var* types were shared between the three patients. One *var* type was shared between PM55 and PM32 while no *var* types were shared between PM55 and PM30. A substantial overlap was observed in PM30 and PM32 with 20 DBL1α types detected in both cases. A shared *var* type in this case does not mean that the entire *var* genes represented by each tag were identical. There were 20% shared *var* types in PM30, and 26% shared *var* types in PM32 comprising 61% and 32% of all clones detected respectively. In the brain 20% of PM30 *var* types (90%) were also in the brain of PM32 including all the dominant *var* types. In the heart 42% of all PM30 (89% of the clones) and 14% of PM32 (24% of all clones) were shared. PM30 and PM32 were from the same malaria season but from separated villages.

Patients described in this study carried genetically complex *P. falciparum* infections. Most of the *var* sequences contained four cysteines with only 12% of the *var* types containing two cysteine residues. The sequences were classified according to previously identified DBL1α motifs such as the number of cysteine residues and positions of limited variance (PoLV) [105].

4.3.4.3 *var sequences expressed in the hearts of Malawian patients are similar to 3D7 var genes*

var types expressed by *P. falciparum* in the heart tissue of PM30 and PM32 were highly similar or identical to 3D7 *var* types. Eleven *var* types from PM30 displayed greater than 70% identity with *var* sequences from the 3D7 genes and eight *var* types showed 80% identity to these genes. In PM32, seven out of twenty five multiple copy *var* types had high similarity with 3D7 *var* types. Twenty four Malawian DBL1α sequences displayed varying levels of similarity to fourteen 3D7 *var* genes.

These findings suggest organ specific sequestration. Dominance in expression of a particular *var* type in the brain and other organs strongly suggests that a particular PfEMP1 type mediates sequestration in these tissues. The organ specificity observed in the *var* types observed in the brain and heart of the malaria patients studied suggests that a form of organ specific *var* expression as seen in placental malaria [106] may also occur in pediatric severe malaria.

4.4 WORK OUTSIDE THE FOUR PAPERS

During the study, there were several small projects which were carried out most of which were either inconclusive or needed further clarification. These projects were not included in the four papers reffered to in the thesis. I hereby give a short summary of these studies.

4.4.1 Invasion inhibition assays with anti-SURFIN$_{4.1}$ antibodies

Invasion inhibition assays using rabbit and rat anti-SURFIN$_{4.1}$-C1 antibody on 3D7S8 and FCR3 parasites were conducted as outlined in the materials and methods section (4.2.9). The experiments were repeated five successive times each with three replicates. There was no difference in the invasion rate between the control culture (without antibody) and in the treated culture with anti-SURFIN$_{4.1}$ antibody after 48hr and 96hr incubations. There was no difference observed in parasite invasion in cultures with pre-immune and immune rabbit and rat sera. The rat sera generally showed lower parasite invasion rates with both immune and pre-immune sera which could indicate that there was a rat specific inhibitory factor within the sera which interfered with parasite growth and or invasion. Both 3D7S8 and FCR3 parasites showed that the anti-SURFIN$_{4.1}$-C1 antibody did not inhibit merozoite invasion in these parasites (Figure 6).

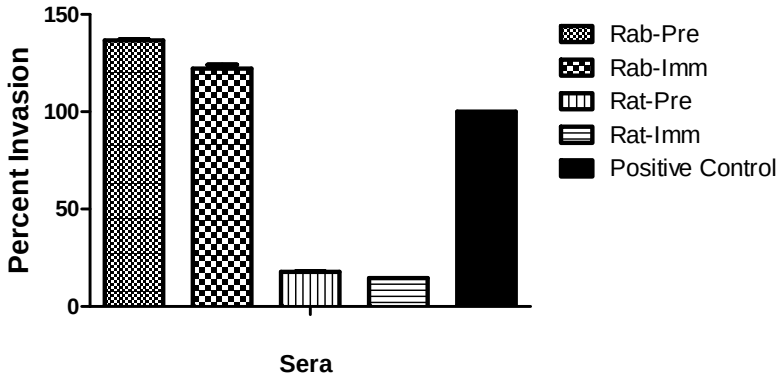

Figure 6: $SURFIN_{4.1}$ Invasion inhibition assays. Graph showing a mean invasion rates of FCR3 and 3D7 parasites in the presence of rabbit and rat anti-$SURFIN_{4.1}$ antibodies from five experiments. The percent invasion was calculated in comparison with the control culture (positive control) set at 100% invasion.

4.4.2 Live IFA

Live IFA were conducted to study if $SURFIN_{4.1}$ and three of GroupB SURFIN members were present on the surface of the pRBC. These results were obtained in several experiments. 3D7S8 and FCR3 late stage parasites were harvested and were incubated with specific anti-SURFIN antibodies for $SURFIN_{4.1}$ and three GroupB members. Two sets of antibodies were used one specific to $SURFIN_{4.1,}$ (rabbit and rat anti-$SURFIN_{4.1}$-C1 antibody) and another antibody specific to three GroupB SURFIN members (rabbit anti-$SURFIN_{GroupB}$ antibody). Both sets of antibodies showed no recognition on the surface of pRBC which were used as control. This outcome indicates that either $SURFIN_{4.1}$ or three Group members ($SURFIN_{1.3}$, $SURFIN_{8.3}$, and $SURFIN_{14.1}$) are not present on the surface of the pRBC during the rings, trophozoites and schizont stages.

The outcome that $SURFIN_{4.1}$ is not on the surface of pRBCs is in agreement with the IFA on air dried monolayers which gave the same result.

Results on IFA on air dried monolayers on three GroupB SURFIN members also is in agreement with the live IFA results that these proteins are not on the pRBC surface. Since the anti-$SURFIN_{GroupB}$ antibody was prepared to recognize three GroupB members it would be interesting to prepare antibodies specific to each of these three SURFINs and study their localization on the pRBC.

4.4.3 Protein binding assay using $SURFIN_{4.1}$ recombinant protein

In order to confirm the results of the IFA, protein binding assays were conducted using $SURFIN_{4.1}$ native and denatured recombinant protein. Uninfected RBCs were incubated with the recombinant protein and the RBCs were denatured in SDS loading buffer. A Western blot was run using the RBC lysate and probed with anti-His antibody, the blot revealed a band in the lane containing the denatured protein but not in the lane containing the native protein. This outcome indicates that there was binding of the $SURFIN_{4.1}$ denatured recombinant protein to the RBC surface. More studies have to be conducted to determine what receptor $SURFIN_{4.1}$ uses on the RBC surface. Red blood cells incubated with only PBS were used as negative control.

4.5 SUMMARY

The function of the SURFIN protein family is not yet known, but the studies compiled in this thesis have unveiled some important facts about the SURFIN family.

- The studies on *surf*$_{4.1}$ revealed that this gene is one complete gene in *P. falciparum* parasites, with gene-copy number polymorphisms existing amongst different clones and strains. The *surf*$_{4.1}$ gene encodes a SURFIN$_{4.1}$ protein of Mw ≈ 258kDa present in the PV and associated with the released merozoite.

- Studies on SURFIN$_{4.2}$ showed that this protein uses a receptor that is resistant to chymotrypsin and the receptor is also sensitive to heparin.

- Revised analysis on the SURFIN family revealed that SURFINs are divided into two major groups; GroupA and GroupB and an intermediate group which is similar to both groups but does not fit into neither of the two groups. The SURFIN grouping was made according to sequence similarity and structure.

- It has been discovered that GroupB SURFINs have conserved domains (SCDs) which have been duplicated and inserted in different locations within the protein.

- Postmortem studies in fatal pediatric cases in Malawi, revealed that *var* gene expression and distribution in the human host is complex but there is a clear dominance of certain *var* types in the brain and the *var* types vary between organs.

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7 APPENDIX 1: TABLES

Table 1: *surf* gene specific primers

Gene	PlasmoDB ID	5' Primer Sequence (Primerset 1) 3'	5' Primer Sequence (Primerset 2) 3'
***surf*$_{1.1}$F**	PFA0625w	GCTTGGATTTTAGGTGC	AATGAAAGTAGCGAATACCCTGTAG
***surf*$_{1.1}$R**	PFA0625w	CTTCCCAAACATACATATAG	TTTTCCATTCTTCTTTTCTAATATCTTC
***surf*$_{1.2}$F**	PFA0650w	CCATCTTGCTCAACCGATGAATGACAT	CCCATTTTATCAATAAAACAAATCAAAG
***surf*$_{1.2}$R**	PFA0650w	CTGCTACTAACACTACTGTAACTTAAATAA	GACACTTTTTATATGTTTATTCATCATAC
***surf*$_{1.3}$F**	PFA0725w	GACATAGTTCTCCAGGTTTA	TCATGCGGAATCTGGAATGTTTGG
***surf*$_{1.3}$R**	PFA0725w	CAGCAGAAGGAACCGC	TTCTATTTTTGTTTCTCTTATGCCTTG
***surf*$_{4.2}$F**	PFD1160w	CTTCCCCTTTACAAATGAATGCTC	AATATTATCAATGTTAGGTTTGTC
***surf*$_{4.2}$R**	PFD1160w	AACATCAACACCTCTACGCCGC	AAAATATATAATCATCTTGATCATC
***surf*$_{8.1}$F**	MAL8P1.1	CCATTCTTCATTTTTCCATTTTTTTTTTTTG	GTCAATAATACACTTGTTCTAGAAGG
***surf*$_{8.1}$R**	MAL8P1.1	CAGCAAGATTGGATTAGGAGAG	TCACTCATCACTTCCAAGTCGTC
***surf*$_{8.3}$F**	MAL8P1.162	GACATAGTTCTCCAGGTTTA	TCATGCGGAATCTGGAATGTTTAG
***surf*$_{8.3}$R**	MAL8P1.162	CGTGAAGAAATGAGAGAAAAAG	TTTCTAATTCTTTTTCTCTCATTTCTTC
***surf*$_{13.1}$F**	PF13_0074, PF13_0075	GACCATGTACGCTTGAAGAATC	ATTACAACAAGATATGTTCCAATTACC
***surf*$_{13.1}$R**	PF13_0074, PF13_0075	CCGTGTAGGGAACCT	TCTTTTATTATATTATCTTCCTCTTGTG
***surf*$_{14.1}$F**	PF14_0747	GACTATTCTGGTTTTCCCTC	TGGATACATTAACATCTGAAAATTCTC
***surf*$_{14.1}$R**	PF14_0747	AGTATGTCCCCGTAGCTTTAGCAGT	TTCTATTTTTGTTTCTCTTATGCCTTG

Table 2: *surf* $_{4.1}$ gene specific primers

Section	5' Primer Sequence 3'
* ***surf*C$_{4.1}$F**	CCTCACAATTTTTCCAGTCGCA
* ***surf*C$_{4.1}$R**	AGTATGTCCCCGTAGCTTTAGCAGT
S$_{4.1}$F	ATGTTGGAAAAAAAGTATAAATGTGAA
S$_{4.1}$R	TCCATATATATTTCTATAACGGTTTTC
5C-S1F	ATGCATTTTGTAGTTGAA
5C-S1R	AATATGTTTAGGTTTCAGCC
5C-S2F	GGCTGAAACCTAAACATATT
5C-S2R	ACTGCTAAAGCTACGGGGACATACT
1C S1F	TGCGACTGGAAAAATTGTGAGG
1C-S1R	GTTTTTTCTTTCCCCTTCTTTC
1C-S2F	GGAAAGAAGGGGAAAGAAAAAAC
1C-S2R	CCTATCCTTTTATTCATTTCC
1C-S3F	GGAAATGAATAAAAGGATAGG
1C-S3R	CACAAGAATTATTAAATATTTCG
§Sintra$_{4.1}$F	GAAGAAGGTATGATGTGA
§Sintra$_{4.1}$R	CTTCACATTTATACTTTTTTTC

Table 3 : Antibodies used in this study

PROTEIN	ANTIBODY	HOST/TYPE	REF
$SURFIN_{4.1}$	Anti-$SURFIN_{4.1}$-C1-HIS	Rabbit Rat	[90]
$SURFIN_{4.2}$ PFD1160 *surf*-gene product	Anti-Surf4.2, Anti-S1.3	Rabbit Rat	[89]
EBA 175 Erythrocyte binding antigen	Anti-EBA 175	Rabbit	MR4 MRA-2) [107] [108]
GROUP B SURFINs PFA0725w, MAL8P1.162, PF14_0747	Anti-GroupB	Rabbit	Mphande et al., PAPER IV /INNOVAGEN A.B

Fingani Mphande, 2009.