PLASTICITY AND DIVERSITY OF THE *PLASMODIUM FALCIPARUM* PLACENTAL MALARIA ANTIGEN VAR2CSA

by

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(Under the Direction of David S. Peterson)

ABSTRACT

As long as humans have walked this earth, malaria may have stalked them. Malaria is a disease of poverty and underdeveloped countries. Today, half of the world population lives in regions at risk for developing malaria and more than half a million deaths occur annually due this disease. Malaria is unforgiving, as most of the morbidity and mortality occurs in pregnant women and children under the age of five years. The majority of malaria-related deaths occur in Sub-Saharan Africa because there is limited access to effective vaccine and treatment. The malaria parasite evades host immune defenses through antigenic variation of proteins called *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1). These proteins are expressed on the surface of infected erythrocytes, which allow them to bind to various host tissues. The proteins are encoded by a highly polymorphic *var* (variant) gene family, consisting of ~60 genes. While polymorphic, one of these genes, *var2csa,* is the most conserved *Pf*EMP1 identified to date. The genes ancient origin and evolutionary significance is indicated by the fact that an ortholog is found in the genome of chimpanzee malaria *P. reichenowi*. VAR2CSA is only expressed during pregnancy. In pregnant women, *Plasmodium falciparum* is able to bind to a unique low sulfated form of chondroitin in the placenta by expressing VAR2CSA on the surface of the infected erythrocyte. The massive sequestration of these erythrocytes in the placenta results in

pregnancy complications, including low-birth weight (LBW), maternal and neonatal death. While women in their first and second pregnancies are at highest risk for developing placental malaria (PM), partial immunity is achieved after multiple pregnancies through protective antibodies. This suggests that a vaccine to protect against placental malaria (PM) may be feasible. The dissertation herein provides detailed information on the genetic complexity of PM and methods of identifying and constructing representative allelic variants of VAR2CSA that may be used as a vaccine to protect mothers and their fetuses from placental malaria.

INDEX WORDS: Malaria, VAR2CSA, Chondroitin Sulfate A, Placenta, Pregnancy

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# DEDICATION

I would like to dedicate this work to my family. I could have not accomplished this work without the continued support of my parents, Jasmina and Hasan Talundzic, and my brother Emir Talundzic. We have had a long and difficult journey. Throughout the years, you sacrificed so much to ensure that Emir and I would have a good life filled with many opportunities. Mom and dad, you are my true heroes. Emir, thank you for always supporting me and for being so patient with your big brother. To my beloved grandmother, Ismeta Strikovic (صلى الله عليه وسلم), who helped raise me and teach me to follow my passion in life, thank you for everything. Lastly, to the rest of my family, including those we lost during our journey (صلى الله عليه وسلم).

(الصالحين مسار في الله وفقنا).

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# CHAPTER 1

# INTRODUCTION

Malaria is a disease with devastating medical complications especially in children and pregnant women in endemic areas. Half the world population (~3.3 billion) lives in areas at risk for malaria transmission. Despite recent intensified international efforts to reduce the malaria burden, an estimated 215 million clinical cases and 655,000 deaths were reported in 2010 [1] . Successful malaria control and elimination programs rely greatly on anti-malarial drugs. However, recent evidence of drug resistance to our last working anti-malarial(s) [2-4], artemisinin and its derivatives, is posing a major threat to the global effort in controlling this devastating disease.

The disease is unforgiving, as it accounts for one in five childhood deaths in Sub-Saharan Africa. A recent pivotal, large-scale Phase III trial of the RTS,S malaria vaccine candidate reported one-third fewer episodes of clinical and severe malaria in infants aged 6-12 months after a third vaccine dose [5]. Unfortunately these results are still disappointing and the vaccine will likely not be used until the efficacy is improved. If the malaria infected child survives past the age of five years, they will acquire partial immunity to malaria during adulthood in the form of inhibitory antibodies. While this partial immunity does not provide sterile protection, it can control the severity of the disease by the clearance of infected erythrocytes (IEs) through the spleen, thus avoiding organ-related pathogenesis.

Malaria in pregnancy, however, is unique. Irrespective of previous immunity to the disease, mothers and their fetuses are extremely susceptible. It is estimated that more than 54 million cases of malaria occur every year in reproductive age women [6]. Mothers in their first and second pregnancies are at highest risk for placental malaria, resulting in severe disease and pregnancy

complications, including low-birth weight (LBW), maternal anemia, and neonatal death. This is caused by the massive sequestration of *Plasmodium falciparum* infected erythrocytes in the intervillous space of the placenta, resulting in placental malaria (PM).

PM is largely attributed to the cytoadherent nature of the infected erythrocytes and the subsequent hyper-inflammatory immune response that causes tissue damage and organ dysfunction. In particular, the parasites express a unique *Plasmodium falciparum* erythrocyte membrane protein-1 (*Pf*EMP1), called VAR2CSA, capable of binding to the low sulfated glycosaminoglycan, chondroitin sulfate A (CSA). Although chondroitin is found throughout the human body, placental CSA is distinctive in that it bears a unique low sulfation pattern – only 10% of the disaccharide repeat units are chondroitin 4-sulfated (C4S) – and is found on the surface of specialized fetal epithelial syncytiotrophoblast (ST) cells. This unique, low sulfated placental Chondroitin-4-sulfate (C4S) is either not available or scarcely present on other endothelial cells, hence VAR2CSA expressing IEs (VAR2CSA-IEs) do not sequester in organs other than the placenta. The expansion and accumulation of IEs leads to the infiltration of mononuclear cells, resulting in inflammation and impairment of the placenta. Eventually these processes result in PM related pathogenesis.

Thus, in pregnant women *Plasmodium falciparum* has evolved to seize the availability of a new organ, the placenta, overcoming any preexisting protective immunity. Interestingly, however, after multiple exposed pregnancies women develop inhibitory antibodies against VAR2CSA and are able to partially overcome the severe outcomes of PM. This suggests VAR2CSA has partially conserved/shared epitopes or that after multiple pregnancies women develop a broad enough immune response against different VAR2CSA variants. Sera from multigravid women from different malaria endemic regions have been shown to inhibit binding of various placental IEs to C4S [7-9]. In addition, multigravid women retain these anti-VAR2CSA inhibitory antibodies [10]. This implies that antibody recognition is not dependent on geographical origin of the VAR2CSA-IEs and that a vaccine to protect against PM may be feasible.

VAR2CSA is part of a family of 200-400kDa antigenically distinct proteins called *Pf*EMP1s, encoded by a repertoire of ~60 *var* (variant) genes. Despite being antigenically variant, *Pf*EMP1s have similar domain sequence arrangements, each consisting of a large extracellular polypeptide and highly conserved cytoplasmic acidic terminus that is linked through a transmembrane segment. The domains vary in their numbers and arrangements for each *Pf*EMP1. While polymorphic, VAR2CSA is the most conserved PfEMP1 identified to date [11-13], a fact that makes it intriguing to study. It is well-known that VAR2CSA is specifically up-regulated during PM or when selected *in vitro* for C4S binding [12,13], and the disruption of the *var2csa* gene leads to loss or significant reduction of IEs adhesion to C4S [8,13].

VAR2CSA consists of six Duffy-binding-like (DBL) domains, a large cysteine-rich inter-domain region (CIDR) between DBL2X and DBL3X, a C-terminal region predicted to be cytoplasmic, and a number of interdomain regions [14]. As reported previously, three of the six DBL domains (DBL2x, DBL3x, and DBL6ε) have been shown to bind CSA [15,16], with the minimal binding region located in the N-terminus [17,18]. Specifically, recent work has shown that the DBL1X-3X fragment has similar C4S binding characteristics as the full-length VAR2CSA and inhibitory antibodies raised against full-length VAR2CSA target principally the DBL3X domain [18,19]. While cross-reactive antibodies can be induced from all the domains to some degree, the DBL3X and DBL4ε domains are the most conserved of the six DBL domains. Taken together with the evidence that the DBL3X domain can induce an inhibitory antibody response against full-length VAR2CSA, and owing to its sequence conservation, make this domain an appealing target for investigation.

**Significance**

The underlying interaction(s) between the pregnant woman and the allelic variants of VAR2CSA that promote susceptibility to PM and associated poor birth outcomes require detailed

study. Although progress has been made in terms of determining the minimal binding region of VAR2CSA and identification of domains that elicit antibody responses, the genetic complexity of placental infections in regards to VAR2CSA still needs further investigation. It remains unclear whether specific motifs within VAR2CSA or genotypes are linked to clinical and pathological outcomes of PM. A detailed understanding of the interaction(s) between the allelic variants of VAR2CSA and placental C4S is fundamental.

In order to design an efficacious vaccine that will provide a broad protective immune response against PM, we first have to address the genetic complexity of *var2csa*. Only through detailed understanding of the parasite genetic background between primigravid and multigravid women will we be able to better understand the biology of PM. It is not well known whether immune pressure selects for genetically unique VAR2CSA-IEs and how this may play a role in the outcomes of PM. By investigating the complex polymorphic nature of this gene will it be possible to understand both the disease and development of immunity to placental malaria (PM). Subsequently, this will provide critical information relevant to designing an effective vaccine able to protect mothers and fetuses from the adverse effects of malaria during pregnancy.

The study here provides detailed information on the genetic complexity of PM in primigravid and multigravid women from holoendemic Kenya, as well as evidence on gravidity mediated selection for unique *var2csa* genotypes. This information is used to identify motifs and parity representative allelic variants that will serve an important role in designing an efficacious PM vaccine. Altogether these data suggests that a PM vaccine should target gravidity-specific epitopes to prevent vaccine-induces strain selection. Lastly, we demonstrate that a multiplex real-time PCR assay can be used for identifying pregnant women with submicroscopic *P. falciparum* infections—which will serve an important role during vaccination and elimination programs.

**Hypothesis and specific aim(s)**

Here we contribute to the advances in prevention and of control PM through identifying factors that may mediate development of immunity in the context understanding genetic complexity of *var2csa* in placental infections. The *central hypothesis* guiding this proposal is that genetic complexity of placental infections is far higher than currently reported, and that specific VAR2CSA types will be linked to clinical and pathological outcomes of disease. The *rationale* for the proposed research is that both the high complexity of *var2csa* genotypes within the placenta and unique sequence types of the gene have implications for the development of immunity and disease pathogenesis.

To fully understand the genetic complexity during PM and identify factors associated with poor birth outcome, we addressed the following objectives:

(1) Identify sequence types associated with pathogenesis and/or gravidity, and
(2) Determine regions under selective pressure, including gravidity associated motifs
(3) Identify critical residues involved in binding and/or immune evasion
(4) Determine the true-genotype complexity and copy number variations (CNV) of *var2csa*,
(5) Investigate any association with gravidity and by extension immune driven selection and
(6) Test new methods for identifying pregnant women with submicroscopic *P. falciparum* infections

To accomplish these objectives we used samples obtained from Kenyan women exposed to intense transmission of malaria, for whom clinical information including birth outcome and placental pathological state are known, and applied a combination of molecular methods and bioinformatics analysis.

We took the following approaches:

A. Cloning and Sanger sequencing of the *var2csa* DBL3X domain to obtain a measure of diversity and identify an association with gravidity, regions under selective pressure, and important amino acid residues that may be critical for immune evasion
B. Next-generation sequencing (454 Roche) of variable regions within *var2csa* DBL3X region to obtain in-depth accurate measure of genotype complexity and identify unique sequence types associated with gravidity
C. Real-time PCR to determine copy number variations (CNV) of *var2csa* and determine associations with pathology and/or parity
D. Multiplex real-time PCR to determine the burden of submicroscopic PM infections in women treated with or without intermittent preventive treatment (IPTp)

To test our central hypothesis and achieve the overall objective(s) of this work, we addressed the following specific aims:

SPECIFIC AIM I: Characterize the genetic complexity of placental infections and identify important amino acid residues that may be critical for immune evasion.

*Working hypothesis:* The genetic complexity during placental malaria is far higher than currently reported and specific sequence types and/or motifs will be implicated in immune evasion.

SPECIFIC AIM II: Determine to what extent gravidity is associated with *var2csa* diversity and identify gravidity-specific epitopes.

*Working hypothesis*: A high *var2csa* genotype complexity due either to high sequence complexity or multicopy nature of *var2csa* in some isolates will be parity associated and an indicator of immune selection.

SPECIFIC AIM III: Identify and test a suitable assay in detecting submicroscopic placental malaria infections in women treated with or without intermittent preventive treatment (IPTp)

*Working hypothesis*: A multiplex real-time PCR assay will be a suitable test for detecting submicroscopic PM infections in both women treated with and without intermittent preventive treatment (IPTp) in a developing world laboratory setting.

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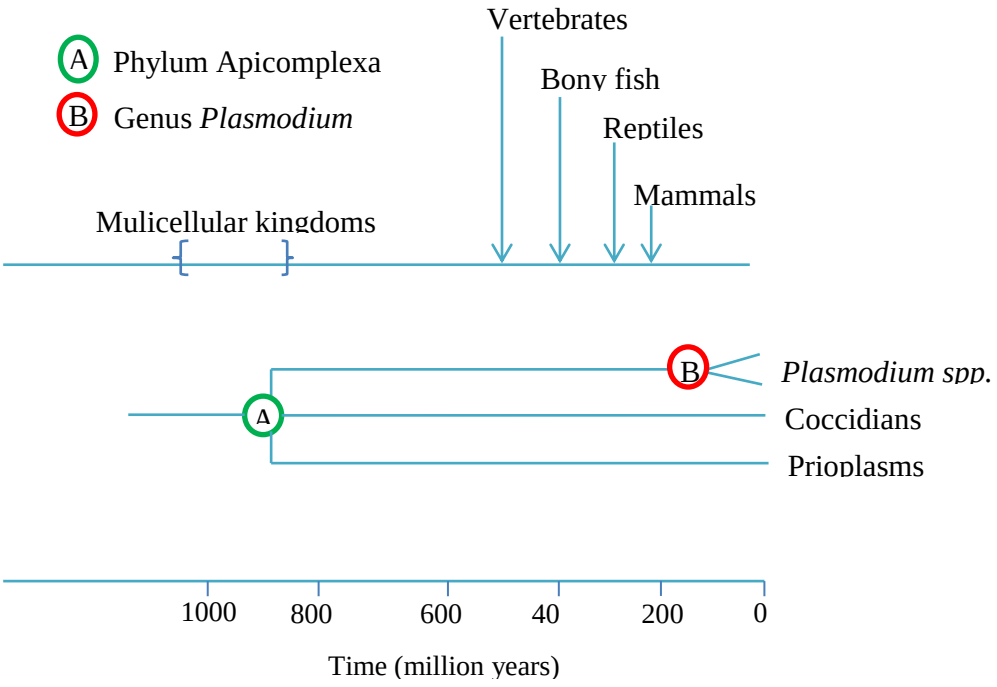
# CHAPTER 2

# LITERATURE REVIEW

## Early history of malaria

The parasite responsible for malaria has been in existence for hundreds of millions of years and belongs to the genus *Plasmodium*, which consists of nearly 200 species that are infectious to birds, reptiles and mammals. *Plasmodium* belongs to the phylum Apicomplexa, characterized by their eponym structure, the apical complex, which plays an important role in host cell invasion. The *Plasmodium* lineage is believed to have diverged from other Apicomplexa parasites around 200 million years ago and perhaps even before the vertebrates originated from their ancestral invertebrate lineage (Figure 2.1) [1].

**Figure 2.1 Simplified phylogeny of *Plasmodium* and related protozoa**




While close relatives of the human malaria parasites are found in chimpanzees, recent evidence suggests that *P. falciparum* may have originated in gorillas [2]. Vertebrates serve as the

intermediate hosts for *Plasmodium*, while invertebrate hosts are the typical definitive hosts or vectors (*anopheles* mosquito). Human malaria is caused by five *Plasmodium* species: *P. falciparum, P. vivax, P. malariae, P. knowlesi* and *P. ovale*. Most mortality and morbidly due to malaria is caused by *P. falciparum* and ninety-percent (90%) occurs in Sub-Saharan Africa, mostly in children and pregnant women.

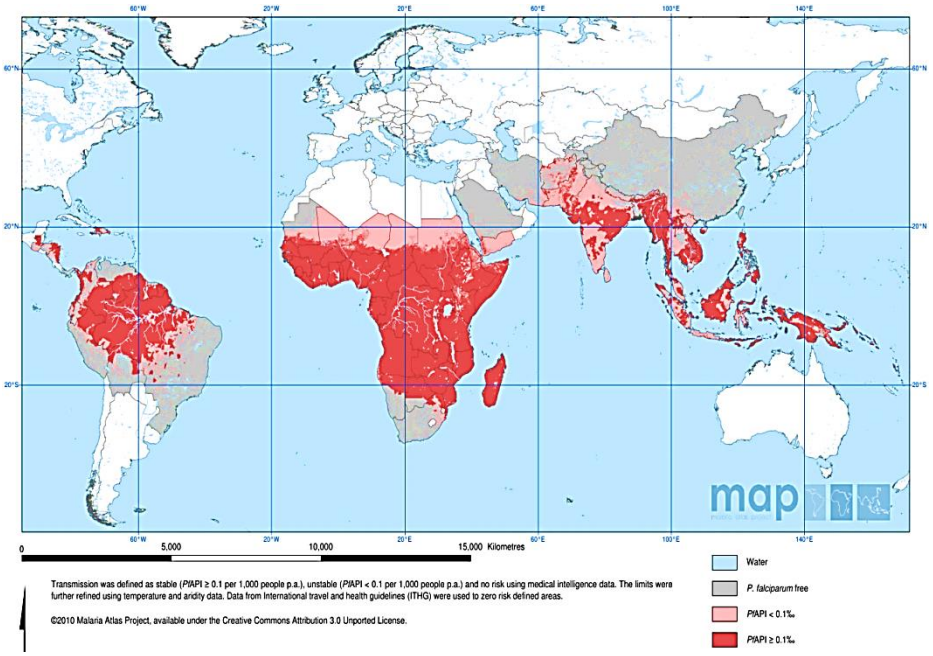
Although the *Plasmodium* parasites have been in existence for millions of years, their numbers did not increase until about 10,000 years ago, concurrently with the advances in the development of human settlements and agriculture [3]. Records of periodic fevers are first found in history in the beginning of 2700 BC in China [4], and later during the Roman empire where it was coined as the "Roman fever" [5]. The term malaria, or *mala aria* meaning "bad air", was first coined during Medieval Italy. Malaria records are found throughout history and ancient literature, including potential treatments for the disease. For example, the Qinghao plant (*Artemisia annua L)* was described as an anti-malarial treatment in 340CE China during the East Yin Dynasty. Today, Qinghao is known as artemisinin and is part of our effective antimalarial treatments.

While artemisinin was only discovered a few decades ago (1970s), the first effective treatment came from the bark of cinchona tree, which contains quinine. The indigenous people of Peru used this extract to control fevers. Its effectiveness in treating malaria was introduced by the Jesuits in Europe around 1640. In 1677 it was considered an effective antimalarial treatment [6]. The active ingredient, quinine, was not identified until 1820 when the French chemists Pierre J. Pelletier and Joseph B. Caventou successfully extracted it from the cinchona bark [7]. Quinine was the predominant antimalarial medication until the 1920s, which two decades later was replaced by chloroquine. Chloroquine continued to be used for the treatment of both uncomplicated and severe malaria until resistance emerged globally in the 1980s. Today, artemisinin in combination with another antimalarial, known as artemisinin combination therapy (ACT), is the recommended treatment for malaria by the WHO.

**Economic and social burden of malaria**

Malaria, one of the most deadly parasitic infections, occurs in over 100 countries and half of the world population (~3.3 billion) lives in areas at risk for malaria transmission (Figure 2.2). Despite recent intensified international efforts to reduce the malaria burden, an estimated 215 million clinical cases and 655,000 deaths due to malaria were reported in 2010 [10]. Population projections show approximately that 450 million births will occur in regions at risk for malaria by 2014.

**Figure 2.2 *Plasmodium falciparum* spatial limits of transmission in 2010**




Close to 90% of all malaria cases occur in Sub-Saharan Africa, where most people become infected during childhood, and the majority of mortality and morbidity occurs in children under the age of 5 years and pregnant women. Although malaria is not commonly perceived to be associated with poverty, evidence suggests it can be a hindrance to economic development [22]. The disease is associated with negative economic effects in endemic regions.

There is strong evidence that suggests where malaria prospers most, human societies prosper the least. This is indicated by the global distribution of per-capita gross domestic product and a

correlation between malaria and poverty, where malaria endemic countries show lower rates of economic growth [23]. Malaria can directly impede development leading to poverty and poverty in turn can increase the risk for malaria, due to financial limitations that can hinder the prevention or treatment of the disease. In endemic countries, the disease may be responsible for greater than 40% of public health spending and responsible for close to 50% of hospital admissions [24]. Malaria demands up to 5% of the per-capita gross domestic product in Sub-Saharan Africa [25].

A severe form of the disease called cerebral malaria often leads to mortality, but can also be the leading cause of neurological disabilities. Evidence shows that cognitive abilities and school performance were drastically impaired in patients with cerebral malaria, uncomplicated malaria or even after recovery when compared to healthy individuals [26]. Consequently, malaria can lead to a wide range of socioeconomic problems that are beyond the effects of the disease alone.

**Malaria drug resistance**

Malaria is once again on the rise globally due to drug resistance. This has resulted in a resurgence of malaria in areas where it was previously under control. Due to the evolutionary plasticity of *Plasmodium falciparum*, drug resistance has evolved and spread rapidly, leading to the failure of choloroquine (CQ) and sulfadoxine-pyrimethamine (SP) as first line treatments in malaria endemic areas. The spread and increased resistance to these generally available and inexpensive drugs has resulted in an increase of malaria related deaths in children under the age of 5 years in Sub-Saharan Africa, during a time when childhood mortality was declining [8,9].

As resistance to monotherapy evolves rapidly, WHO recommends artemisinin based combination therapy (ACT) as an effective strategy for combating the evolution of drug resistance [10]. Artemisinin (including its derivatives) has a short half-life and it is combined with a second drug with a long half-life (several days to weeks) to make the combination therapy efficacious. It is

widely believed that the success of ACT will be compromised if one of the partner drugs fails to produce the expected cure rate.

Although ACT was introduced relatively recently (2000) in Southeast Asia and subsequently in Africa and other parts of the world, recent studies suggest the potential emergence of resistance to artemisinin in Cambodia and Thailand [11-19]. It has become apparent from these studies that artemisinin efficacy is significantly reduced in Southeast Asia as time to parasite clearance has increased from about 24 hours to more than 48 hours. This delay in parasite clearance is considered a first warning signal for the emergence of resistance and is alarming as it may lead to ACT failure. Currently, the global malaria community is treating this delayed clearance of *P. falciparum* to artemisinin combination therapy as a surrogate for resistance. Therefore, delayed clearance can be defined as the first signs of resistance to artemisinin.

These recent findings have raised serious concern for the potential spread of resistance to ACT outside of Southeast Asia, as there are very few alternative drugs available to treat ACT-resistant *P. falciparum* parasites. Historically, CQ and SP resistant *P. falciparum* parasites emerged in Southeast Asia and spread to Africa [20,21]. As a result, there are global efforts to prevent a similar spread of artemisinin resistant parasites to other parts of world.

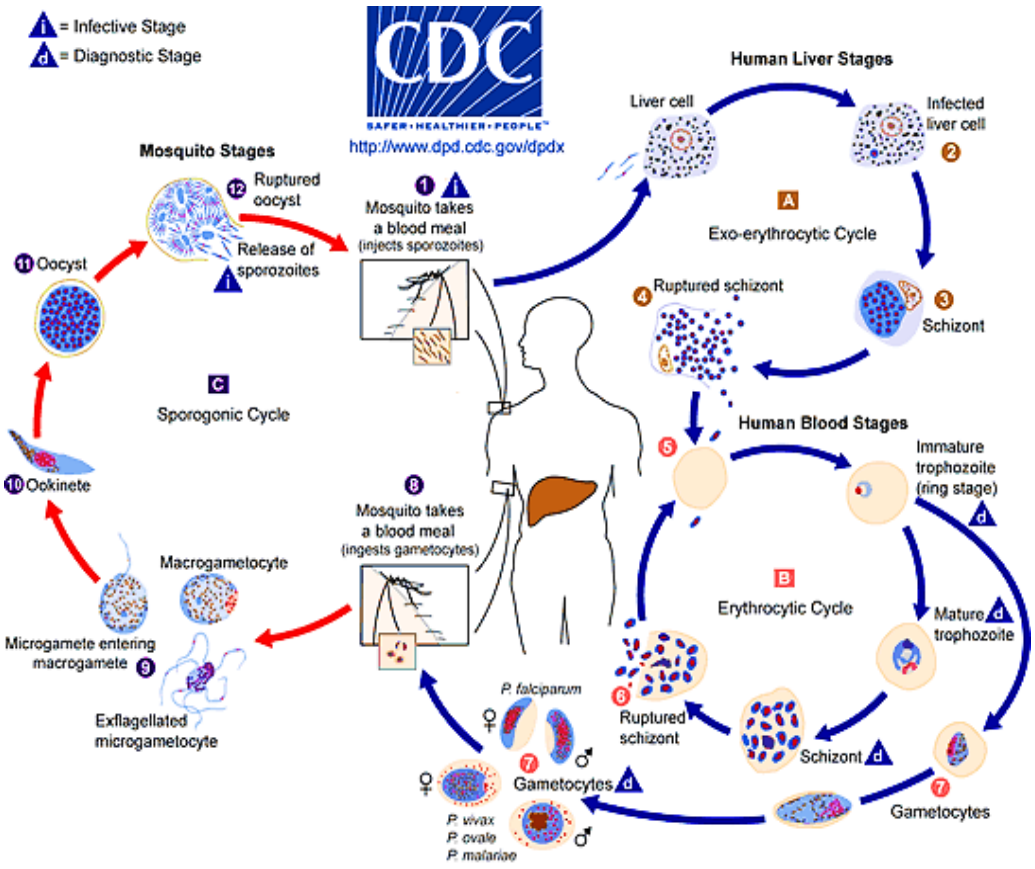
There is growing optimism that this goal can be accomplished, as recent whole genome association studies, performed on clinical samples diagnosed with malaria in the Cambodia and Thailand region, show strong evidence of positive selection of genes found on chromosome 13 and 10 [18,19]. Cheeseman et al. identified a 35kb region within chromosome 13 showing evidence of positive selection of delayed clearance attributable to artemisinin treatment. The most recent study by Takala-Harrison et al. identified a single nucleotide polymorphism (SNP) in a gene located in the same region of chromosome 13 (MAL13-1718319) and another polymorphism located in chromosome 10 (MAL10-688956) to be associated with delayed clearance in response to artemisinin treatment. While these studies only provide preliminary evidence that these markers are potentially

relevant for tracking delayed clearance for artemisinin treatment, validation of these markers in other regions with a well-characterized cohort population is critical for conclusively establishing them in drug resistance.

**Malaria life cycle**

Malaria is caused by the protozoan parasite of the genus *Plasmodium*. Of the approximately 200 species of *Plasmodium*, five are infectious to humans: *P. falciparum, P. vivax, P. malariae, P. knowlesi* and *P. ovale*. *Plasmodium falciparum* is the most virulent of the five and accounts for the majority of severe infections in humans, predominantly in Sub-Saharan Africa. The malaria life cycle is complex and consists of multifaceted stages within the intermediate host, humans, and definitive host or vector, the *Anopheles* mosquito (Figure 2.3) [27].

**Figure 2.3 Malaria life cycle**



The life cycle begins when an infectious female *Anopheles* mosquito takes a blood meal, injecting from its salivary glands 15-200 *sporozoites* into the human host [28]. Under the skin, the motile *sporozoites* can remain in the dermal tissue up to 6 hours [29], although some evidence suggests they can remain in the skin up to 24 hours or in immunoprivileged hair follicles for weeks [30]. The majority of the *sporozoites* will take the blood route and some the lymphatic route [31], the latter end their journey in the first draining lymph node. In the lymph node, they will be degraded by dendritic cells (DC) within a few hours [31], but some will develop into small exo-erythrocytic forms [30]. *Sporozoites* in the bloodstream eventually will reach the liver where they invade the hepatocytes through the sinusoidal lining and Kuppfer cells, ultimately entering the infectious liver stage [32,33].

Within the hepatocytes, the *sporozoites* will go to through a replication process for 5-7 days, undergoing multiple rounds of mitosis to produce hepatocytic *schizonts*. The liver-stage *schizonts* mature into blood infective *merozoites*, rupturing the hepatocyte and releasing thousands of *merozoites* into the blood stream where they invade erythrocytes and begin the blood-stage life cycle.

At this stage, the *merozoites* undergo multiple rounds of mitosis again (erythrocytic schizonogy) [34], transforming into immature *trophozoites* or ring forms, then either develop into mature *trophozoites* or the sexual forms called *gametocytes*. The mature *trophozoites* will grow into *schizonts*. In a synchronous mass division step up to 30 *merozoites* assemble on the surface of the mother *schizont* and rupture [35], each able to invade a new erythrocyte. This erythrocytic stage is repeated causing symptoms associated with malaria periodic fevers which include fatigue, vomiting, and anemia due to destruction of erythrocytes and an inflammatory immune response [36].

Although the signal for the initiation of *gametocytogenesis* remains unknown [37], one potential hypothesis could be that the parasites are able to utilize a “quorum-like sensing” mechanism to communicate with infected erythrocytes (IEs) and/or sense when it is time to exit the host. More specifically, the parasites may be able to communicate through microvesicles released by IEs

allowing them to sense (concentration dependent signal) when the parasitemia is either too high or low, initiating differentiation into female and male *gametocytes.* This is hypothesis should be addressed by future studies. Currently, some evidence suggests that *gametocytes* appear around 7-15 days post the liver stage [38].

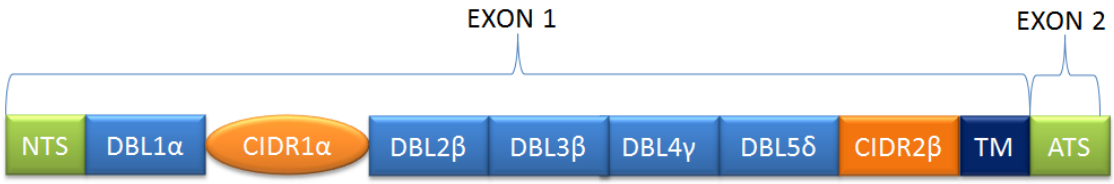
The life cycle continues when the *Anopheles* mosquito ingests the *gametocytes* during a blood meal, initiating the mosquito stage. In the mosquito midgut, the male *gametocytes* undergo a series of mitotic nuclear divisions, forming into 8 flagellated *microgametes* [39]. These *microgametes* then fuse with female *macrogametes* to form a *zygote* [40]. Here the malaria parasite will go through its only meiosis stage within a few hours of the formation of the diploid *zygote* [41], which then differentiates into mobile *ookinete* that attaches to the mosquito gut wall. Following attachment, the *ookinete* over the course of several days transforms into *oocytes* where schizonogy produces thousands of haploid *sporozoites* [42]. The *oocytes* rupture releasing the *sporozoites*, allowing them to migrate and penetrate the salivary glands of the *Anopheles* mosquito. Here the *sporozoites* remain dormant until the next blood meal, waiting to gain transmission to the vertebrate human host [43], thus completing the malaria life cycle.

In conclusion, there are four critical parts in the malaria life cycle in which a small number of parasites multiply to generate vast and antigenically unique populations. These include *gametocytogenesis* in the vertebrate host [44], uptake of *gametocytes* by the vector and formation of *sporozoites* [45], release of *sporozoites* back into the intermediate host and subsequent invasion of hepatocytes (liver-stage development) [46], followed by the erythrocytic stage and asexual mitotic reproduction [46,47]. During each of the stages, the parasites increase their numbers using multiple rounds of mitosis [48], and through antigenic variations avoid host mediated immune clearance.

**Antigenic variation, host cell hijacking and pathogenesis**

Splenic clearance during malaria infections is considered the major mechanism of parasite growth regulation. However, the parasite has developed a highly complex system to evade immune mediated clearance by expressing variant antigens called *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1) on the surface of IEs [49]. These parasite protein(s) are encoded by a highly polymorphic *var* gene family, consisting of ~60 *var* (variant) genes. *Var* genes consist of two-exon structures that are between 6-13kb in size, with the first exon encoding the diverse extracellular region and the second exon the more conserved cytoplasmic tail [50]. The N-terminal extracellular region followed by Duffy-binding-like (DBL) domains, a large cysteine-rich inter-domain region (CIDR) and short transmembrane (TM) sequence are encoded by Exon I, while the conserved acidic terminal sequence by Exon II (Figure 2.4). This architecture is similar for all *var* genes, but the number of combinations of domains varies considerably between different *Pf*EMP1s.

**Figure 2.4 Schematic structure of *var* gene(s) and the encoded *Pf*EMP1 protein [51]**




The variant antigenic repertoire of *Pf*EMP1s is maintained through gene recombination events between *var* paralogs [52] in the telomeric chromosome clusters [53] during meiosis and at a low frequency in mitosis [54]. In a natural infection there is essentially an unlimited potential for antigenic variation within each *Pf*EMP1 [55]. The switching between the different *Pf*EMP1s is a non-random, highly structured and balanced process of parasite-intrinsic switching and immune mediated-selection [56] . The antigenic variability and the vast adhesive phenotypes within the *Pf*EMP1 repertoire make it the primary virulence factor during malaria infections.

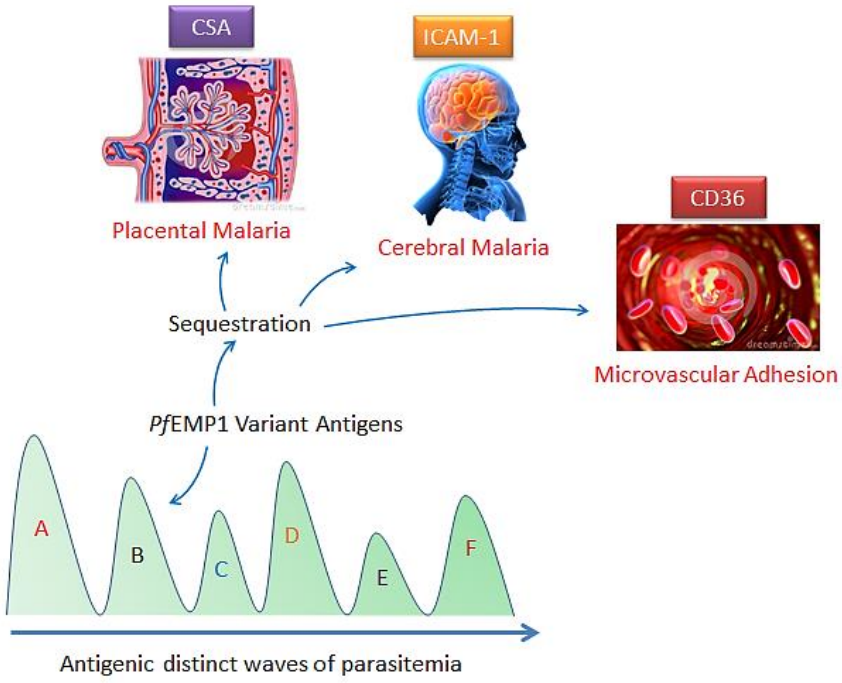
*Pf*EMP1s are expressed during the late erythrocytic stage on the surface of IEs within 16 to 18 hours post erythrocyte invasion [57,58], in association with knob structures known as Knob Associated Histidine Rich Proteins (KAHRP) [59]. Following the invasion of the host erythrocyte, the parasite modifies the host cell morphology, creating a unique protein construction niche. The modified IE now consists of its own pararasitophorous vacuole (PV) surrounded by a PV membrane (PVM) that a provides a barrier to the host cell [60]. During this incredibly amazing process, the malaria parasite exports hundreds of proteins into the IE and "takes over" the host cell [61]. By drastically changing the erythrocyte membrane and cytoskeleton, the parasite is able to set up nutrient acquisition system(s) necessary for its survival and eventual egress.

The translocation of *Pf*EMP1s is somewhat complex as the parasite has to export the protein from the endoplasmic reticulum (ER) and beyond the confines of its own plasma membrane (PM) and PVM into the IEs cytosol to promote virulence. The *Pf*EMP1 is brought to an export cargo receptor that recognizes the special TM domain and undergoes through the secretory system to the PM, where a PVM chaperone takes it to the translocon for export to the IE cytosol [62]. Once in the IEs cytosol, the *Pf*EMP1 is directed to the parasite derived protein sorting site called Maurer's cleft (MC) and then finally trafficked to the IEs surface [63].

Now that the *Pf*EMP1 is expressed on the surface of the hijacked host cell, the IEs are able to sequester to various endothelial tissues enabling the parasite to complete its life cycle, increase in numbers, and re-invade new erythrocytes without being cleared by the spleen. The variable extracellular domains, CIDR and DBL-domains, of the *Pf*EMP1 can bind to various host receptors including cluster of differentiation 36 (CD36), intercellular adhesion molecule 1 (ICAM1), thromobospondin (TSP), complement receptor 1 (CR1), and chondroitin sulfate A (CSA) [50]. *Pf*EMP1-IEs (*Pf*EMP1-expressing infected erythrocytes) may also bind to other IEs or form the so-called "rosettes" by adhering to non-infected erythrocytes [64] .

The specificity of *Pf*EMP1 binding is determined by the unique DBL-domains and CIDR components, which ultimately are associated with specific disease pathologies. *Pf*EMP1 mediated cytoadherance of IEs to the hosts receptors is associated with severe disease outcomes such as cerebral malaria or placental malaria [50]. For example, sequestration of IEs to ICAM-1 in the brain post-capillary venules results in cerebral malaria, which is mediated by a sub-set of *Pf*EMP1 proteins that contain the DBLβ domain [65]. The host receptor CD36, which is abundant in various tissues including the heart, liver, lungs, kidneys and muscle binds to most *Pf*EMP1-IEs and often is associated with severe malaria in children, including cerebral malaria [66]. In the placenta, a low-sulfated form of CSA can bind to a unique *Pf*EMP1 called VAR2CSA, resulting in severe pregnancy complications [67] (Figure 2.5).

**Figure 2.5 *Pf*EMP1 adhesion to host cell receptors and related pathogenesis [68]**

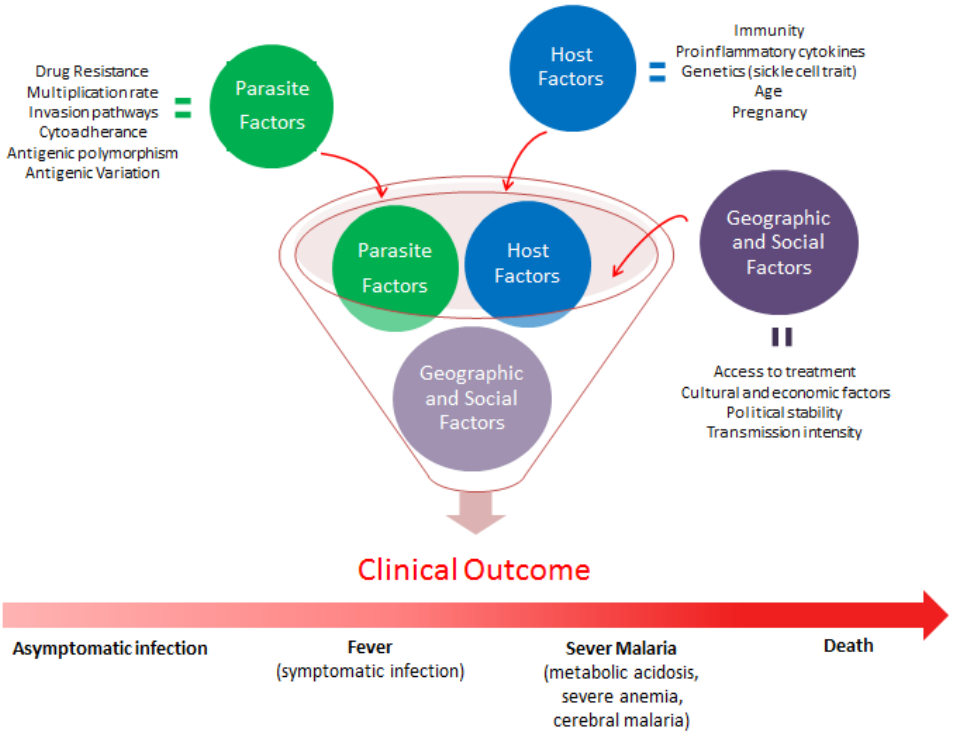



Clinical manifestations are the result of *Pf*EMP1-IEs sequestering to host cell tissues, and subsequent rupturing and re-invasion of new erythrocytes. During the early developing stages the parasite digests hemoglobin and converts the product to haemozoin in the digestive vacuole (DV).

Upon rupture, toxic compounds such as haemozoin, *alias* malaria pigment, and Glycosyl-phosphatidylinositol (GPI) anchored proteins are released into the host environment. Taken together, these "left-overs" initiate an inflammatory immune response, including direct stimulation of cytokines, associated with tissue and organ damage. Additionally, recent evidence suggests that the released DV membrane can activate two major enzymes cascades in the blood, complement and coagulation [69].

Metabolic acidosis is one of the principal pathological features associated with classical clinical symptoms of severe malaria [70], which can lead to respiratory distress [71] and contribute to reduced oxygen delivery to tissues [72]. Sequestered IEs can also cause direct obstruction to tissue perfusion and by the destruction of erythrocytes further compromise oxygen delivery, resulting in severe anemia. The clinical and pathological outcomes during a malaria infection depend on multiple factors, including host genetics. A summary is provided in Figure 2.6.

**Figure 2.6 Factors influencing the clinical outcomes during a malaria infection [68]**



Host genetics can influence how we respond to an infectious disease. A classic example of this can be seen where sickle cell anemia (SCA) meets malaria. Resistance to severe malaria can be attributed to the SCA trait (genotype HbAS) [73-75]. SCA is caused by an abnormal type of hemoglobin called hemoglobin S, which changes the shape of the erythrocyte into rigid crescents or sickles. This sickle-shape leads to enhanced clearance by the spleen of erythrocytes including IEs and an increase in the innate and acquired immunity [76,77]. SCA disease occurs more commonly in people (or their descendants) from Sub-Saharan regions where malaria has been common for centuries—likely the result of natural selection over generations [78]. Another example is an erythrocyte deficiency in the enzyme glucose-6-phosphate dehydrogenase, which increases vulnerability to oxidative stress and subsequent protection against parasitization [79-81]. Many other published polymorphisms that are associated with susceptibility and resistance to malaria infections have been reported, summarized in the review by Driss et al. [82]. It is becoming clearer that a dual process of natural selection is occurring between the parasite and human host. The majority of these polymorphisms are found in genes directly or indirectly involved in host immunity, including cytokine genes, complement regulatory genes, endothelial receptor genes, and human leukocyte antigen (HLA) genes.

**Malaria, the immune system and vaccines**

Malaria continues to be a severe public health problem, which will not disappear without the introduction of an efficacious vaccine. Data indicating that significant immune protection can be achieved after repeated infections [83] or that protection can be transferred by transfused antibodies [84] provides support for the view that a malaria vaccine is conceivable. Nonetheless, understanding the immune responses at both the pre-erythrocytic (PE) and erythrocytic stages (ES) of malaria infection will be critical in achieving this goal.

When an infected female *Anopheles* mosquito takes a blood meal, it injects 10-200 sporozoites into the skin of the host, which over the next few minutes or hours enter the blood vessels and are carried to the liver. It is well known that strong protective immunity can be achieved after immunization with radiation-attenuated sporozoites (RAS) [85-87] which are able to invade hepatocytes, but are arrested at the early liver stage [88]. The surface of the sporozoites are dense with protein(s), the most abundant being the circumsporzoite (CS) protein, which is shed as the motile sporozoites undergo their journey to the liver [89]. The CS protein and other antigen(s) (Ag) are believed to be presented on the infected hepatocytes to T cells [90], or by dendritic cells (DC) that either take up the sporozoites directly or ingest it through another pathway. The only malaria vaccine candidate to date to enter Phase III trials, the RTS,S vaccine [91], is based on the sporozoite CS-protein.

The CS-protein plays a critical role in the humoral immune response that confers some protection against the pre-erythrocytic stage. The mechanism facilitating this protection likely involves the inhibition of hepatocyte invasion and/or opsonization of sporozoites by antibodies (Ab), ultimately leading to macrophage (Mϕ) clearance. Previous work has demonstrated that passive transfer of monoclonal Abs specific for the CS-protein can protect against sporozoites challenge in mice [92] and immune serum from sporozoite-immunized individuals can block *Pf* sporozoites invasion of hepatocytes [93]. Immunization with radiation-attenuated sporozoites provides lasting immunity up to 10 months [85,86], suggesting that the Ab mediated response has to be maintained at some level, perhaps by long-lived plasma cells (PC) in the bone marrow.

Adaptive immunity in the form of $CD4^+$ T cells is believed to be involved during the pre-erythrocytic stage as well. It was believed that CD4+T cells may provide protection through the production of interferon-gamma (IFN-γ), a potent inhibitor of intracellular parasites [94], but recent work suggests the involvement of various other immune cells including B cells, Mϕ, DC and CD8 T cells. Although CD4+T cells are generally believed to coordinate the different arms of the immune

system, they can directly have potent cytolytic activity. The role of $CD4^+$ T cells in the hepatocytic stage is demonstrated by the fact that CS protein activated $CD4^+$ T cells can eliminate infected hepatocytes *in vitro* and the protection is transferable *in vivo* [95]. While $CD4^+$ T cells and Abs can contribute to controlling the liver-stage infection, $CD8^+$ T cells have been found in the blood of both naturally exposed individuals and RAS-immunized volunteers [96,97]. The exact mechanism of activation is still somewhat elusive and controversial.

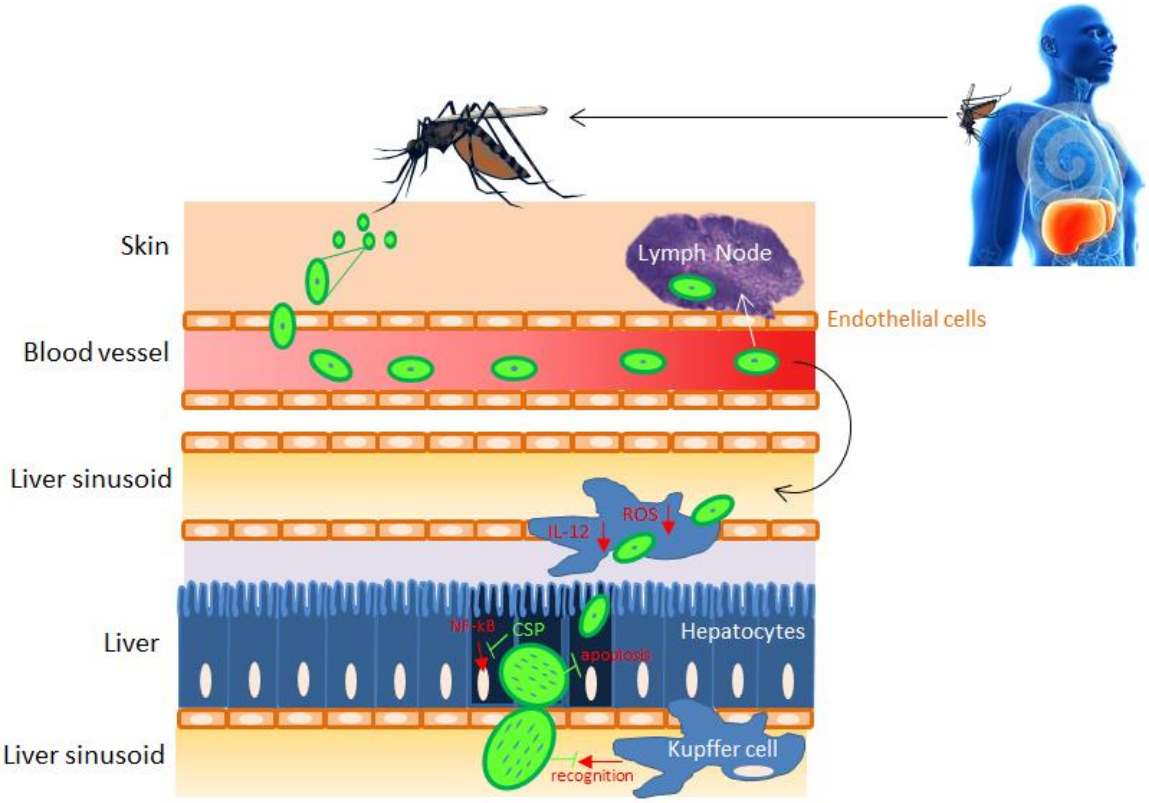
Native sporozoites are not able to activate CD8 T cells when compared to attenuated sporozoites [98]. One explanation for this phenomenon could be that the attenuated sporozoites eventually undergo apoptosis within the hepatocytes and are taken up by $CD8^+$ -DC cells and then presented to CD8 T cells [99], while the native sporozoites, continue their life-cycle post the liver-stage and are able to inhibit apoptosis. As discussed previously, some of the native sporozoites travel to the lymph nodes and it is perhaps here where they active CD8 T cells. This could explain why $CD8^+$ T cells are found in both naturally exposed and RAS-immunized individuals. Thus, both the humoral and adaptive arms of the immune system have an important role during the PE stage of infection.

Even with both arms of the immune system primed, in a natural infection the sporozoites continue to produce blood infective merozoites, rupturing the hepatocytes and releasing thousands into the blood stream where they invade erythrocytes and begin the erythrocytic stage (ES). A growing body of evidence suggests that sporozoites have developed ways to both suppress and escape the immune responses described above.

The rapid migration of sporozoites helps them escape phagocytic clearance and once gaining access, via the Kupffer cells, to the hepatocytes [100], host immune defenses seem to be repressed—creating a unique protective niche for the parasite. Some recent work suggests that the secreted CS-protein is able to suppress multiple host defensive mechanisms including the blocking of oxidative stress (a key defense mechanism in the Mϕ arsenal), suppression of pro-inflammatory signals through

interfering with NF-kB and induction of anti-inflammatory enzymes [101-103]. The developing sporozoite further evades host immune defenses by inhibiting apoptosis of the hepatocytes [104], unlike irradiated sporozoites. The CS-protein utilizes the PEXEL motif, as previously discussed in *Pf*EMP1export, to reach the cytoplasm and interfere with host immunity. A model for the pre-erythrocytic stage of infection and repression of host immune defense is shown in Figure 2.7.

**Figure 2.7 Sporozoite journey to the hepatocyte and immune evasion [105]**




By interfering with host defenses, the sporozoites are able to freely develop over the next 5-7 days into merozoites. The newly formed merozoites are released into the blood stream covered by the host cell membrane [106], providing yet another layer of protection from the host immune system. The blood infective merozoites then invade erythrocytes and begin the erythrocytic stage (ES) of infection. At this stage, innate immunity is critical in controlling the expansion of parasites and mediating the initiation of adaptive immunity. One of the first lines of defense against the ES is splenic clearance of infected erythrocytes (IEs).

The spleen is a vital organ for both the mechanical removal of IEs and the development of immune responses to malaria. Blood flow in the spleen is complex and consists of numerous circulation pathways that allows for filtration and immune clearance of erythrocytes. Within the spleen, different sections contain various immune cell populations, including T-cells, dendritic cells (DC), B cells, and macrophages (Mϕ) [107]. The activation of these immune cells is achieved through the recognition of malaria derived components both on the IEs surface and when freed in the blood. Interestingly, recent reports show signs of splenic remodeling as a result of a *P. yoelii* infection [108], suggesting that just like in SCA, the spleen of individuals living in endemic regions may be under selective pressure to change due to malaria infections.

The IEs that are not cleared by the spleen harbor developing merozoites, which within the next 16-18 hours will successfully differentiated into mature trophozoites. At this stage the main malaria virulence antigen, *Pf*EMP1, will be expressed on the surface of the IE [109], allowing them to adhere to various ligands in the host. The sequestered IEs will avoid splenic clearance and undergo clonal expansion to an antigenically privileged parasite population, which likely will not be recognized by the activated immune cells from the spleen. The parasite has to maintain a delicate equilibrium between a hyper-immune response, which often leads to organ failure, and anti-inflammatory response to allow for survival, expansion and transmission to the mosquito.

These sequestered IEs are not completely safe from the host immune system. The initial innate immune response is critical in this early stage of the infection, as recognition molecules, such as Toll-like receptors (TLRs) and scavenger receptors can interact with the sequestered IEs, including *Pf*EMP1s, and parasite components post erythrocyte egress [110,111] . These TLRs are non-catalytic proteins expressed on various host tissues, Mϕ and DCs, capable of triggering a pro-inflammatory immune response. While the host immune response is figuring out what to do with this new threat, the cell bursts and releases newly formed merozoites into the blood stream. Post egress, the released GPI and haemozoin “left-overs” both activate TLR2 and TLR4 [112-114] , and get

phagocytosed by Mϕ and different tissues [115]. This results in the production of pro-inflammatory or immunosuppressive cytokines [116]—leaving the host immune system in a state of frenzy.

At this stage of the infection, the innate immune system continues to releases a plethora of cytokines such as IFN-γ, Tumor necrosis-factor-alpha (TNF-α) and various interleukins, including IL-1, IL-6, and IL-12 [117-120]. These are important in cytosolic-dependent recognition by Mϕ, Natural Killer (NK) cells, and DC of IEs [121,122], but in excess can lead to malaria associated pathology [123-125]. Of particular importance are NK cells, as they are able to detect stressed cells in the absence of antibodies and "self" markers of the major histocompatibility complex (MHC) 1 [122]. In both murine and human models of malaria, NK cells are critical during this early phase of infection, as they are a major source of IFN-γ [126].

IFN-γ is a major macrophage-activating factor. Innate immune cells of the monocyte lineage, including monocytes, DCs, and Mϕ are crucial during the ES infection. These cells, referred to herein as monocytes/ Mϕ, play a protective role in malaria through phagocytosis of IEs, antibody-dependent cell inhibition, and cytokine production [127]. IFN-γ stimulated macrophages will produce IL-12, a response that can lead to the protection against both PE and ES infection [128,129], TNF-α which has been linked to severe malaria outcomes [130], and nitric oxide synthase (iNOS) [131]. The released merozoites are also targeted by the activated monocytes/ Mϕ.

Phagocytosis of the IEs is one of the primary mechanisms by which parasitemia is reduced during the ES infection. An important factor mediating successful phagocytosis, are opsonins such as complement fragments and antibodies. Complement fragments are small proteins found in the blood, generally synthesized by the liver, circulating as inactive precursors. In the early phases of ES, the alternative pathway of activation is triggered when the C3 protein (complement component 3) directly binds to the IEs, its components or merozoites. Complement-opsonized IEs are phagocytosed by monocytes/ Mϕ via the complement receptor 1 (CR1 or CD35) [132], while antibody-opsonized IEs and merozoites are phagocytosed via FcγR [133].

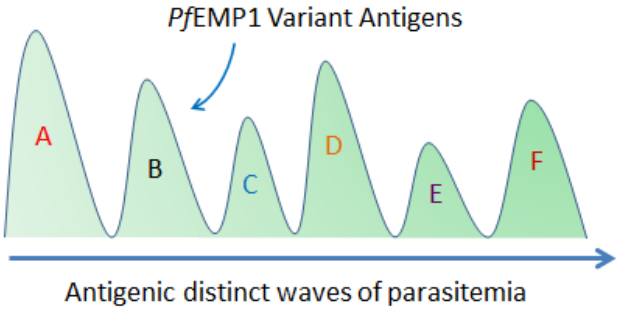
At this point, the innate immune system has interacted with the different components of the IEs, including *Pf*EMP1, and began to activate the adaptive branch of immunity. Monocytes/ Mϕ that have digested the *Pf*EMP1 and other IEs contents are presenting the peptides via class II MHC to specific T cells, resulting in the proliferation of effector and memory T cells. Helper T cells, also known as $CD4^{+}$T cells, then activate specific B cells that subsequently produce antibodies which assist in recognizing *Pf*EMP1-IEs.

Antibody mediated immunity is an important protective mechanism associated with reduced parasite burden [134] and protection from subsequent malaria infections [135-138].

At his stage of the infection, B cells are secreting antibodies into the bloodstream specific for the IEs components and *Pf*EMP1s. Immunoglobulin G (IgG) is the main antibody isotype found in the blood and the principal Abs facilitating protection against the erythrocytic stage (ES) through inhibiting merozoites proliferation [84,139] and mediating opsonization of IEs [140]. In fact, experimental animal models and passive transfer studies of antibodies from malaria-immune hosts can be used to treat those with severe malaria [141,142].

Nonetheless, in malaria-naïve individuals the host immune system is constantly trying to catch up with recognizing newly and antigenically different *Pf*EMP1s. Every newly invaded erythrocyte can express a unique *Pf*EMP1, evading antibody-mediated clearance. This results in antigenically distinct waves of parasitemia, Figure 2.8.

**Figure 2.8 Antigenic variations of *Pf*EMP1s and related parasite burden**



This antigenic variation contributes to the virulence and pathogenesis of malaria during the erythrocytic stage of infection. Over time, however, in malaria endemic regions individuals who survive until adulthood, acquire partial immunity to malaria (or anti-disease immunity) [43,143]. Although it is possible to develop sterile immunity in some cases [43,133,144], most adults will become clinically ill, but not develop severe disease or die—with the exception of pregnant woman.

**Malaria in Pregnancy**

Over 54 million cases of malaria occur every year in pregnant women, and thousands of these women will die or have adverse birth outcomes [145]. Women in their first and second pregnancies are at highest risk for developing placental malaria (PM), which is a major risk factor for the mother and her unborn fetus and is implicated in 75,000 – 200,000 infant deaths annually [146]. Irrespective of previous exposure or partially acquired immunity, selective accumulation of *Plasmodium falciparum* infected erythrocytes in the intervillous space of the placenta results in low birth weight (LBW) [147-150] through preterm delivery (PTD) [151], maternal anemia as a result of erythrocyte loss [152,153], and intrauterine growth restriction (IUGR) due to blockage of adequate nutrient/gas exchange between fetus and mother [147,151,154].

PM infection is an example of a severe malaria syndrome, largely attributable to the cytoadherent nature of the infected erythrocytes and the subsequent hyper-inflammatory immune response that causes tissue damage and impairment of the placenta. Unlike non-pregnant individuals, who are immune to these parasites, pregnant women are at highest risk for developing PM regardless of previous exposure to *P. falciparum* and are more attractive to mosquitoes [155].

Pregnant women that develop PM in high-transmission areas are less often symptomatic then those in low-transmission regions who invariably show signs of fever, headache, abdominal pain, and frequent vomiting [149,156]. The assumption for pregnant women living in endemic regions is that natural immunity will control the infection, however, if left untreated the infection can rapidly

become severe and lead to fetal and maternal death [156]. In symptomatic women, prompt diagnosis and management can reduce pregnancy complications [157,158]. Fever is known to induce IUGR and increase the chances of PTD [159,160]; both these factors have been attributed to severe pregnancy complications [161]. Peripheral parasitemia is at its peak during the third trimester of pregnancy [148], during a time when fetal development is at its highest.

PM affects women in a gravidity dependent manner, where primigravidae (PG) women are more susceptible than multigravidae (MG) [162,163]. In areas of low transmission there is a slight trend towards lower parasitemia with increasing gravidity [149], whereas in regions of high transmission, PG are clearly at greater risk for PM and adverse clinical outcomes [163]. Maternal age and trimester of presentation may also have an association with the prevalence of PM [164], with younger maternal age being an independent risk factor.

Regardless of endemicity, maternal anemia is common during placental malaria [165]; erythrocyte destruction induced by the infection is the underlying reason for anemia and/or hemoglobinopathy [166]. Severe anemia most frequently results in hypoglycaemia and pulmonary oedema [167]. At this stage fetal loss is inevitable. In endemic regions, low birth weight (LBW) is always associated with malaria during pregnancy [168], which can result from both IUGR and PTD. Without prompt treatment, pregnant women with PM will be at higher risk for pregnancy complications that may lead to neonatal death.

**Treatment for placental malaria**

Placental malaria prophylaxis is becoming increasingly more difficult with the emergence of drug resistance. For decades, choloroquine was used as treatment during pregnancy. Prior to the spread of drug resistance to choloroquine [169,170], good adherence to the drug regimen could free the woman of parasites completely during pregnancy. Once the use of choloroquine (CQ) diminished, sulfadoxine-pyrimethamine (SP) and mefloquine (MQ) became the critical treatment.

However, SP and MQ resistance occurred rapidly as CQ [13,171]. Following the resistance to SP and MQ, aggressive detection and management were utilized to avoid severe disease along with our last working treatment available, artemisinin combination therapy (ACT). Although ACT treatment during pregnancy is efficacious [172], recent studies suggest the potential emergence of resistance to artemisinin in Cambodia and Thailand [11-19]. This is alarming as the Cambodia/Thailand region is the focal point of where all previous malaria drug resistance emerged.

Pregnant women infected with *P. falciparum* must be treated promptly, whether they are symptomatic or not, to avoid progression to severe malaria and associated outcomes. Insecticide-treated nets and intermittent presumptive treatment (IPT) with SP (IPT-SP) or (IPT-AZ) artesunate derivatives is currently the recommended treatment for prevention of PM in endemic areas [25]. IPT consists of two doses of a working anti-malarial drug during pregnancy. Although IPT-SP treatment remains effective in West Africa in higher doses [173], this data has to be considered with caution as a recent review demonstrated limited protection from infection against PM with IPT-SP [174]. IPT-SP is no longer effective in some regions, but it is still widely prescribed because it is cheap and easily available. There is an urgent need for alternative antimalarials in pregnancy. .

**Placental malaria antigen and ligand: VAR-2-CSA**

In pregnant women *Plasmodium falciparum* has evolved to seize the availability of a new organ, the placenta. During pregnancy, selection for adhesion in the placenta and immune selective pressure drive the emergence of a unique IE binding phenotype. In particular, the parasites express a unique *Plasmodium falciparum* erythrocyte membrane protein-1 (*Pf*EMP1), called VAR2CSA, capable of binding chondroitin sulfate A (CSA) [67,175-178]. In contrast to parasites from non-pregnant women, PM-parasites that adhere to CSA show an absence of binding to ICAM-1 and CD36 [67,179]. Panning of VAR2CSA-IEs on CSA *in vitro* results in the loss of binding to CD36 and ICAM-1, suggesting that *var2csa* expression may be mutually exclusive.

While several *var* genes can be transcribed during the ring stage, only one full-length mRNA is transcribed, translated into protein and expressed on the surface of the IE [178,180]. Thus, *Pf*EMP1 expression is believed to be mutually exclusive [180,181]. However, there is some evidence in the case of *var2csa* that challenges this general dogma of *Pf*EMP1 expression. A recent study by Brolin et al., provided compelling evidence that multiple *var2csa* genes can be transcribed simultaneously, suggesting that *var2csa* expression is not a mutually exclusive process [182]. The gene was only recently identified to be duplicated in culture adapted *P. falciparum* HB3 parasites [183] and located at different chromosomal loci in many isolates [184].

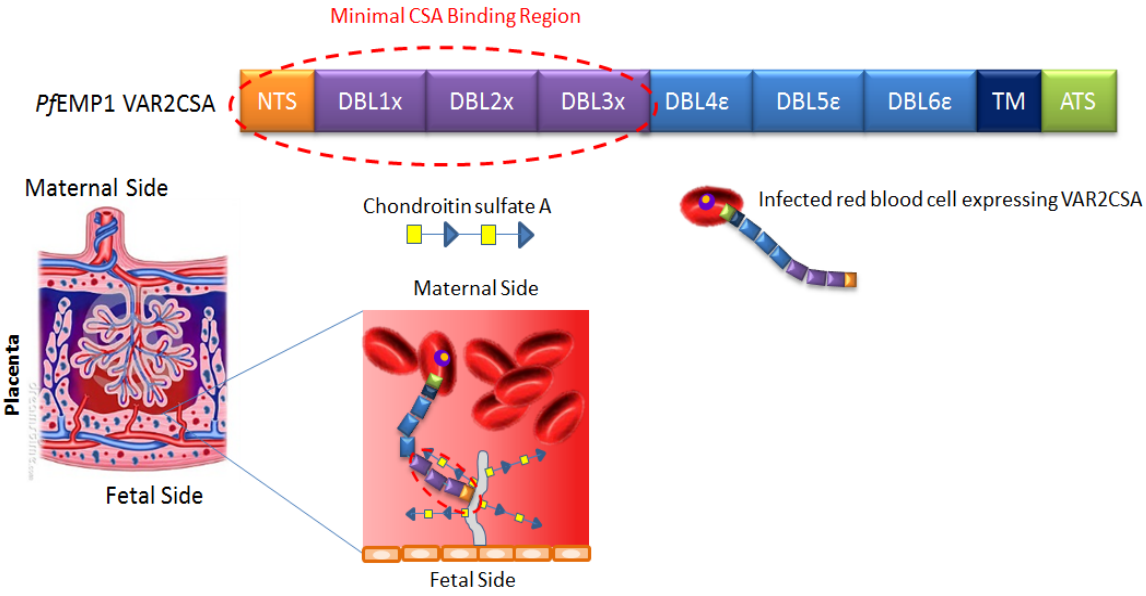
The *var2csa* gene is found in almost all *P. falciparum* isolates [185]. A *var2csa* ortholog is found in the genome of chimpanzee malaria *P. reichenowi*, indicating its ancient origin and evolutionary importance. *Var2csa* is part of a repertoire of ~60 *var* (variant) genes and is the most conserved *Pf*EMP1 identified to date [178,186], with diversification associated with gene conversion events and segmental gene recombination [183,185,187]. Sequence analysis between the different *var2csa* genes reveals polymorphic sites to group into segments of limited diversity, in which a few basic types within each segment characterize the majority of isolates [188].

The encoded VAR2CSA protein consists of six Duffy-binding-like (DBL) domains, a large cysteine-rich inter-domain region (CIDR) between DBL2X and DBL3X, a C-terminal region predicted to be cytoplasmic, and a number of interdomains [189]. Three of the six DBL domains (DBL2x, DBL3x, and DBL6ε) are known to bind CSA [190,191], with the DBL3X and DBL4ε domains being the most conserved of the six DBL domains.

The minimal binding region is located in the N-terminus of the protein including the DBL2X domain and flanking regions [192,193]. Recent work demonstrated that the DBL1X-3X fragment has similar CSA binding characteristics as the full-length VAR2CSA and inhibitory antibodies raised against full-length VAR2CSA target principally the DBL3X domain [193,194]. A summary of the

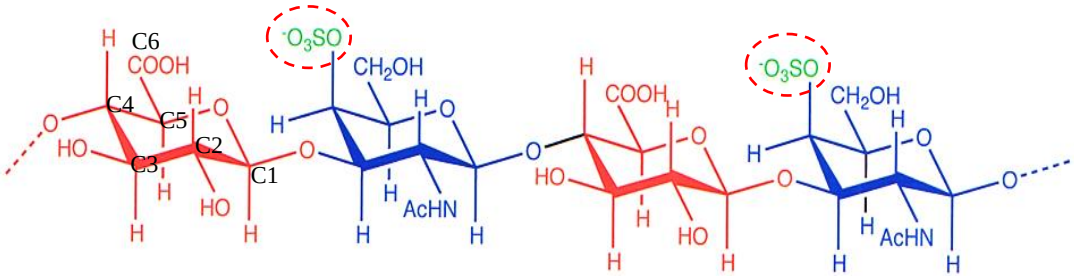
DBL domains implicated in interacting with CSA and the VAR2CSA protein structure is shown in Figure 2.9.

**Figure 2.9 VAR2CSA protein structure and predicted binding of DBL domains [195]**




Although chondroitin is found throughout the human body, placental CSA is distinctive in that it bears a unique sulfation pattern: only 10% of the disaccharide repeats units are sulfated, exclusively at the N-acetylgalactosamine *O*-4 position [196], Figure 2.11.

**Figure 2.11 Unique sulfation at *O*-4 position of placental C4S**




Chondroitin 4-sulfate (C4S) is found on the surface of specialized fetal epithelial syncytiotrophoblast (ST) cells and in the intervillous space of the placenta. C4S is a member of glycosaminoglycan (GAG) family of polysaccharides, which are covalently attached to a proteoglycan (PG) or "core protein" such as CD44 [197]. Interestingly, only about 30% of chondroitin sulfate proteoglycans

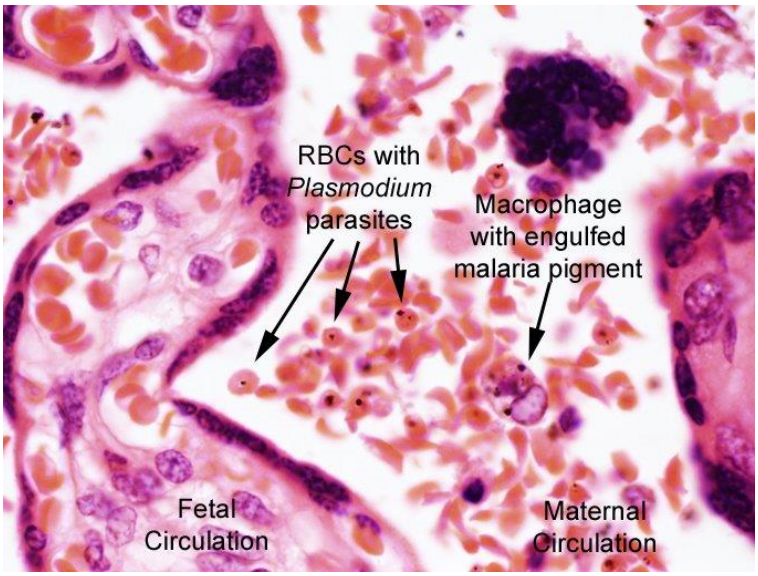
(CSPGs) are 4-sulfated, ~15% are 6-sulfated and the rest are non-sulfated [198]. Nonetheless, both ST cells and the intervillous space CSPGs bind effectively VAR2CSA-IEs [199]. The C4S in the placenta consists of sulfate groups that are clustered at a density of 2 sulfate groups per 6-14 disaccharides, equivalent to ~28% sulfate content [200]. Inhibition studies using varying disaccharide chain lengths and sulfate content of C4S show that a dodecasaccharide with at least two 4-sulfated disaccharide moieties is optimal for binding VAR2CSA-IEs [201,202]. Furthermore, the correct conformational orientation (equatorial) of C4S is important when interacting with VAR2CSA-IEs [198].

The unique low sulfation pattern of placental Chondroitin-4-sulfate (C4S) is likely not available on other endothelial cells, ensuring that VAR2CSA expressing IEs (VAR2CSA-IEs) only sequester in the placenta. This sequestration leads to the expansion of VAR2CSA-IEs, which leads to the infiltration of mononuclear cells, resulting in inflammation and impairment of the placenta. Eventually this accumulation of IEs results in PM related pathogenesis.

**Placental pathology during a malaria infection**

Characteristic feature of placental malaria (PM) pathology include the expansion and accumulation of VAR2CSA-IEs, deposition of the malaria pigment (haemozoin), fibrinoid necrosis, and infiltration of mononuclear cells [203]. During PM infections, the placenta can harbor a tremendous number of parasites, macrophages (Mϕ) and malaria pigment as seen in thick smears of placental blood [204]. Parasites are frequently detected within the intervillous space of the placenta and within the cytoplasm of Mϕs [205]. Pregnant women with IEs and malaria pigment in monocytes or fibrin have a higher prevalence of LBW and maternal anemia [156]. See Figure 2.11 on next page for cross section of PM infected placenta.

**Figure 2.11 Cross section of malaria infected placenta [206]**




Haemozoin deposition is present in the maternal part of the placenta in the intervillous space at relatively high concentrations, within perivillous fibrin and in the cytoplasm of maternal Mϕs [207]. Haemozoin is the product of haemoglobin digestion by the parasite [208]. Haemoglobin is a major nutrient source for the parasites, especially at the early stages of development. However, soluble haemozoin is toxic to the parasite. Thus, the parasite converts it into insoluble microcrystalline haemozoin through biocryztallization to avoid toxicity. This process is highly specific for the malaria parasites. Heme biocryztallization is an important avenue of research in terms of using it as a drug target and for malaria diagnosis.

Interestingly, malaria products and IEs have not been shown to accumulate on the fetal side or structures [209,210], suggesting that the placental barrier protects the fetus until delivery. Only the maternal side will have an accumulation of infected erythrocytes [211,212]. Ultimately, the sequestration of IEs in the placenta will lead to a wide spectrum of immune responses that can be both protective and harmful to the mother and fetus.

**Placental malaria immune responses**

Placental malaria (PM) is unique in that the host immune system is altered to accept the fetus while maintaining host defenses against a malaria infection. This provides the parasite not only with a new organ for sequestration, the placenta, but a host that is more susceptible to the infection.

Two types of effector $CD4^+$T helper cell responses, type 1 helper T-cell (Th1) and type 2 helper T-cell (Th2), play a critical role during placental malaria. The Th1 response is characterized by the production of interferon-gamma (IFN-γ), which activates monocytes/ Mϕ and induces B cells to produce antibodies, leading to cell-mediated immunity. The Th2 response is characterized by interleukin 4 (IL-4), a cytokine that induces differentiation of naïve helper T cells to Th2 cells, and activates humoral immunity through stimulating B cells proliferation and antibody class switching.

The placenta is a major anti-inflammatory organ, able to suppress cell-mediated responses (Th1 response) through Th2 pathway with anti-inflammatory cytokines such interleukin-10 (IL-10) [213,214]. A Th2 response is important in maintaining pregnancy, while the expression of Th1 responses is associated with spontaneous abortions [215]. However, a Th1 pro-inflammatory response is critical during the blastocyst stage of pregnancy[216]; IFN-γ is important in remodeling the arteries to help with adequate placental blood flow [217] and towards the end of the pregnancy tumor necrosis factor-alpha (TNF- α) is needed to induce labour [218].

During a malaria infection, however, this delicate balance of the Th1/Th2 is shifted prematurely towards the Th1 pathway through the production of IFN-γ and TNF-α [219]. This inflammatory response along with the parasite burden in the placenta is associated with poor pregnancy outcomes, especially for women in their first and second pregnancies.

The Th2 response during the second trimester of pregnancy creates a temporary state of "limited immunity" [215], allowing parasite persistence and a subsequent rise in parasitemia [148]. The accumulation of IEs results in the activation of both circulating and resident Mϕs, that release a Th1 cytokine response [220], to aid with phagocytosis through the production of nitric oxide (NO).

The Th1 response leads to IFN-γ production, which will ultimately induce B cells to produce cytophilic antibodies and complement-fixing antibodies. Foetal ST are also able to directly produce IFN-γ [220], likely through the stimulation of TLRs, although this is somewhat questionable. The elevated IFN-γ levels have been associated with gravidity dependent protection [221]. In MG women the IFN-γ levels are able to spike and then subsequently drop, whereas in PG women this cytokine peaks and remains there [220]. This difference is likely due to the fact that MG women are able to clear the IEs more efficiently due to previous exposure to VAR2CSA-IEs, whereas PG lack a broad enough immune response for all the VAR2CSA variants. The direct sequestration of IEs can induce local production of TNF-α [222]; the main source of this cytokine is believed to be from malaria pigment activated Mϕs [223]. TNF-α can promote monocyte recruitment [224] and change the placental structure through hormonal regulation [225].

The other key cytokine is the migration inhibitory factor (MIF), which can be secreted directly from STs as a result of VAR2CSA binding to CSA [226]. MIF is able to suppress Mϕs anti-inflammatory responses, thus ensuring the continuing stimulation of IEs phagocytosis [227]. As anticipated, in PG women, where the parasite burden is higher and more difficult to control, MIF levels are higher when compared to MG women [228].

The Th1 response seems to be robust as long as the parasite burden is severe and Th2 dominance is not restored until the parasitemia is reduced—likely using a positive feedback system. Women with PM tend to have a dominant Th2 response regardless of parity or age, but as long as parasite density is kept consistently low [229]. Local upregulation of IL-10 has been shown to inhibit pro-inflammatory response of Mϕs during PM [220,230].

Mϕs phagocytosis is further enhanced through complement activation. However, excessive levels of complement can directly contribute to pathogenesis [231]. Nonetheless, complement can play a supplementary role in IE clearance as discussed previously under “malaria, the immune system and vaccines”. In the placenta, complement component 3 (C3) is kept at reduced levels by

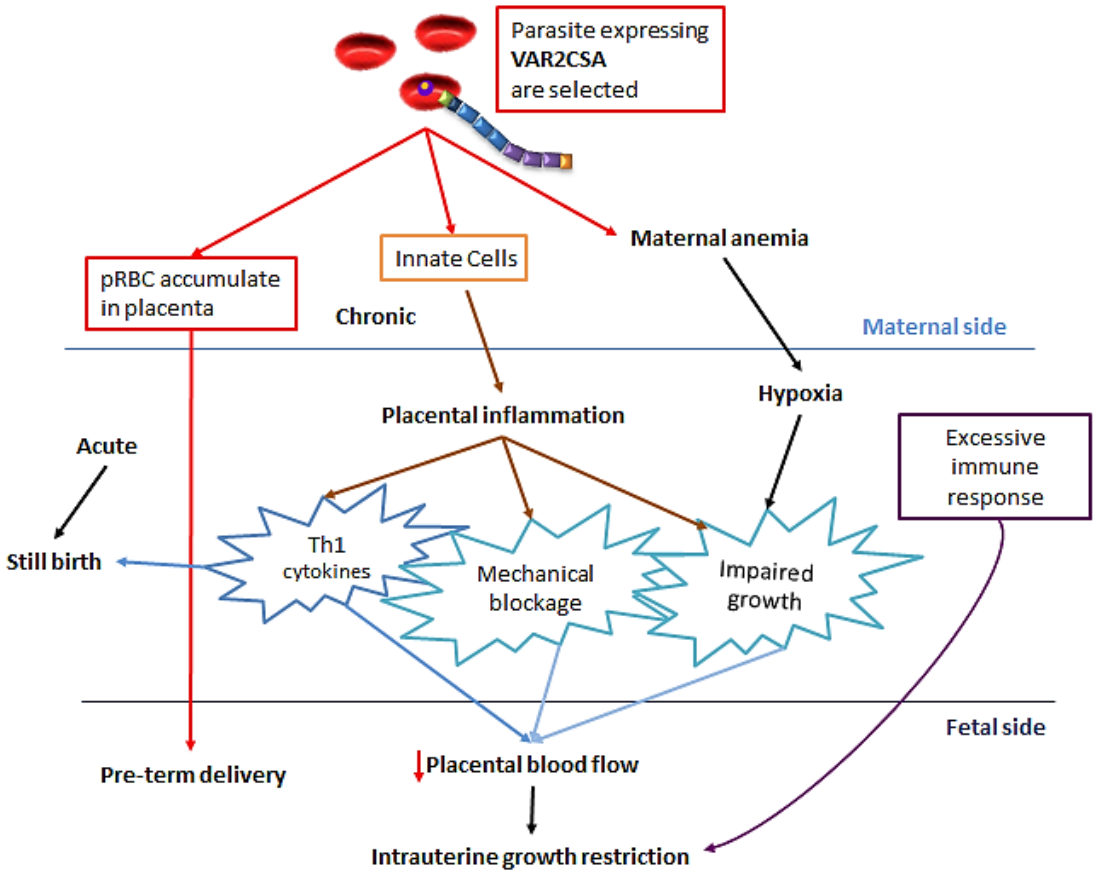
the expression of CD59 and cofactor proteins [232]. Excessive levels of complement 5a (C5a), activated by C3a, are associated with both placental and fetal injury in mice, resulting in IUGR and PTD [231]. Taken together, both complement and cytokines can mediate monocytes/ Mϕ recruitment and subsequent activation of the adaptive immune system including antibody production. Arguably, antibody mediated protection is perhaps the most critical aspect of immunity in regards to preventing PM associated outcomes for the mother and fetus.

PG women are not exposed to VAR2CSA-IEs prior to becoming pregnant and thus lack specific protective antibodies [176,219]. Sera from multigravid (MG) women from different malaria endemic regions have been shown to inhibit binding of various placental VAR2CSA-IEs isolates to C4S [219,233-237]. In addition, multigravid women retain these anti-VAR2CSA inhibitory antibodies [238], as indicated by the fact that MG women are protected from adverse pregnancy outcomes due to PM. The levels of anti-VAR2CSA antibodies correlate directly with the degree of inhibition of IEs binding to CSA [233]. As expected, after multiple pregnancies and an increase of exposure to variant VAR2CSA-IEs, the levels of protective antibodies increase [219,237]. Thus, levels of protective antibodies increase with gravidity. Although anti-VAR2CSA antibodies are found in PG women [238,239], their antibody repertoire is not broad enough to confer protection against PM.

If the pregnant woman is not able to clear IEs, she will be at higher risk for developing severe anemia and PM related pathogenesis. Immunoglobulin G (IgG), specifically the sub-classes IgG1 and IgG3, are important in conferring protection against PM [240]. As discussed previously, IgG can directly promote splenic removal of IEs through anti-adhesion properties and influence cytophilic clearance through opsonization [241,242]. Serum from MG women is both cross-reactive [243] and cross-inhibitory [219], the latter is thought to be due to conserved epitopes or polymorphic epitopes found on all VAR2CSA isolates. VAR2CSA-IgG's are detected up to six months after delivery [239], and only one in 4,000 B cells is specific for polymorphic VAR2CSA epitopes [244]. The

exact mechanism maintaining B cell memory in MG is still not known, especially since there is a lack of antigenic exposure post-delivery. A summary of immune interactions during PM is shown in Figure 2.12.

**Figure 2.12 Interplay of immune interactions during placental malaria [245]**




**Evolutionary importance of *var2csa* and placental malaria**

The extremely high diversity of the *var* multi-gene family, generated by recombination within genes and between genomes [246,247], is one of the major factors why anti-disease immunity to malaria develops only after numerous infections. Even then, the infected individual may be harboring a chronic infection due to the ability of the parasite to persist by clonal antigenic variation, which results in successive waves of parasitemia mediated by antigenic switching.

*Var* genes are traditionally classified by upstream promoter sequences into five distinct types (UpsA thru. UpsE), corresponding to the direction of transcription and chromosomal location [248], as well as by conserved fragments within DBLα [249]. While the majorities of Ups-var genes are highly polymorphic and not found in all isolates, the UpsE-*var2csa* gene is exceptionally conserved across all parasite isolates. Interestingly, the gene is found in the sub-telomeric regions—known to be highly recombinogenic and rapidly evolving [250]. Even more fascinating, *P.falciparum var2csa* shares a common ancestor with chimpanzee *P. reichenowi*. It appears that the gene evolved from a common progenitor several millions of years ago. The *var2csa* ortholog in *P.reichenowi* has several polymorphic blocks that are related or nearly identical to *P.falcparum* at both the nucleotide and amino acid level [185].

This degree of gene conversation, especially for a *Pf*EMP1 variant antigen, is unprecedented. This suggests that parasites are under strong selective pressure to retain the *var2csa* gene, and that it may plays a critical role in the parasite population as a whole. One interpretation for this unusual gene conservation maintenance, over millions of years, is that pregnant women serve an essential role as a reservoir for parasite transmission. In fact, it seems this gene has been around as long as pregnant women have walked the earth. The real question is then, what will happen if we eliminate placental malaria?

**Summary and gaps in knowledge**

While our understanding of placental malaria (PM) has increased substantially over the years, there are a number of areas that require further research to increase our complete understanding of PM pathogenesis. A lot of progress has been made, especially in the last few years, in determining the interactions between the individual VAR2CSA domains and placental CSA.

The initial identification that *var2csa* retains between 54-94% of its amino acid sequence between isolates [188], ignited a race in the research community for the development of a PM

vaccine. However, vaccine development was set-back initially due to the difficulty of expressing full-length recombinant protein. As a result, multiple groups studied the individual domains and reported that three out of the six DBL domains (DBL2x, DBL3x, and DBL6ε) are able to bind CSA, while the remaining three (DBL1x, DBL4ε, and DLB5ε) show limited affinity for binding [251,252]. The early results obtained from these studies [253-258] have proven difficult to interpret for the following reason(s): (1) different protein expression systems were used, including mammalian systems which glycosylate proteins, whereas *P. falciparum* is not known to glycosylate *Pf*EMP1s; (2) adhesion inhibition was measured using assays of CSA bound to plastic mounts; and, (3) most studies used varying sources of CSA that do not contain the correct sulfation pattern, the low-sulfated form of C4S, which is critical in the interaction with VAR2CSA. Taken together, the experimental methods were not representative of the likely interactions observed *in vivo.*

Further, antibodies for the different recombinant domains have been raised in mice and goats [256,259-261]. Antibodies elicited by the DBL3X and DBL5ε domains were able to recognize various VAR2CSA strains from diverse geographical regions, whereas the same cross-reactivity was not observed with DBL1x, DBL4ε and DBL6ε. Unfortunately, in the same studies the DBL3X and DBL5ε antibodies were not able to inhibit binding. The most effective anti-adhesion results with antibodies induced in rats is seen with the DBL4ε domain [257], however, the study used both non-placental C4S and the ligand was bound to petri dishes. Although the results are interesting, it is not known whether the same antibodies would be induced *in vivo* during a natural malaria infection. A recent study reported that immunization and antibody responses varied greatly and unpredictably between mice, rabbits and rats [262]. Thus, these immunization studies have to be interpreted with great caution.

Recent progress has been made in expressing the full-length VAR2CSA protein [259]; however, the full-length protein induces highly strain specific anti-adhesion antibodies [261]. This suggests that the individual domains may be better in inducing cross-inhibitory antibodies and

mediate binding [258]. Further, due to the fact that VAR2CSA is a large protein, ~350kDA, using it as immunogen will be difficult in both terms of mass production and administration. Convincing evidence is emerging to suggest that the DBL1X-3X fragment has similar CSA binding characteristics as the full-length VAR2CSA and inhibitory antibodies target principally the DBL3X domain [193,194]. While finding a globally conserved epitope may prove impossible, expressing recombinant gravidity representative domain(s) might be enough to induce cytophilic IgG antibodies—which have been shown to be effective in conferring protection during PM [241].

Taken together, these studies have greatly contributed to our understanding of PM mediated cytoadhesion and the limitations of particular experimental methods. In spite of this data, there are relatively few published reports characterizing the degree of diversity of *var2csa* among parasite isolates from various geographical regions. The most in depth study on *var2csa* polymorphism looked at 18 full-length genes from geographically distinct isolates, identifying segments that undergo frequent recombination [188]. While this work was significant, it was performed on culture adapted isolates and it was not possible to relate it to clinical correlates of PM. Another study [185] performed on 22 sequences from culture adapted 3D7 strains, identified extensive gene mosaicism within polymorphic blocks and a 54-94% amino acid identity in the extracellular binding region. Two other studies [263,264] characterized the diversity of the DBL3X domain from Malawian and Senegalese women. Both studies showed relatively low sequence diversity, with one study [264] identifying parity associated motifs and regions under diversifying selection. The most recent study by Sander et al. [184], using 54 placental blood samples, identified a dimorphic motif within the DBL2X region and subsequently identified *var2csa* to be a multi-copy gene. Thus, several critical aspects in regards to *var2csa* diversity remain still unknown:

(1) What is the degree of diversity within a more confined endemic region?

(2) Are there particular motifs or sequences that are associated with severe outcomes of PM? If so, are these motifs geographically confined or globally conserved?

(3) What is the genotype complexity within each pregnant woman and is there an association with gravidity?

(4) How does *var2csa* copy number relate to gravidity and immune status?

The goal of this dissertation is to answer these questions in an effort to help select optimally protective antigens and ascertain whether a few or many such antigens confer protective immunity through identifying correlates with clinical outcomes of PM. More specifically, this data can be used to design recombinant regions of *var2csa* in an effort to elicit a sufficient protective response against genetically diverse parasites. Again, sterile immunity is likely not achievable, but a protective response similar to that observed in MG women may be.

The traditional approaches of target antigen identification through human and animal models, such as represented by irradiated sporozoite immunization, are powerful models for the development of vaccines. However, the specific target antigens and epitopes that elicit protection *in vivo* are largely unknown and correlates of protection after experimental immunization are not clear. In a pre-genomic era, these traditional approaches are useful and powerful. Today, with the wealth of proteomic, genomic and transcriptomic datasets new approaches can be utilized. In particular, with the right questions and experimental design(s) in terms of collecting this data, we have a great opportunity to critically and rationally identify targets for antigen selection.

A recombinant protein that is able to induce a protective response against a few variants may be beneficial for women in their first or second pregnancies by helping reduce the parasite burden to manageable levels, as observed in multigravid women. Naturally, it only takes one to two pregnancies to develop inhibitory antibodies against VAR2CSA-IEs and by identifying epitopes associated with PM, we could achieve potentially even better protection than that which is acquired during a natural infection. Importantly, in combination with the knowledge offered by pathogen genomic sequences, a systematic approach for antigen selection is used in this study.

Importantly, the correct protein expression system must be used, including the ability to produce a correctly folded protein by taking into account ribosomal pausing as observed in *P. falciparum*. Furthermore, low-sulfated placental C4S ligand and the correct conformational orientation must be used during the binding studies. For antibody mediated inhibition, serum from pregnant women living in endemic regions needs to be used instead of immunized animals.

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# CHAPTER 3

# SEQUENCE POLYMORPHISM, SEGMENTAL RECOMBINATION AND TOGGLING AMINO ACID RESIDUES WITHIN THE DBL3X DOMAIN OF THE VAR2CSA PLACENTAL MALARIA ANTIGEN

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**Abstract**

*Plasmodium falciparum* malaria remains one of the world's foremost health problems, primarily in highly endemic regions such as Sub-Saharan Africa, where it is responsible for substantial morbidity, mortality and economic losses. Malaria is a significant cause of severe disease and death in pregnant women and newborns, with pathogenesis being associated with expression of a unique variant of the multidomain *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) called VAR2CSA. Here, we characterize the polymorphism of the DBL3X domain of VAR2CSA and identify regions under selective pressure among placental parasites from women living in endemic western Kenya. In addition to significant levels of polymorphism, our analysis reveals a strikingly high multiplicity of infection (MOI) not previously reported. Furthermore, we were able to identify novel DBL3X sequence motifs that were associated with gravidity, suggesting that parasites expressing these particular motifs may represent responses to immune selective pressure. Interestingly, we also identified a number of critical residues implicated in immune evasion through switching (or toggling) to alternative amino acids, including an arginine to glutamine or glutamic acid within the binding pocket in subdomain III, which was previously implicated in binding to placental CSA. Overall, these findings are important for understanding parasite diversity in pregnant women and will be useful for identifying epitopes and variants of DBL3X to be included in a vaccine against placental malaria.

**Introduction**

Malaria in pregnancy is a disease syndrome with devastating social and medical complications requiring multidimensional solutions. Despite intensified international efforts to reduce the malaria burden in the developing world, it is estimated that more than 54 million cases of malaria occur every year in reproductive age women [1]. Placental malaria (PM) results from massive sequestration of *Plasmodium falciparum* infected erythrocytes in the intervillous space of the placenta, causing severe clinical symptoms that result in maternal morbidity, low birth weight and/or neonatal death [2].

Malaria-induced morbidity and mortality is largely attributable to the cytoadherent nature of *Plasmodium falciparum* infected erythrocytes. This cytoadherence is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a parasite protein encoded by the highly polymorphic *var* gene family and expressed at the surface of the infected erythrocytes. During pregnancy, expression of a single member of the *var* gene family, *var2csa*, mediates binding to a form of chondroitin sulfate A (CSA) unique to the placenta [3-8]. Several studies have shown that, after multiple pregnancies, women in malaria endemic regions develop antibodies that inhibit infected erythrocyte binding to CSA [4,9-11], suggesting that placenta-sequestering parasites express surface molecules that are partially conserved or contain shared epitopes. Further evidence for the presence of a conserved antigen in CSA-adherent parasites has been demonstrated by the fact that plasma and parasites from pregnant women from different malaria endemic regions show cross-reactivity [11,12]. This implies that antibody recognition is not dependent on geographical origin of the parasites and that a vaccine to protect against PM is feasible.

Interestingly, while polymorphic compared to most malarial antigens, *var2csa* is the most conserved PfEMP1 identified to date [3,13-15], a fact that suggests it as a candidate for vaccine development. It is well-known that VAR2CSA is specifically up-regulated during PM or when selected *in vitro* for CSA binding [16], and the disruption of the *var2csa* gene leads to loss or

significant reduction of infected erythrocyte adhesion to CSA [9,15]. VAR2CSA consists of six Duffy-binding-like (DBL) domains, a large interdomain region (ID2) and a C-terminal region predicted to be cytoplasmic [17]. As reported previously, four of the six DBL domains (DBL2x, DBL3x, DBL5ε, and DBL6ε) have been shown to bind CSA [18,19], although this is controversial [20,21]. In order to design molecular interventions against PM, it is important to determine the minimal CSA binding regions and understand how variability is distributed throughout the individual domains of VAR2CSA.

The DBL3X domain has been shown to be the most conserved of the six DBL domains of VAR2CSA [22], and antibodies raised against the recombinant proteins of this domain show the most cross-reactivity with heterologous parasites compared to the other binding domains [23]. Furthermore, as suggested by Barfod et al. [24], antibody-mediated immunity is predominantly acquired by malaria-exposed women to DBL3X and DBL5ε exclusively. Lastly, Srivastava et al. [21] demonstrated recently that DBL1X-3X shows similar CSA binding characteristics as the full-length VAR2CSA and inhibitory antibodies raised against full-length VAR2CSA target principally the DBL3X domain. Collectively, these studies provide good evidence that the DBL3X domain can induce strong protective immunity and, owing to its sequence conservation, make it an excellent target for vaccine development.

The focus of this study was to characterize the complexity of *var2csa* genotypes within naturally infected placentae and investigate patterns of sequence polymorphism within the DBL3X domain. Here we determined the sequence of *var2csa* DBL3X amplified from Kenyan placental parasites and applied novel computational and molecular modeling methods to investigate polymorphism within this domain.

**Results**

*Var2csa DBL3X domain shows a high degree of complexity within individuals*

To enable complete characterization of *var2csa* polymorphism in placental parasite populations, the DBL3X region from 12 maternal placental blood samples was amplified. Five samples were from primigravid women, two from secundigravid, and five from multigravid women. Complete sequences from an average of nine clones per sample were obtained. As a control three independent plasmids containing the FCR3 DBL3X domain were also sequenced. These yielded identical sequences, with 100% sequence identity with the FCR3 *var2csa* DBL3X sequence in GenBank. Overall, 108 sequences were obtained, of which 79 were unique at the nucleotide level when compared to these samples and existing DBL3X sequences in GenBank (Table 3.1). 76 of these 79 sequences were also unique at the amino acid level, demonstrating a very high ratio of nonsynonymous to synonymous mutations. In support of these sequences being unique, we found that most of the sequences obtained differed by 5 or more nucleotides in pairwise comparisons. Indeed of the 3081 possible pairwise comparisons between these sequences, only 73 demonstrated 4 or fewer nucleotide differences. The most interesting finding obtained from this data is the surprising degree of complexity observed within individual patient samples. Among the 12 placental samples, half showed nine or more unique *var2*csa DBL3X sequence types, a level of multiplicity of infection (MOI) not previously reported. There was no apparent relationship between gravidity and MOI. It is clear from this work that the majority of the placental infections are highly complex with regard to *var2csa* genotypes, arguably the most relevant measure of parasite diversity for PM studies. It is interesting to compare these findings with other studies conducted to assess MOI during pregnancy in Africa. Some of these studies used gel based PCR-RFLP analysis methods, and reported MOIs ranging from 1.5 to 2.8 [25]. Another study used a quantitative Gene scan method and found a MOI of 5.6, suggesting that more sensitive methods are likely to detect higher MOIs [26]. The studies conflicted on the relationship between gravidity and MOI, with one study finding a negative

correlation [26], and another finding no association [25]. Three other studies, two in Gabon [27,28] and one in Ghana [29], also reported relatively low MOI (range  of mean MOI 1.5-3). Again there was disagreement as to a correlation between gravidity and MOI as one study found decreased MOI with increasing gravidity [29] while the other found no correlation between MOI and age (an approximation for gravidity) [27]. Taken together, these studies found a relatively low MOI for placental parasite populations but arrived at this conclusion via techniques that did not assay the most relevant polymorphic molecule for PM, VAR2CSA. We demonstrate here significantly higher MOIs in western Kenya as measured by sequence analysis of *var2csa*. It is possible that the high genetic complexity at the var2csa locus we demonstrate here is due in part to parasites with multiple var2csa genes. While haploid in the human host, recent work has demonstrated that some isolates of *P. falciparum* contain more than one copy of a var2csa-type gene [30], and that such parasites may be selected for during the course of infection in pregnant women [31]. While no correlation between genetic complexity within var2csa and gravidity was observed in our Kenyan samples, such an association, whether due to a higher multiplicity of infection or the multicopy nature of the var2csa gene in some isolates, likely needs to be tested by deeper analysis of the degree of complexity of placental infections.

Using these data, the 79 unique DBL3x sequences were further characterized by constructing a maximum likelihood tree (Figure 3.1). One interesting feature of the tree is that sequences from the same patient sample tend to group together in one to three clades.  With the exception of one sequence, 0786-A09 (denoted with an asterisk), no sequences from one patient grouped in a clade with those of another patient. This result is in conflict with a similar analysis of 43 DBL3x sequences from 24 Senegalese placental samples by Dahlback et al. [32], who reported that sequences from different patient samples cluster together in distinct clades. Although it is difficult to know the precise reason for this discrepancy, the observed differences are likely due to the differences in study sample populations, transmission rates and the time span over which the samples were collected. In

particular, one interpretation of this sequence grouping by patient among Kenyan samples is that the women came from different villages separated enough geographically to have distinct parasite populations. Although patient data collection did not include location of residence, it would be very interesting to assess this in future studies.

*Selective pressure in the DBL3X domain shows high sequence diversity and positive selection*

Analysis of sequence polymorphism at the population level is an effective tool for detecting influence of continuing selection. Sites experiencing selection show either a decrease or increase in the density of polymorphism. To further investigate DBL3X polymorphism and regions under selective pressure, the pairwise diversity across the sequences was calculated, and Tajima's Neutrality Test was applied to the sequence to reveal selection hotspots (see Materials and Methods for details). The observed average pairwise diversity (л) values for primigravidae ($\pi = 0.0582$) and multigravidae ($\pi = 0.0507$) are similar (Table 3.2) suggesting that there is no influence of gravidity or, by extension, host immunity on the overall diversity of *var2csa* in placental infections. Still, this observed pairwise diversity is considerably higher than observed for other malarial antigens such as *eba-175* ($\pi = 0.00366$), *ebl-1* ($\pi = 0.00321$), and *eba-140* ($\pi = 0.001$) [33,34] This highlights that while *var2csa* is a conserved member of the *var* gene family, it is highly polymorphic when compared to other malarial antigens considered to be viable vaccine candidate antigens. When $\pi$ is calculated for each position in an alignment of DBL3X sequences it is clear that the diversity in this domain is primarily found in the first and second variable regions (V1 and V2), and to a lesser degree the third variable region (V3) (Figure 3.2). While Tajima's D is not >1 when calculated over the entire DBL3X domain (Table 3.2), certain regions of this domain do show evidence of positive selection; that is, Tajima's D (departure from neutrality) is >1 for those regions exhibiting the highest degree of pairwise diversity. This is not surprising as positive selection will favor escape mutations.

These results are in agreement with other studies that have examined diversity in DBL3X [32,35], and provide good evidence that this domain is a target of immune selection.

*Novel motifs associated with host immune status in Kenyan samples*

Amino acid sequence alignments are frequently used in the analysis of protein structure, function and evolutionary relationships. Analysis of positional amino acid conservation of sequences can aid in identifying critical motifs and structurally important residues. A previous study by Dahlback et al [32] reported that the sequence motif EIEKD at amino acid positions 1423-1427 in V2 was overrepresented in primigravidae, while EIERE/EIEGE/GIERE and GIEGE were predominantly found in multigravid women. Four of these motifs, GIERE, EIEKD, EIERE, and GIEGE, were present in the Kenyan DBL3X sequences, but the motif EIEGE was not observed. GIEGE was somewhat overrepresented in multigravidae, with 69% versus 31% in primigravidae. The motif EIEKD was not conclusively gravidity-associated in these samples. Furthermore, in direct contrast to the findings of Dahlback et al. [32], the motifs EIERE/GIERE were more commonly associated with primigravidae than multigravidae. Finally, unique to the Kenyan samples, the motif TKQN at amino acid positions 1401-1404 in V2 was mainly found in primigravidae and TKTK/PQQK in multigravidae (Table 3.3). These motifs, therefore, show strong association with host immune status. Overall, these results suggest that the Senegalese and Kenyan parasite populations are separated enough geographically to have developed distinct motifs that provide a biological advantage based upon gravidity. It is also possible that host genotypic differences in immune-relevant function have provided unique selective pressures on local parasites, thus reflecting geographically unique host/parasite co-evolution.

*Lowest sequence conservation in the DBL3X domain observed near the predicted binding pocket of CSA and the critical amino acid residue arginine implicated in binding to placental CSA is not absolutely conserved*

Conservation analysis has proven to be a powerful indicator of functional importance and has been used to determine residues implicated in ligand binding [36,37], protein-protein interactions [38,39], and functional specificity [39,40]. Although many computation methods are available for determining the functional importance of residues, it has been found that conservation is one of the most powerful attributes in these applications [41]. Mapping this information on a 3D structure can help visualize potential functional surfaces (e.g. such as surfaces exposed to host humoral immunity). Toward this end, the protein structure of DBL3X (Protein Data Bank: 3CPZ, [42]) was used as a template for structural modeling of the 79 unique DBL3X sequences and analysis of sequence conservation performed using the UCSF Chimera molecular analysis program [43] (see Materials and Methods for details). The structure of the DBL3X domain is made up of three subdomains [42,44]. Subdomain I (S1) (Figure 3.3, green), primarily forms a large loop with little secondary structure. Subdomain II (S2) (Figure 3.3, dodger blue), comprises four helices connected by loops. Subdomain III (S3) (Figure 3.3, magenta), is comprised of two anti-parallel helices. S3 has recently been identified as a minimal CSA binding region and mutations of residues within this subdomain result in substantial reduction of binding to placental CSA [45].

Conservation analysis shows variable regions primarily focused around the predicted binding pocket of DBL3X, implying that these regions may be more accessible in the native protein structure to immune surveillance (Figure 3.4). Epitopes exposed on the surface of the native protein may therefore be accessible to immunoglobulins and, as a result, be under strong selective pressure for escape mutations. While host immune responses exert strong selective pressures resulting in amino acid positions that exhibit a pattern of escape mutations, there can be functional consequences to these changes which can select for reversion mutations in a naïve host. Such mutations within or

flanking functionally important epitopes can have two effects: (1) immune evasion through mutation from a wild type sequence driven by selective pressure and (2) changing ligand binding for potentially improved or reduced affinity. This process of mutation and reversion has been termed "toggling" in a recent study by Delport et al. [46] who used a probabilistic model of protein sequences in HIV-1 to demonstrate that there is a large number of sites evolving under selective pressure, but which also exhibit low sequence diversity. These toggling sites, typically, were found to switch between just 2 or 3 amino acids when comparing different viral isolates. That observation prompted an evaluation of the Kenyan samples to determine the extent to which toggling between wild type and escape amino acids is evident and whether or not such toggling sites can be used to identify specific regions of DBL3X that are subject to immune pressure and, therefore, likely to encode functionally important amino acids. Toward this end, the 79 unique DBL3x sequences were submitted for toggling analysis to the Datamonkey server (see Materials and Methods for details) and the results mapped to the protein structure of DBL3X as above. This analysis yielded a total of 27 toggling amino acids, primarily found on domains S1 and S2 (Figure 3.3). It is interesting to note that an arginine residue ($Arg^{1467}$) on S3, previously implicated in binding to the placental receptor CSA [17,42,45], toggles between arginine (R), glutamine (Q), and glutamic acid (E). A recent study by Khunrae et al.[17] suggests that CSA binding is mediated by the positively charged patch in the binding pocket. This would imply that the toggling between a positive (R) – negative (Q) – positive (E) charge modulates the binding affinity to placental CSA and reflects the pressure on the parasite to forego efficient binding in the face of acquired immune clearance mechanisms. Of note, a single amino acid substitution in *P. vivax* Duffy-binding protein (PvDBP) was found to dramatically alter both its binding affinity and antigenic character [47]. To characterize the individual sites relative to immune status, relative proportions of toggling amino acids at each site in primigravidae and multigravidae were compared (Supplementary Table 3.4**)**. As shown in figure 3.5, nine of the 27 toggling sites were found to toggle differentially as a function of gravidity. Since host immune

responses exert strong selective pressures favoring mutations that prevent immune recognition, these toggling sites (especially Arginine$^{1467}$ on S3) may represent important epitopes that, when targeted by host antibody responses, force the parasite to choose between efficient binding to CSA and evasion of these functionally important immune responses that, among other roles, may block the binding of infected erythrocytes to placental CSA.

**Discussion**

Numerous studies have addressed the sequence polymorphism of *var2csa*. After performing similar alignment, tree and structural analyses as previous studies, the present results reveal agreements and discrepancies with the available published data. This analysis revealed an surprisingly high level of parasite genetic complexity at the *var2csa* locus, which may reflect a level of multiplicity of infection (MOI) not previously reported in pregnant women. Given that an average of nine clones per sample were examined, the true MOI may in fact be considerably higher and will require additional investigation. While it is possible that the multicopy nature of the *var2csa* gene in some isolates may account for some of the observed complexity, the number of unique genotypes observed is striking. Several strong gravidity-associated motifs were identified in the Kenyan samples, but the patterns and identity of the motifs differ from those previously reported in Senegalese samples [32]. An important novel advance in this study was the application of structural analysis methods to identify functionally important positions that toggle between a limited set of amino acids that may impact binding to placental CSA. The most interesting finding from this *in silico* structural analysis is that an arginine residue (Arg$^{1467}$) on S3, which is implicated in binding to placental CSA, toggles between arginine, glutamine, and glutamic acid, likely modulating the binding affinity to CSA. Overall, the nine toggling positions that vary significantly between primigravidae and multigravidae in terms of which amino acid is preferred provide indirect evidence that acquired immunity to CSA-binding parasites that is elicited by repeated exposure to PM yields parasites with different sequences

than those used by parasites occupying an immunologically naïve environment. Further study will be required to evaluate the extent to which the toggling at these sites is linked to immune responses and affects efficiency of binding to CSA.

As already observed, detailed sequence analysis coupled with 3D modeling of DBL3X reveals that this domain is very complex. Our results confirm the polymorphic nature of DBL3X to a degree not previously shown. Indeed, previous attempts, using other less polymorphic targets, to define MOI in pregnant women are likely to have been significant underestimates of the true diversity of infection. While this degree of polymorphism is potentially intimidating for workers engaged in attempts to identify immunologic targets within DBL3X and elsewhere in VAR2CSA for inclusion in a vaccine, it is noteworthy that some regions of this protein are constrained. The toggling amino acid sites identified in this study show that successful parasites have limited choices for some residues within VAR2CSA. The immune pressure in a multigravid host selects for escape mutations that later revert back under selection for binding function in a naïve, primigravid host. Should these sites be confirmed as targets for protective antibodies, then such information could be used in the design of a VAR2CSA vaccine construct that targets different serological variants of these regions.

**Materials and methods**

*Sample collection*

The samples used in this study were derived from a project designed to assess gravidity-dependent acquisition of cell-mediated immunity to PM as previously described [48]. In brief, samples were collected from women delivering at Siaya District Hospital in western Kenya. This is an area with stable malaria transmission all year round with reported entomological inoculation rates (EIR) estimated as high as 31 infective bites/ person/ year [49]. Informed consent was obtained from potential clients in agreement with Kenya Medical Research Institute Ethical Review Committee, the

Institutional Review Boards of the University of Georgia, the Centers for Disease Control and Prevention, and the National Institutes of Health. All participants provided informed, written consent under the auspices of these approved protocols. Immediately post-delivery, placentas were collected and maternal placental blood obtained either by the prick method [50] or perfusion [51] under aseptic conditions. Placental parasitemia was calculated by counting the number of parasites on a Giemsa-stained thick smear of maternal placental prick blood and is reported as parasitemia per 300 white blood cells. Each of the collected samples is well defined by clinical data (age, gravidity, birth weight). Samples for this study were collected over a time span of 1.5 years.

*Amplification and sequencing*

DNA was extracted from placental blood samples using the illustra™ blood genomicPrep™ Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer's instructions. Primers to conserved regions of the 5' (BacdiDBL3F - CACCATGAATTATATTCGTGGGTGTCAA) and 3' (BacdiDBL3R – ATTTGCTGATATACATTCAGG) boundaries of the var2csa DBL3X domain were designed from an alignment of 5 *var2csa* sequences. For each sample, the DBL3Xdomain was amplified and cloned via the TOPO reaction into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). The samples were chosen to include a range of gravidities with 5 primigravid, 2 secundigravid and 5 multigravid women. The TOPO reactions were transformed into competent *E. coli* and plated to obtain cloned plasmids. Colonies containing plasmids with a DBL3X insert were identified via colony PCR. Multiple clones were obtained for each sample and submitted for sequencing. A total of 6 overlapping reads were obtained for each sequence using vector primers and four sequence specific primers (DBL3F.2 TGATTATAAGAATATGATTTTGGGTAC, DBL3R.2 TTTATATCTTCTTGTAATTTTCCAATG, DBL3F.3 GAATACCACGATAAAGGTACAGC, DBL3R.3 CCACGAAACGAACTGATCCTC).

Following sequencing, all chromatograms were checked for accuracy and quality, and reads in both directions were assembled into full length contigs for each clone using the Geneious software package (Biomatters Ltd, Auckland NZ). We obtained between 7 and 13 complete DBL3X sequences per sample, with the exception of one sample for which a single sequence was obtained. As a control, the amplification of DBL3Xregion from the *var2csa* of isolate FCR3/IT4 was used. All three FCR3 sequences that were obtained perfectly matched the reported sequence (GenBank accession number AY372123), suggesting a very low error rate. From the 10 placental blood samples a total of 108 sequences were obtained. Taking a conservative approach, sequences were only considered unique if they differed by more than 2 base pairs.

*Phylogenetic analysis and neutrality Test*

To characterize the 79 unique DBL3X sequences, along with the FCR3/IT4 sequence, alignments were done in Geneious Pro using the ClustalW algorithm and phylogenetic analysis was performed in MEGA [52]. Tajima's D test as implemented in both MEGA and DnaSP [53] was used to test 35 multigravidae and 30 primigravidae DBL3X sequences for departure from neutrality in the nucleotide frequency distributions (secundigravidae were excluded from analysis since only 15 sequences from 2 individuals were available). This is determined by divergence in the values of $\pi$ (observed average pairwise nucleotide diversity) and $\theta$ (expected nucleotide diversity under neutrality derived from the number of segregating sites, $S$). Elevated $\pi$ and positive values to D would confer a balancing selection of nucleotide sites that would be maintained at intermediate frequencies.

*3D structure modeling, percent sequence conservation*

The protein structure of DBL3X (Protein Data Bank: 3CPZ, [42]) was used as a template for structure modeling. Structural visualizations and labeling was done using the UCSF Chimera

molecular analysis program [43]. Briefly, the 79 unique DBL3x sequences including the FCR3/IT4 sequence was aligned using Chimera's Multialign View feature and sequence conservation was calculated using the mavPercentConservation method. The mavPercentConservation method is based on the AL2CO algorithm. The algorithm of AL2CO program performs calculations in two steps. First, amino acid frequencies at each position are estimated and then the conservation index is calculated from these frequencies. The results were then mapped to the protein structure of DBL3x (Protein Data Bank: 3CPZ, [42]) using the following color parameters: lowest (40%) and highest (100%) sequence conservation are represented in yellow and red, respectively. Insufficient data (shown in grey) indicates amino acid locations that were missing from our sequences.

*Toggling amino acid analysis and 3D structure mapping*

Toggling amino acid analysis was performed on 79 sequences obtained from 12 placental blood samples using Datamonkey: a suite of phylogenetic analysis tools for evolutionary biology [54]. Briefly, the sequences were uploaded on the Datamonkey server and analysis was performed using the following four steps: First, a nucleotide model was fitted to the DBL3X sequences using maximum likelihood to obtain branch lengths and substitution rates. Second, the data were fitted to obtain a global $\omega$=dN/dS ratio. Next, codon ancestral sequences were constructed site by site using maximum likelihood. Lastly, a test of escape and reversion from/to the wild type was conducted and results reported with the following summary statistics: the likelihood ratio test statistic, *P*-value, toggling rate and the proportion of time each site "spends" in the wild-type, single-step and multiple-step escape amino acid states. Each of the toggling amino acids was then mapped to the template sequence of the protein structure of DBL3X (Protein Data Bank: 3CPZ, [42]) and analyzed using the UCSF Chimera molecular analysis program.

*Statistical analysis*

The chi-square test (or Fisher's exact test when appropriate) was used to compare toggling amino acid proportions between multigravidae and primigravidae. Statistically significant ($P$ <0.05) sites were mapped using WebLogo.

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**Table 3.1**: **Summary of patient gravidity, total number of DBL3X sequences obtained, the number of clones sequenced and among these the number that were unique, and the nucleotide diversity.** The value of π denotes the observed average pairwise nucleotide diversity.

| Sample | Gravidity | Parasitemia Per 300 WBCs | | Clones | Unique (nt/aa) | π | Pairwise Identity (%) |
|---|---|---|---|---|---|---|---|
| | | Peripheral | Placental | | | | |
| 0593 | P | 24 | 3942 | 9 | 9/8 | 0.0047 | 99.5 |
| 0626 | P | 5 | 251 | 7 | 6/5 | 0.0401 | 94.4 |
| 0659 | P | 871 | 1745 | 8 | 7/6 | 0.0342 | 96.3 |
| 0696 | P | 798 | 1353 | 13 | 11/11 | 0.0447 | 93.1 |
| 0895 | P | 200 | 2534 | 11 | 2/2 | - | 99.9 |
| 0661 | S | 147 | 5429 | 10 | 9/9 | 0.0419 | 93.0 |
| 0786 | S | 563 | 4097 | 12 | 5/5 | 0.0366 | 95.8 |
| 0551 | M | 18 | 4 | 8 | 2/2 | - | 99.0 |
| 0608 | M | 650 | 929 | 9 | 9/9 | 0.0346 | 95.1 |
| 0694 | M | 242 | 325 | 11 | 9/9 | 0.0199 | 95.3 |
| 0833 | M | 3 | 15 | 9 | 9/9 | 0.0539 | 94.1 |
| 0855 | M | 3 | 72 | 1 | 1/1 | - | - |
| | Totals: | | | 108 | **79/76** | | |

**Table 3.2**. **Results from Tajima's Neutrality Test.** The Tajima test statistic [1] was estimated using MEGA4 [2]. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The abbreviations used are as follows: m = number of sites, S = Number of segregating sites, $p_s = S/m$, $\Theta = p_s/a_1$, and $\pi$ = nucleotide diversity. D is the Tajima test statistic

| | **m** | **S** | **$p_s$** | **$\Theta$** | **$\pi$** | **D** |
|---|---|---|---|---|---|---|
| **Primigravid** | 35 | 172 | 0.195011 | 0.047353 | 0.058227 | 0.866659 |
| **Multigravid** | 30 | 178 | 0.201130 | 0.050769 | 0.059910 | 0.695683 |

**Table 3.3**. **Novel motifs identified in Kenyan samples are associated with host immune status (gravidity).** Motifs TKTK/PQQK are predominately found in primigravidae and TKQN in multigravidae. Sequence motifs (highlighted in grey) identified in Dahlback et al [33], show no strong parity association in Kenyan samples. * $P < 0.001$, by $c^2$ test.

| **Sequence Motif** | **Primigravid** | **Multigravid** |
|---|---|---|
| EIKD | 59% | 41% |
| EIERE/GIERE | 41% | 59% |
| GIEGE | 31% | 69% |
| TKQN* | 28% | 72% |
| TKTK* | 80% | 20% |
| PQQK* | 75% | 25% |

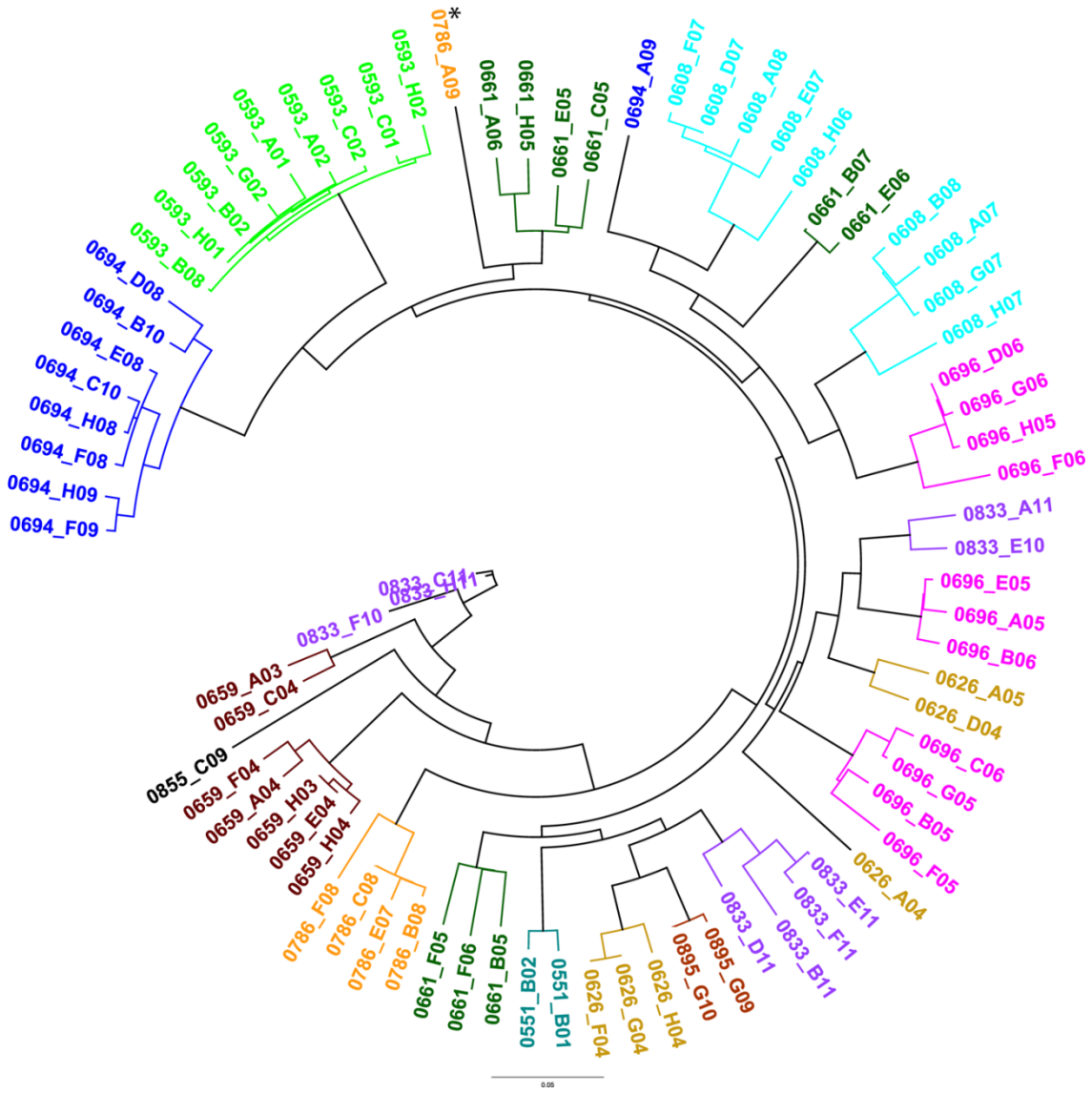


**Figure 3.1 VAR2CSA in placental parasites shows unprecendented diversity with no interindividual clustering.** Maxiumum Liklihood Tree of 79 Unique DBL3X Sequences. Sequences were aligned by translation. Consensus tree shown, with 50% cutoff. Taxa colored by placental sample. Sequences from the same patient sample tend to group together in one to three clades with the exception of 0786-A09, denoted by an asterisk.

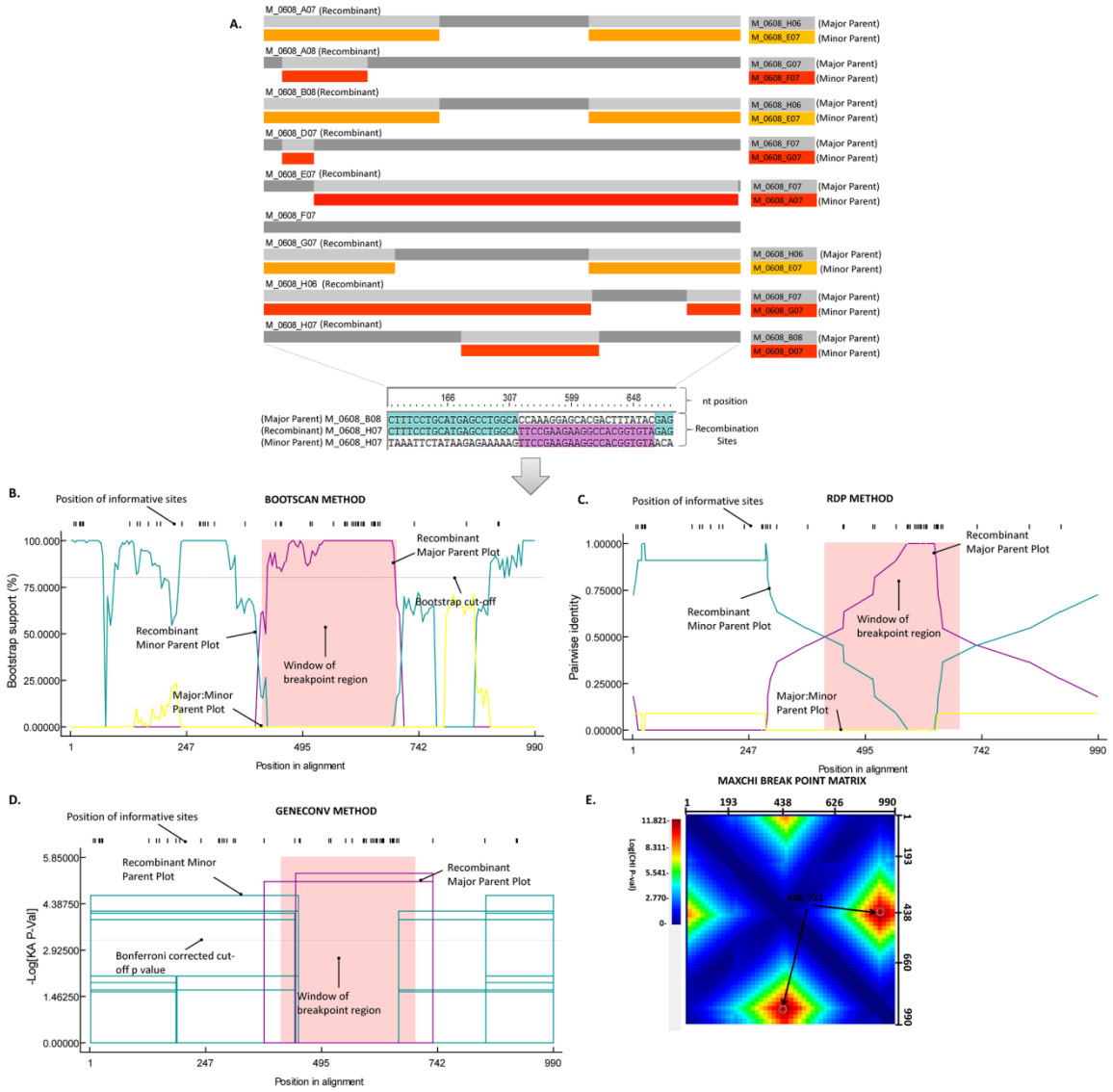


**Figure 3.2. Identification of recombination events in a single patient and general protocol used for identifying recombination in all patient samples using RDP4.**

(A) RDP4 generated recombination schematic indicating evidence of intra-segmental recombination. Regions of recombination events, and corresponding insertions from donor sequences are depicted. Significant sites including nucleotide position(s) involved in recombination are shown for the last recombinant (M_0608_H07). Significant nucleotide positions involved in recombination were checked in a similar fashion for all other recombinants. Recombination events were only considered if at least three recombination analysis methods were in agreement with the obtained results, a *p*-

value of 0.05 or better was obtained, and the consensus recombination score was above the recommended 0.60 value. An example for recombinant M_0608_HO7 showing recombination signals identified by the (B) BOOTSCAN method, (C) RDP method, and (D) GENECONV method are shown. Lastly, the MAXCHI matrices tool when necessary was used to determine the optimal locations of breakpoint pairs, shown in (E). These graphically represent the probabilities of all potential breakpoint pairs that have the best associated *p*-values displayed by the color key beside the matrix. Dark red peaks indicate the most probable positions of breakpoint pairs, for recombinant M_0608_HO7 these were at nucleotide positions 438 and 733.

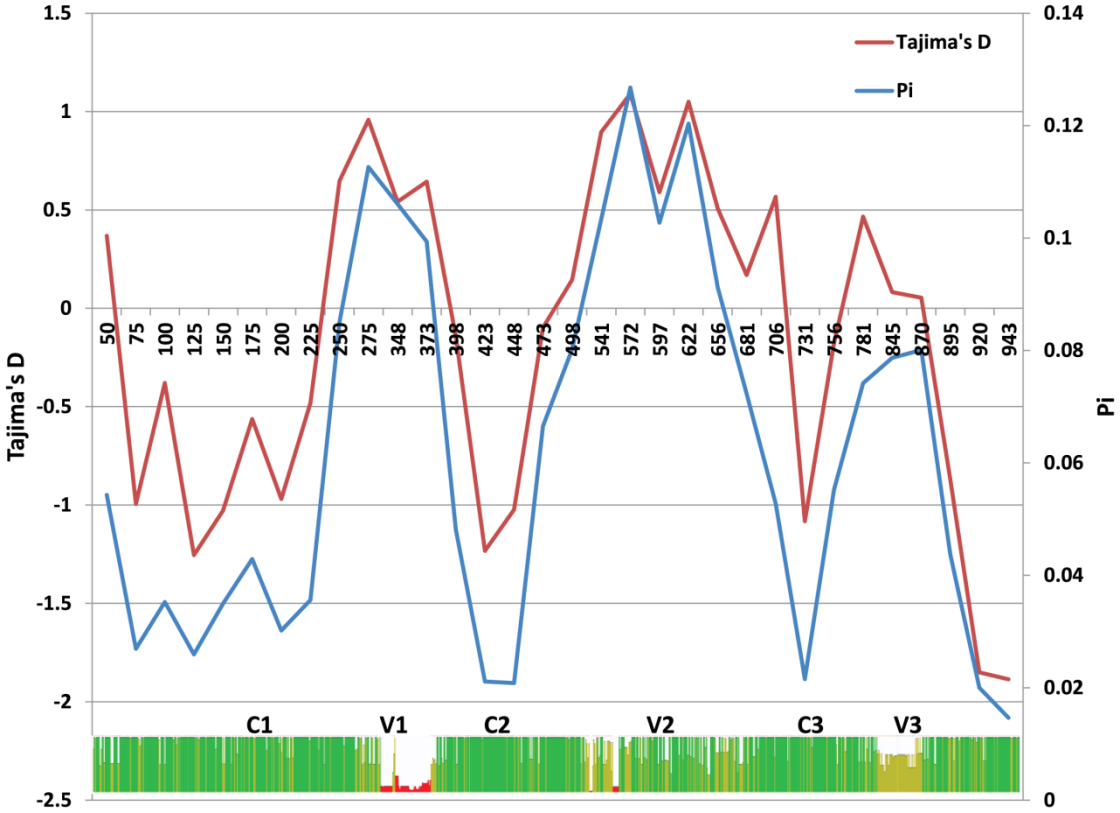


**Figure 3.3. Evidence for selective pressure in regions of the DBL3X domain showing the highest sequence diversity**. Overview of sequence diversity among DBL3X of Primigravidae and Multigravidae. The average pairwise similarity ($\pi$) and Tajima's D for the aligned sequences, over an 870 nucleotide window including Conserved (C1-4) and variable (V1-3) regions are shown.

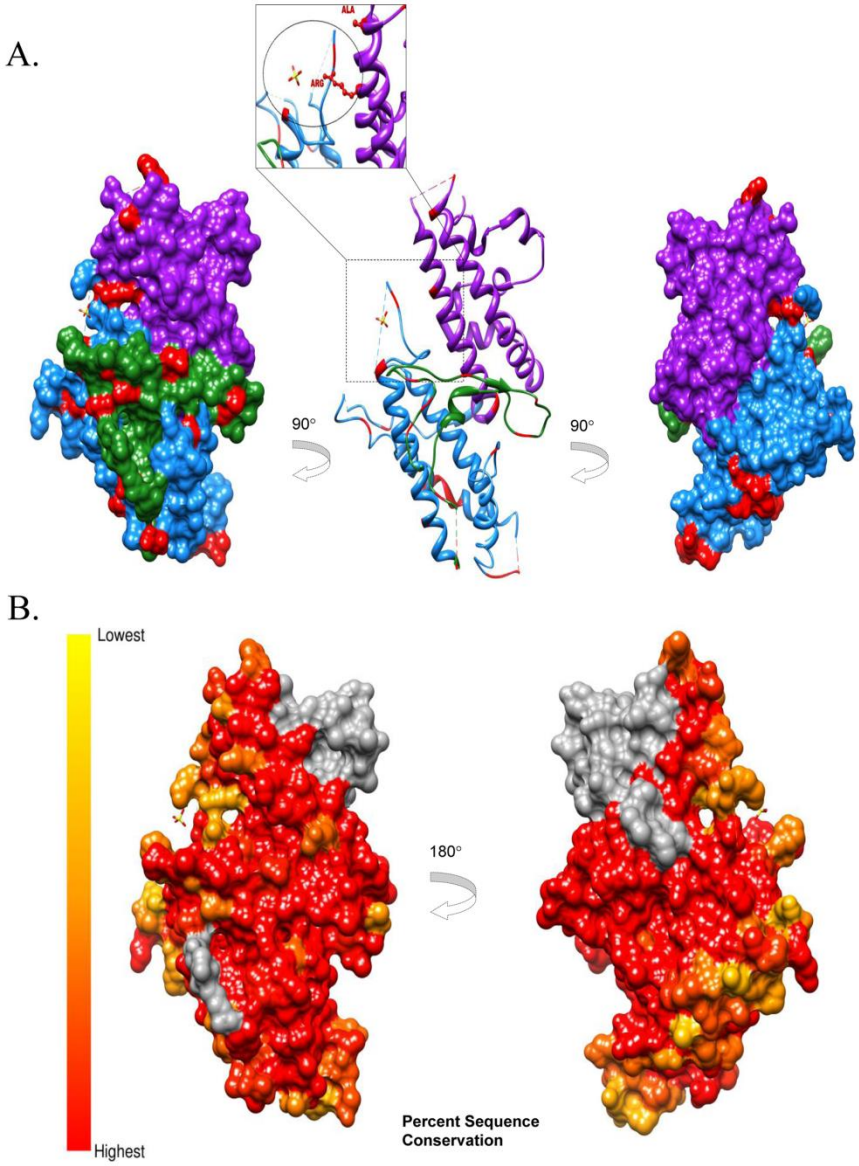


**Figure 3.4 Analysis of Amino Acid Conservation of VAR2CSA DBL3X domain**

Percent sequence conservation from highest 100% (red) to lowest 40% (yellow) based on an alignment of 79 DBL3X sequences isolated from twelve Kenyan placental blood samples is shown on a DBL3X surface based model. Dotted circles indicate the predicted binding pocket region for CSA, shown with an $SO_4$ molecule. Insufficient sequence data for analysis is depicted in grey.

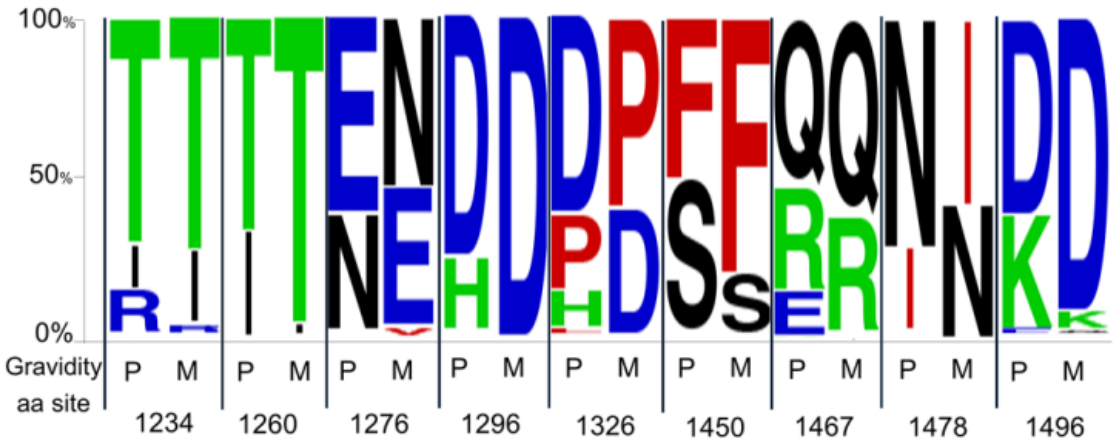


**Figure 3.5 Toggling amino acids with gravidity-associated preferences.** A total of nine sites were identified as varying significant between primigravidae (P) and multigravidae (M), shown here in relative proportion of toggling amino acids at each site (see Supplemental Table 1 for details and other toggling sites). Amino acids are colored according to their chemical properties: polar amino acids (G,S,T,Y,C,Q,N) are green; basic (K,R,H); blue; acidic (D,E), red; and hydrophobic (A,V,L,I,P,W,F,M), black. The chi-square test (or Fisher's exact test when appropriate) was used; all sites, $P < 0.05$.

# CHAPTER 4

# GRAVIDITY ASSOCIATED SELECTION DRIVES ANTIGENIC VARIATION IN PLACENTAL MALARIA

[2]

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**Abstract**

Malaria parasite-infected erythrocytes accumulate within the placenta by expressing a *Plasmodium falciparum* erythrocyte membrane protein-1 (*Pf*EMP1) variant called VAR2CSA. This protein interacts with a placenta-specific, low-sulfated form of chondroitin sulfate A (CSA). Placental malaria (PM) is a severe malaria syndrome that develops as a result of this interaction and has important repercussions for the health of both mother and fetus, with the first and second pregnancies being at highest risk for placental pathology and maternal and fetal morbidity and mortality. While partial immunity against VAR2CSA, and therefore protection against pathogenesis, may be achieved after multiple PM-exposed pregnancies, the importance of VAR2CSA diversity for susceptibility to severe disease remains largely unknown. In this study the genetic complexity of the *var2csa* gene from placental blood samples obtained from women living in a malaria holoendemic region of Kenya was characterized. We confirm that a remarkably high number of unique sequence types are found within both primigravid and multigravid women. However, higher copy numbers of *var2csa* in parasites infecting multigravidae results in reduced clonal index in this group, suggesting that, overall, infections are more diverse in primigravidae. Finally, we report divergent, gravidity-specific sequence patterns in the DBL3x domain of this critically relevant immunogenic target. These results provide compelling evidence that previous exposure to PM induces selection for unique VAR2CSA types in parasites bearing multiple copies of the gene.

**Introduction**

In pregnant women, *Plasmodium falciparum* has evolved the ability to utilize a novel receptor, VAR2CSA, to anchor infected erythrocytes (IE) within the placenta. Placental malaria (PM) is an example of a severe malaria syndrome that can lead to adverse pregnancy complications in association with organ-specific IE sequestration [1]. Of the over 54 million cases of malaria that occur in pregnant women every year, thousands will die or have adverse birth outcomes [2]. Women in their first and second pregnancies are at highest risk for developing PM, which is implicated in 75,000 – 200,000 infant deaths annually [3].

Irrespective of previous malaria exposure or partially acquired immunity, selective accumulation of *Plasmodium falciparum* IE in the intervillous space of the placenta results in low birth weight [4-7] by means of preterm delivery [8], maternal anemia [9,10], and intrauterine growth restriction due to blockage of adequate nutrient/gas exchange between mother and fetus [4,8,11]. Unlike non-pregnant individuals, in whom there is no selective advantage for VAR2CSA-expressing parasites due to lack of low-sulfated placental CSA, pregnant women are uniquely susceptible to PM [12].

PM affects women in a gravidity-dependent manner, whereby primigravid women are more susceptible than multigravidae [13,14]. Importantly, after multiple pregnancies, women develop inhibitory antibodies against VAR2CSA and are able to overcome the severe outcomes of PM [15-18]. Although anti-VAR2CSA antibodies do develop in primigravidae [18,19], their antibody repertoire is not broad enough to confer protection against PM [18].

Recent work suggests that inhibitory antibodies raised against the full-length VAR2CSA antigen target primarily the DBL3X domain [20,21]. Because this domain is one of the most highly conserved DBL domains [17], yet appears to be an important target of immunity, we investigated the extent to which parasites bearing specific genotypes or motifs within this domain are associated with gravidity among women naturally exposed to malaria.

Pyrosequencing and *var2csa* copy number analysis reveal (i) a high number of unique DBL3X sequence types in both primigravid and multigravid women, (ii) selection for parasites with increased *var2csa* copy numbers in multigravid women, and (iii) several sequence types representing a putative loop region near the predicted binding pocket that are strictly gravidity-specific.

**Results**

*High number of unique sequence types detected in both primigravid and multigravid women*

Using a cloning and Sanger sequencing approach, we previously identified a relatively high number of unique sequence types (e.g. eleven) within the DBL3X domain, including regions implicated in immune evasion through switching (or toggling) to alternative amino acids [22]. To overcome the limitation of low sequence coverage/depth from that work, a total of 166,601 sequence reads from 50 patients were obtained, of which 122,850 reads (73%) were used for downstream analysis. The two highly variable (V1-V2) and conserved (C2) region of the DBL3X domain were sequenced, Figure S1. Sequences that were below the expected read length (320bp-420bp), did not match *P. falciparum* BLAST results, contained an incomplete barcode, or lacked forward and reverse primers, were omitted (*Materials and Methods)*. For primigravidae (N = 25) and multigravidae (N = 25) the average number of sequence reads per patient was 2,394 (SD =1475) and 2,520 (SD = 1486), respectively, Table S1 and S2.

To identify unique sequence types within each patient, a *de novo* contig assembly analysis approach was developed (*Materials and Methods)*. A total of 503 contigs, or unique sequence types, were identified. BlastN analysis of these unique sequences revealed 53/503 (11%) to have a perfect match with previously reported *var2csa* sequences. The average variant consensus sequence length was 354 base pairs (range, 320–420 bp, reflecting the presence of indels in the sequenced region). Among 25 primigravid women, an average of thirteen (13) unique sequence types per patient were

detected, ranging from 1 to 33. Interestingly, multigravid women showed similar numbers of variants, ranging between 2 and 30, with an average of eleven (11), Figure 1.

Due to the relatively narrow sequencing region (320- 420 bp), the actual number of unique sequence types per patient may be higher. In our previous work [22], the sequenced region was twice the size (1000 bp) and yielded an average of 10 sequences from each of 12 patient samples.

*Selection for multiple copies of var2csa in multigravid women*

Changes in gene copy numbers can drastically affect the fitness and subsistence of parasites. Gene copy number variability in *Plasmodium falciparum* has been associated with drug resistance [23], loss of cytoadherance by infected erythrocytes [24,25], and alterations to erythrocyte invasion [26]. Changes in gene copy numbers provide a simple way for the parasite to alter its phenotype without requiring a change in sequence. Recently, Sander et al. identified the *var2csa* gene to be duplicated [27], and showed evidence of selection for multiple copies of *var2csa* during the course of a natural infection [28].

Using the $2^{-\Delta\Delta}$ Ct method of relative gene copy number quantitation [29], a clear difference is seen in the Kenyan samples between the primigravid and multigravid populations. In primigravidae, the estimated *var2csa* copy number is 2.93 (SD = 1.97) compared to 4.85 (SD = 2.29) in multigravidae ($p = 0.002$), indicating selection for parasites with higher *var2csa* copy numbers in the latter group, Figure 1.

Using the copy number results, we assessed the potential parasite burden in each patient by estimating the number of parasite clones per patient; defined herein as the clonal index (clonal index = number of unique *var2csa* sequence types divided by *var2csa* gene copy number). Using this approach, primigravid and multigravid women differed, with an average clonal index of 7.24 (SD = 6.21) and 2.68 (SD = 1.68), respectively ($p=0.012$; Figure 1).

These results suggest that in primigravid women, in the context of little to no immune pressure, a higher number of parasite clones with fewer *var2csa* copy numbers can be sustained. In contrast, in multigravidae, who likely have developed broad immune recognition of diverse VAR2CSA types, parasite clones bearing multiple copies of *var2csa* may have an immune evasion advantage via simultaneous usage of several unique VAR2CSA types. Our data along that of Sander et al. [28], support the notion that immune pressure drives selection of parasite clones with multiple copies of *var2csa*.

*Phylogenetic analysis reveals strict gravidity-associated clustering of unique sequence types*

In general, the number of unique sequence types discovered tends to be contingent upon the sampling effort. In this context, as we increase the number of samples tested by repeated sequencing, the number of unique sequence types will increase. This is apparent in our sample set, Figure 2. The accumulation curve does not reach its asymptote, suggesting that repeated sampling will result in the identification of additional variants.

Using the 503 unique sequence types, we constructed a phylogenetic tree by the minimum-evolution (ME) method [30]. Remarkably, all 503 unique sequences clustered strictly based on gravidity (Figure 3), suggesting the presence of gravidity-specific motifs.

To identify selection hotspots within the unique sequences, Tajima's neutrality test was performed. The Tajima's D index is close to 3 in the first 150 bp; that is, departure from neutrality and evidence for positive selection was very high in this sequence region, suggesting immune-driven selection, Figure 4.

Analysis of the consensus alignment of the first 250bp of each sequence revealed distinct clustering on the basis on the presence or absence of indels plus specific sequence motifs, prompting the grouping of sequences into six types, each containing contributions from multiple patients, Figure 5. Five of the six types, representing 56% (283/503) of the unique sequences, were exclusively

found in primigravidae or multigravidae but not both. The remaining 220/503 (44%) sequences were found in both gravidity groups at similar ratios.

*Gravidity-associated sequence differences map to the loop region found next to the predicted binding pocket*

Recent work has provided compelling evidence for the involvement of the DBL3X domain in both CSA binding and inducing protective antibodies [20,21,31]. Toward this end, we asked whether the gravidity-associated sequence types had any functional importance at the protein level. Interestingly, the region of the sequence that exhibited the highest Tajima's D index (e.g., positive selection) mapped directly to the loop region next to the predicted binding pocket for CSA, Figure 6**.** This loop region differed between primigravid and multigravid women on the basis of size and amino acid composition.

**Discussion**

This study is the first to use deep sequencing to describe the diversity of *var2csa* in placental malaria parasites obtained from women naturally exposed to endemic *P. falciparum.* This method is particularly useful since it presents a more accurate measure of parasite diversity and allows insight into minority variants, which would not be identified using traditional Sanger sequencing. The ability to accurately and quantitatively describe the in-host diversity of placental malaria infections is critical for understanding the selective pressures exerted by host immunity on the parasite population and their ability to overcome this pressure. Here we describe, (i) a high number of unique sequence types in both primigravid and multigravid women; (ii) selection for parasites with increased *var2csa* copy number in multigravid women; and (iii) identification of gravidity-associated sequence features that translate to the loop region near the predicted CSA binding pocket in the DBL3X domain.

We identified a total of 503 unique sequence types, of which 56% (283/503) were gravidity associated and 53/503 (11%) had a perfect match to previously reported sequences. However, the rarefaction analysis suggests that this is only a portion of the actual number of unique sequence types in the population and that repeated sequencing would identify additional variants. While there is extensive diversity within *var2csa*, our findings show a gravidity-associated pattern in the DBL3X domain. Given that these mutations are nonsynonymous substitutions, we suggest that it may be a combination of directional and balancing selection. This would maintain diversity while keeping particular epitopes at higher frequency on the basis of survival fitness. The identified loop region next to the predicted binding pocket of the DBL3X domain likely suggests a functional interplay with the host immune system.

While it is well known that multigravid women are able to partially overcome the severe outcomes of PM through inhibitory antibodies [15-18], it is not known whether this leads to selection of specific sequence types in the parasite population. The data presented herein is the first to show clear evidence for gravidity-associated selection at the sequence level.

Interestingly, we were able to identify similar sequence types in our previously published work [22] and that of Dahlback et al. [32]. Unfortunately, in the paper by Dahlback et al. [32], the published sequences had no information on gravidity. The gravidity-associated pattern in the DBL3X domain was likely missed in previous work due to insufficient sequencing depth, and the fact that the phylogenetic signal which produces the gravidity associated grouping seen here is overwhelmed by nonspecific polymorphism in the longer region sequenced.

We suggest that the changes in the size of the loop region and its amino acid composition, which are gravidity-associated, may constitute a novel mechanism of parasite immune evasion, ultimately playing a role in parasite survival. Interestingly, RDP4 recombination analysis [33] of all published full-length *var2csa* sequences reveals the same loop region to be a major recombination

breakpoint (data not shown). Future work will evaluate this hypothesis by testing the different sequence types for gravidity-based antibody recognition.

Our data are consistent with the hypothesis that immune pressure selects for escape mutations in multigravid women that later revert back under selection in a naive, primigravid host. This may be achieved through altering the loop region next to the binding pocket, varying *var2csa* copy numbers, and/or by toggling critical amino acids (previously shown in [22]). Altogether this information suggests that a vaccine against PM is feasible and should target gravidity-specific epitopes (e.g., serological variants). This will prevent vaccine-induced strain selection and increase the efficacy of a potential PM-vaccine.

## Materials and methods

### *Study population*

Samples from 50 adult women (age range, 15-35 years; mean age 22 ± 5.6 years) were collected at either Siaya or Kisumu District Hospital. Informed consent was obtained from potential clients in agreement with Kenya Medical Research Institute Ethical Review Committee, the Institutional Review Boards of the University of Georgia, the Centers for Disease Control and Prevention, and the National Institutes of Health. All participants provided informed, written consent under the auspices of these approved protocols. Immediately post-delivery, placentas were collected and maternal placental blood obtained either by the prick method [34] or perfusion [35] under aseptic conditions. Placental parasitemia was calculated by counting the number of parasites on a Giemsa-stained thick smear of maternal placental prick blood and is reported as parasitemia per 300 white blood cells. Each of the collected samples is well defined by clinical data. Samples for this study were collected over a time span of 1.5 years.

*Estimation of var2csa copy number per parasite genome using the $2^{-\Delta\Delta}$ Ct method of relative quantitation*

DNA was extracted from placental blood samples using the QIAamp DNA Blood Kit (Cat.no 51104). The $2^{-\Delta\Delta}$ Ct method of relative quantitation [29], was adapted to estimate the mean *var2csa* gene copy number per *P. falciparum* genome in placental blood samples. A complete description of primers used has been published previously [28], and the method was slightly modified. Briefly, the copy number of *var2csa* was determined by normalizing it against a house-keeping gene that has a constant copy number. The *fructose-bisphosphate aldolase* (PF14_0425_v5.5, PlasmoDB) was used as the house-keeping gene. A FCR3 and 3D7 clone with 1 copy of both the *var2csa* and *aldolase* gene was used for calibrating the parasite genome for each qPCR run. The following formulas were used to estimate *var2csa* copy number:

$$(1)\ \Delta\Delta Ct = (Ct\ var2csa – Ct\ aldolase)\chi – (Ct\ var2csa – Ct\ aldolase)y$$

where $\chi$ = unknown sample and $y$ = FCR3/3D7, and

$$(2)\ 2^{-\Delta\Delta Ct}$$

such that *var2csa* copy number is expressed as N-fold changes compared to *aldolase* gene copies. Each sample was run in quadruplicate in 3 separate experiments. The *var2csa* copy number was expressed as the mean between the 3 experiments. Samples with a Ct value >30 and a coefficient of variation >1% between the samples were omitted.

*Amplification and sequencing of var2csa DBL3X domain*

The variable region of the DBL3X domain of *var2csa* was amplified from extracted DNA by using previously described primers [22]. The primers were modified for 454 sequencing by including a multiplex identifier (MID), linker, and tag sequence. Each patient sample was amplified in triplicate using the modified primers. Amplicons were pooled and sequenced using the 454 GS Junior at the

Georgia Genomics Facility at The University of Georgia. Further details are provided in the Supplementary Materials (Figures S1 and S2).

*Data quality filtering*

A total of 166,601 sequence reads were obtained. All sequences were BLAST searched for *P. falciparum* and non-hits were discarded. Remaining sequences were separated by unique (multiplex identifiers) MIDs from the pooled data into patient-specific sequences. For each patient data set, sequences were trimmed of MIDs, tags, and primers. Low-quality reads were removed on the basis of a defined cut-off (e.g., length and quality scores). The length cut-off was determined based on all published DBL3X sequences at the time, and sequences below 320bp or above 420bp were removed. Additionally, sequences with a quality score of less than 80 were discarded. Two separate and independent approaches were used for quality filtering: (1) Geneious v6.2 [36] and (2) a modified QIIME script [37]; both approaches yielded identical results.

*Data analysis and unique sequence types*

For each patient sample, a mean of 2457 (SD = 1480) amplicons were obtained. A *de novo* contig assembly approach was used to identify unique sequence types using Geneious v6.2 [36] and CAP3 [38] independently. Each method yielded the same number of unique contigs. For this analysis, each contig is defined as the number of unique sequence types. Briefly, for each patient, generation of contigs (eg, unique sequence types) was obtained through the computation of overlaps between reads, removal of false overlaps, and construction of multiple sequence alignments. A minimum/maximum overlap of 320bp-420bp and identity of 98% was selected to account for the expected read length and the intrinsic 454 sequencing error of 1%. We obtained between 5 and 2540 (Median = 351) reads per patient per contig. Contigs with less than 5 reads were omitted. To validate this approach in determining unique sequence types, a 3D7 clonal linage was included as a

control in each 454 run, which always yielded a single contig (e.g., one unique sequence type). Additionally, we previously showed that patient 0895 is a single clone by Sanger Sequencing [22], which served as a second internal control. Patient 0895 along with the lab adapted 3D7 clonal strain were included in each 454 run and in all cases yielded one contig (e.g., one unique sequence type). Representative consensus sequences for each unique contig were used to obtain a DNA alignment and construct a phylogentic tree using the minimum evolution (ME) method [30]. Sequence alignments and phylogentic tree analyses were performed in Geneious v6.2 [36] and MEGA5 [39], respectively. The final tree was visualized using FigTree [40]. Diversity and rarefaction curves were determined using EstimatesS v9 [41]. Additional information about the data and unique sequence types is provided in the Supplementary Materials.

*3D structural modeling of the loop region*

The protein structure of the DBL3X domain (Protein Data Bank: 3CPZ [42]) was used as a template for structure modeling. The gravidity associated loop regions of the DBL3X domain were generated using MODELLER [43] and superimposed via the UCSF molecular analysis program [44].

*Statistical analysis and graph generation*

All statistical analysis and graph generation was performed in R [45].

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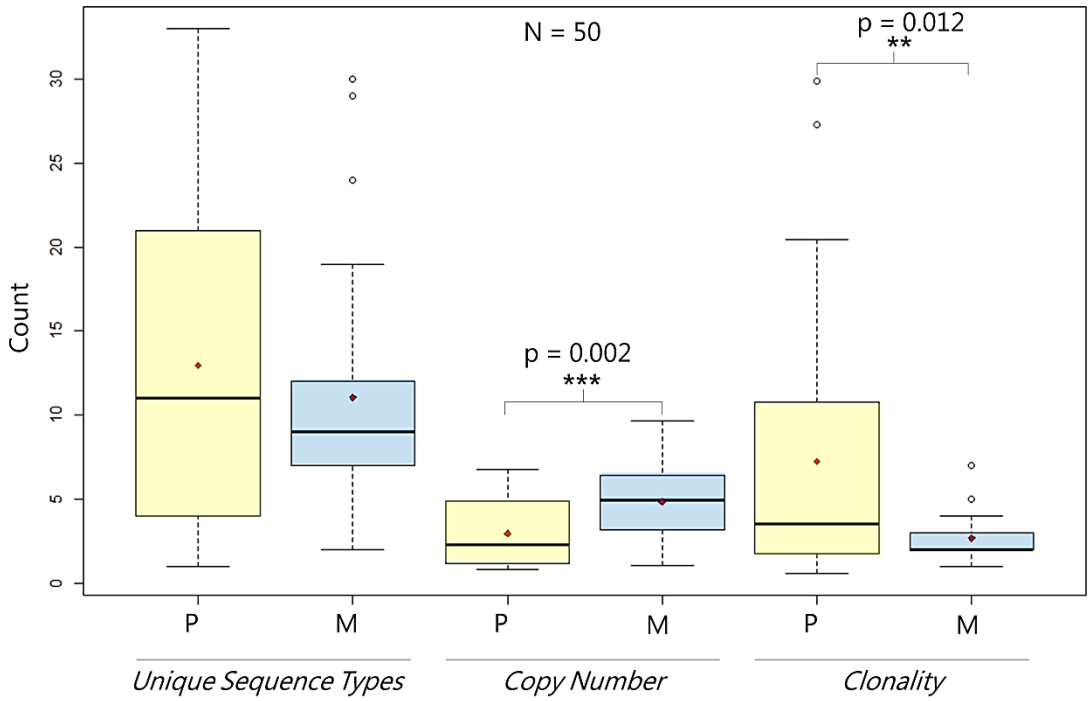


**Figure 4.1. Comparison of clonality, *var2csa* copy number variation, and unique sequence types between primigravid (P) and multigravid (M) women living in endemic Kenya.** The data show selection for multiple copies of *var2csa* (mean = 4.84) in multigravidae (M) as compared to primigravidae (P) (mean = 2.90, p = 0.002). The estimated clonal index is higher in primigravidae as compared to multigravidae, with means of 7.24 and 2.59, respectively (p = 0.012). No statistical difference was observed in the number of unique sequence types present in each group. Bars indicate the 25$^{th}$ and 75$^{th}$ percentiles, mean is shown by red diamond dots, and median is shown by black bold lines in the middle of the box plots.

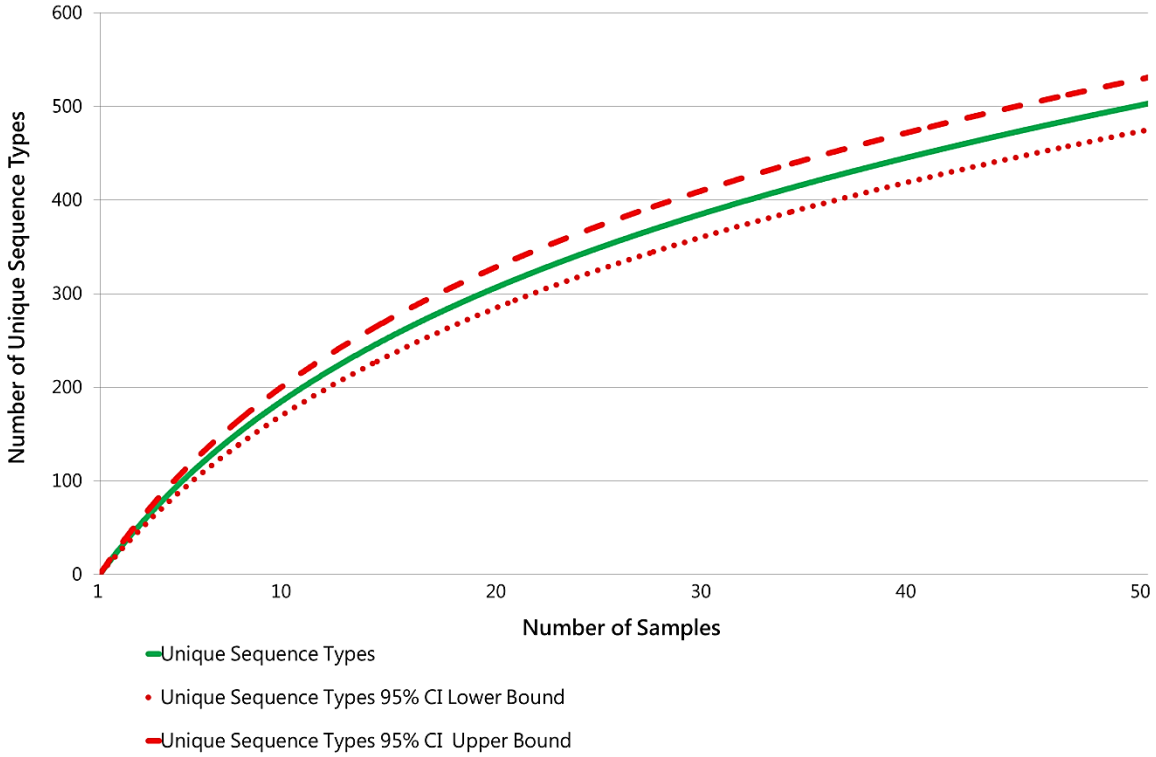


**Figure 4.2. Rarefaction curve of *var2csa* unique sequence types for entire study population (N =50).** The computed refraction curve (in solid green) shows the expected average rate of unique sequence types that would be produced as a result of repeated deep sequencing. Upper and lower 95% confidence intervals (CI) for species richness are shown by dashed and dotted red lines, respectively.

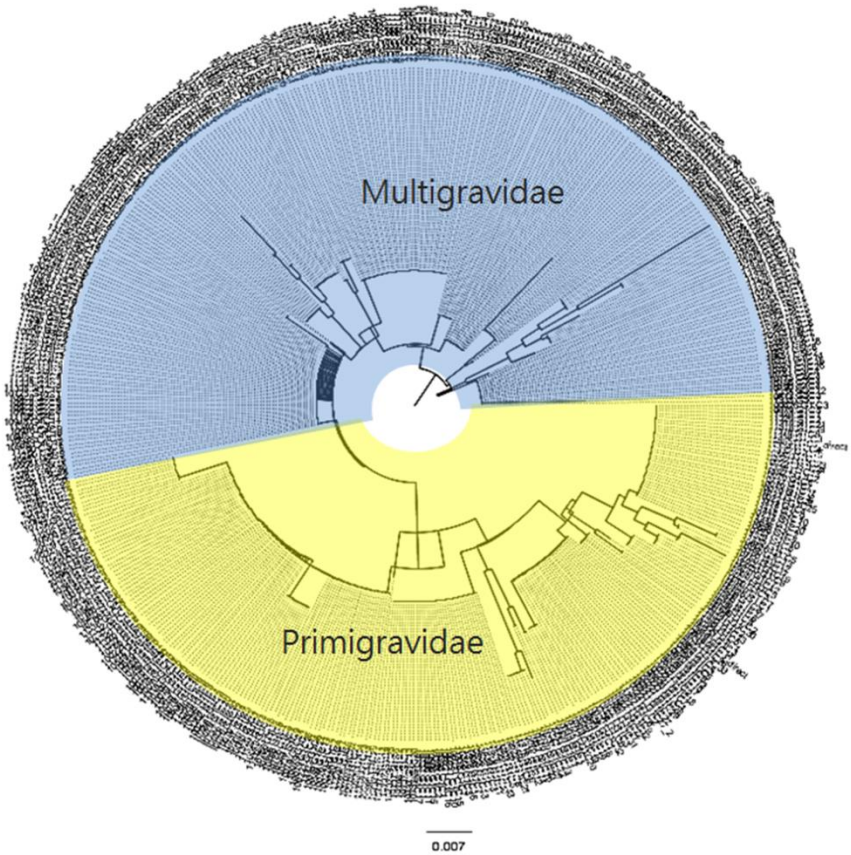


**Figure 4.3. Phylogenetic analysis reveals strict gravidity-associated clustering of unique sequence types.**

Phylogenetic tree of 503 unique sequence types was constructed using the minimum evolution (ME) method [30]. Full-length sequences were used (320bp-420bp). The confidence probability was calculated using the interior branch method with a bootstrap test of 10,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method [46]. 95% of all indels/gaps were allowed at any position. Evolutionary analysis was performed in MEGA5 [39] and the final tree visualized using FigTree [40]. The tree shown has a bootstrap cut-off value of 50.

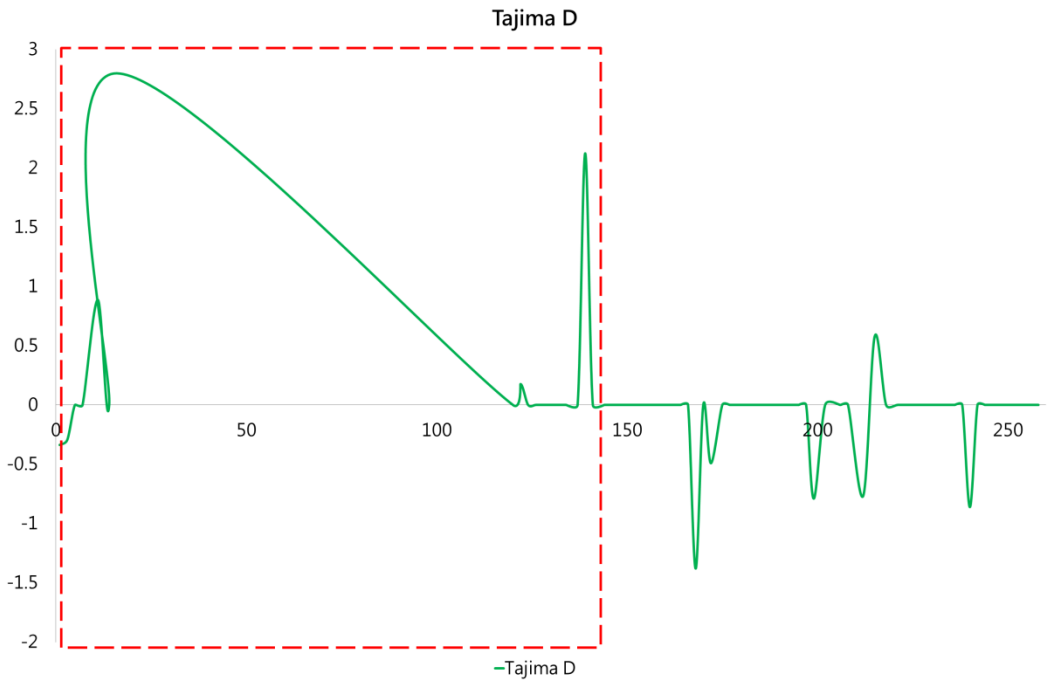


**Figure 4.4. Evidence of strong selective pressure in the beginning sequence region of the 503 unique sequence types.**

Results of Tajima D over the first 250 nucleotides of the unique sequence types identified using a sliding window of 10 base-pairs.

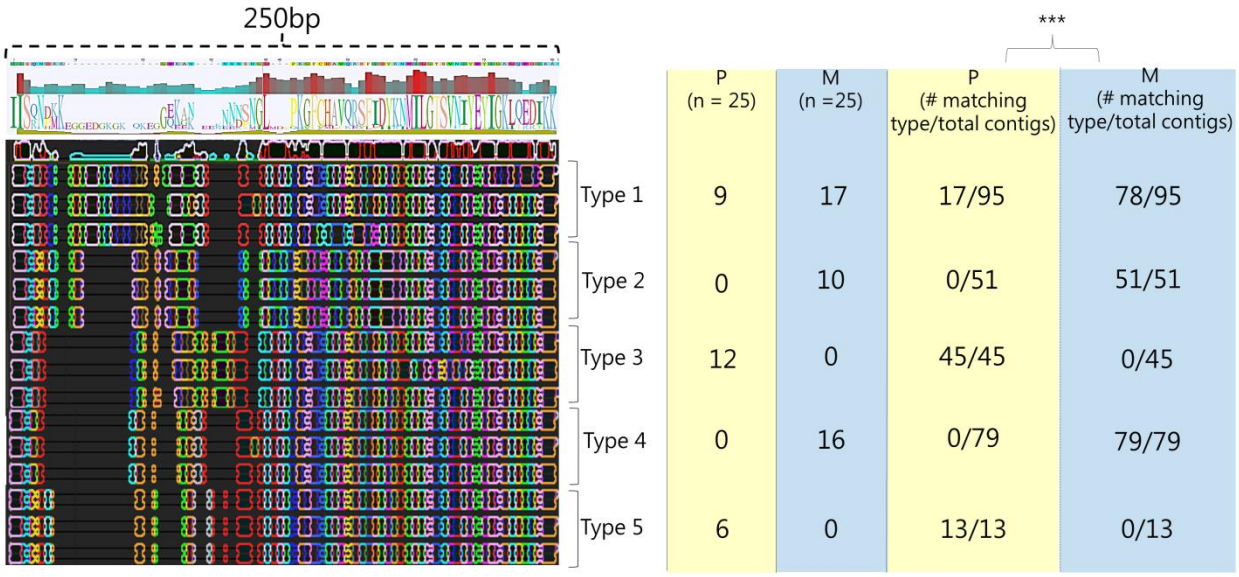


**Figure 4.5. Consensus alignment of unique sequences reveals clustering into sequence types and strict gravidity association.**

Alignment is condensed in order to show the representative sequence types and clear differences on the basis of the presence or absence of indels plus specific sequence motifs. Each gravidity-associated type differed from another type by more than 6 nucleotides or 2 amino acids, with the exception of type 2, which was unique at more than 8 amino acid positions. Each type has an average of 536 reads. P = Primigravidae and M = Multigravidae. *** = < 0.001.

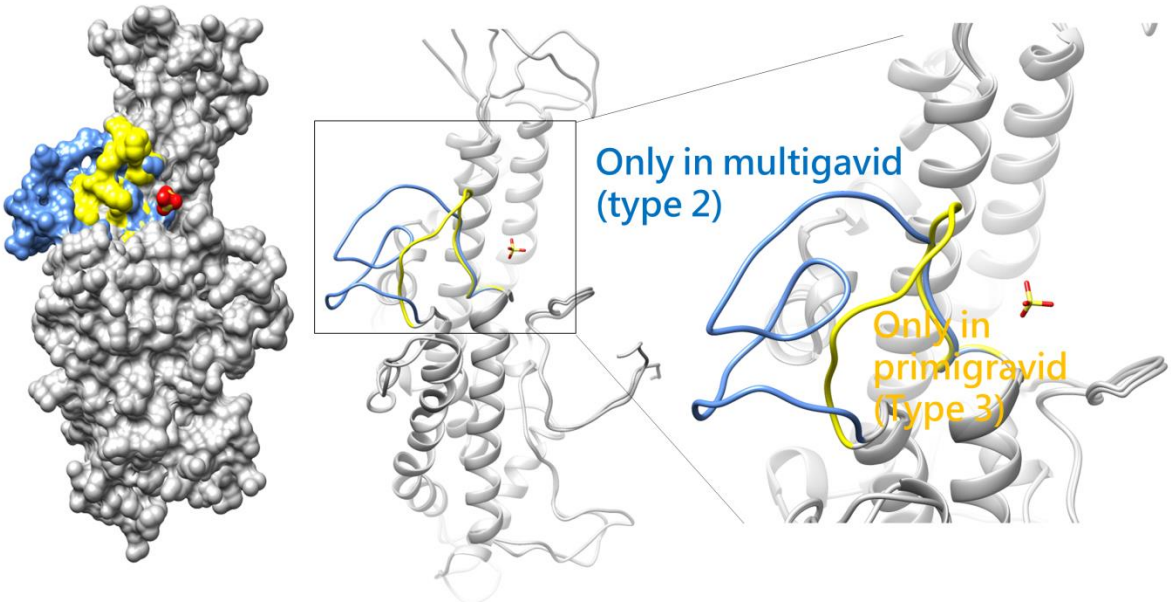


**Figure 4.6. Gravidity-associated sequence differences map to the loop region found next to the predicted binding pocket.**

Multigravid loop region is shown in blue and primigravid in yellow. The sulfate ion ($SO_4$) represents the predicted binding pocket. Sequence types 1 and 3 are shown for comparison reasons. Changes in both the size of the loop region and its amino acid composition are seen between primigravid and multigravid women.

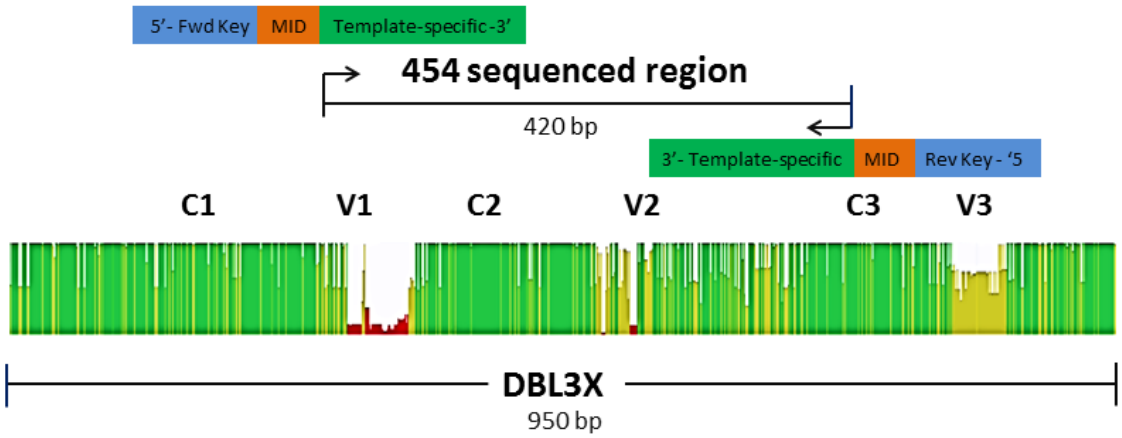


**Figure 4.7. DBL3X region pyrosequenced.** The two highly variable (V1-V2) and conserved region (C2) of the DBL3X domain were sequenced. The blue key identifies the GS FLX Titanium forward and reverse primer, yellow the Multiplex Identifier (MID) tag, and green the template-specific region.

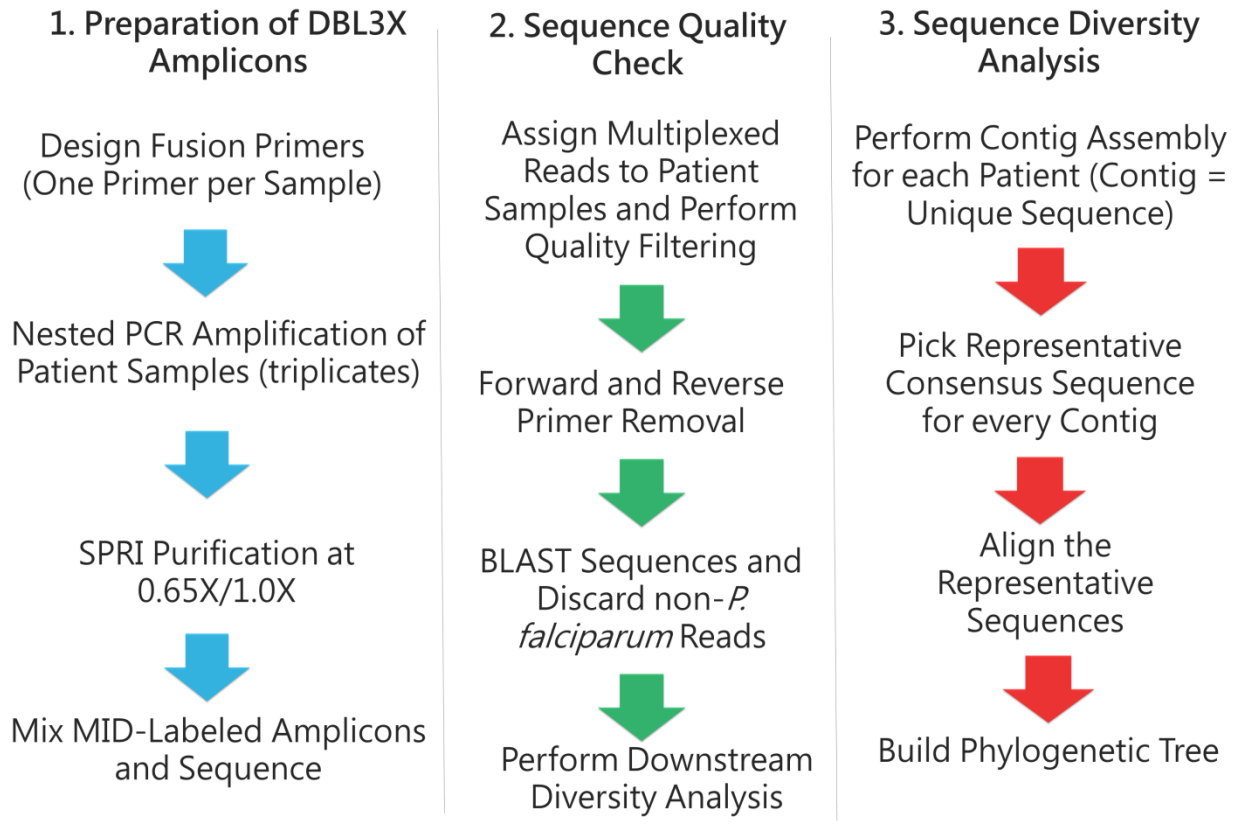


**Figure 4.8. Schematic overview of the overall sequencing process**

**Table 4.1. Summary of data obtained for primigravid women.** Total number of sequences obtained for each 454 run is shown, including sequences used for downstream analysis (AnalysisReads). Clonal index = unique *var2csa* sequences / *var2csa* copy number. Please refer to Materials and Methods section for detailed information on procedures about sequence clean-up and quality assessment.

| Gravidity | Patient | Unique Seq | Copy # | Clonal Index | Reads | Qreads | Analysis Reads | Unused Qreads | Unused Reads |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 10049 | 2 | 1.01 | 2 | 3158 | 3142 | 3129 | 13 | 16 |
| 1 | 10079 | 32 | 1.07 | 30 | 5113 | 5103 | 4391 | 712 | 10 |
| 1 | 10221 | 33 | 1.21 | 27 | 3662 | 3616 | 3296 | 320 | 46 |
| 1 | 10224 | 4 | 2.85 | 1 | 5946 | 5930 | 5884 | 46 | 16 |
| 1 | 10327 | 23 | 2.13 | 11 | 4777 | 4764 | 4541 | 223 | 13 |
| 1 | 10340 | 1 | 4.14 | 1 | 2503 | 2441 | 2426 | 15 | 62 |
| 1 | 10398 | 5 | 2.91 | 2 | 1975 | 1961 | 1894 | 67 | 14 |
| 1 | 10450 | 7 | 6.76 | 1 | 2298 | 2235 | 2206 | 29 | 63 |
| 1 | 10460 | 3 | 5.1 | 1 | 2443 | 2421 | 2398 | 23 | 22 |
| 1 | 10465 | 1 | 6.68 | 1 | 2330 | 2319 | 2303 | 16 | 11 |
| 1 | 10506 | 17 | 4.86 | 3 | 4326 | 4318 | 4125 | 193 | 8 |
| 1 | 10549 | 23 | 1.91 | 12 | 1354 | 1173 | 848 | 325 | 181 |
| 1 | 10608 | 22 | 5.08 | 4 | 3419 | 1861 | 1514 | 347 | 1558 |
| 1 | 10626 | 7 | 2.01 | 3 | 2134 | 1472 | 1298 | 174 | 662 |
| 1 | 10696 | 19 | 5.39 | 4 | 1837 | 1642 | 1419 | 223 | 195 |
| 1 | 10737 | 28 | 2.95 | 9 | 5449 | 5433 | 5206 | 227 | 16 |
| 1 | 10786 | 13 | 1.14 | 11 | 1443 | 1434 | 980 | 454 | 9 |
| 1 | 10858 | 4 | 2.30 | 2 | 8311 | 956 | 940 | 16 | 7355 |
| 1 | 10895 | 1 | 1.10 | 1 | 2538 | 2091 | 2071 | 20 | 447 |
| 1 | 11202 | 6 | 1.27 | 5 | 1037 | 1027 | 983 | 44 | 10 |
| 1 | 11318 | 21 | 6.10 | 3 | 1249 | 1237 | 936 | 301 | 12 |
| 1 | 11320 | 18 | 1.06 | 17 | 8137 | 1922 | 1758 | 164 | 6215 |
| 1 | 11332 | 11 | 2.80 | 4 | 2225 | 588 | 472 | 116 | 1637 |
| 1 | 11354 | 19 | 0.93 | 20 | 4043 | 1511 | 1403 | 108 | 2532 |
| 1 | 11434 | 4 | 1.00 | 4 | 1956 | 1904 | 1844 | 60 | 52 |
| | Average: | **13** | **2.95** | **7** | **3347** | **2500** | **2331** | **169** | **846** |
| | Median: | 11 | 2 | 4 | 2503 | 1961 | 1894 | 116 | 46 |
| | | | | **Sum** | **83663** | **62501** | **58268** | **4238** | **20892** |

**Table 4.2. Summary of data obtained for primigravid women.** Total number of sequences obtained for each 454 run is shown, including sequences used for downstream analysis (AnalysisReads). Clonal index = unique *var2csa* sequences / *var2csa* copy number. Please refer to Materials and Methods section for detailed information on procedures about sequence clean-up and quality assessment.

| Gravidity | Patient | Unique Seq | Copy # | Clonal Index | Reads | Qreads | Analysis Reads | Unused Qreads | Unused Reads |
|---|---|---|---|---|---|---|---|---|---|
| 10 | 10125 | 4 | 1.43 | 3 | 3155 | 3137 | 3101 | 36 | 18 |
| 4 | 10324 | 19 | 6.68 | 3 | 1541 | 1524 | 773 | 751 | 17 |
| 4 | 10353 | 11 | 2.20 | 5 | 5639 | 5625 | 5547 | 78 | 14 |
| 3 | 10380 | 7 | 1.02 | 7 | 2815 | 2784 | 2771 | 13 | 31 |
| 8 | 10473 | 14 | 5.54 | 3 | 2342 | 2306 | 2270 | 36 | 36 |
| 3 | 10486 | 7 | 3.71 | 2 | 5445 | 5360 | 5242 | 118 | 85 |
| 3 | 10490 | 7 | 3.18 | 2 | 4550 | 4535 | 4477 | 58 | 15 |
| 3 | 10521 | 10 | 7.21 | 1 | 2331 | 2322 | 2248 | 74 | 9 |
| 4 | 10551 | 24 | 5.98 | 4 | 2478 | 1064 | 873 | 191 | 1414 |
| 3 | 10580 | 30 | 9.63 | 3 | 4280 | 4037 | 3799 | 238 | 243 |
| 3 | 10612 | 3 | 4.23 | 1 | 3143 | 2853 | 2826 | 27 | 290 |
| 9 | 10694 | 10 | 4.95 | 2 | 2039 | 1354 | 1282 | 72 | 685 |
| 4 | 10745 | 9 | 2.45 | 4 | 5355 | 5339 | 5259 | 80 | 16 |
| 2 | 10781 | 29 | 4.14 | 7 | 3413 | 3392 | 3139 | 253 | 21 |
| 5 | 10899 | 2 | 2.20 | 1 | 2256 | 2193 | 2182 | 11 | 63 |
| 4 | 10948 | 11 | 6.92 | 2 | 4280 | 4270 | 4224 | 46 | 10 |
| 4 | 11186 | 9 | 8.40 | 1 | 1608 | 1528 | 1477 | 51 | 80 |
| 3 | 11290 | 12 | 5.62 | 2 | 1291 | 1274 | 1078 | 196 | 17 |
| 3 | 11295 | 7 | 4.55 | 2 | 4289 | 1866 | 1749 | 117 | 2423 |
| 3 | 11322 | 7 | 7.41 | 1 | 1584 | 1529 | 1421 | 108 | 55 |
| 3 | 11331 | 4 | 1.28 | 3 | 2028 | 1982 | 1960 | 22 | 46 |
| 3 | 11335 | 15 | 6.40 | 2 | 1555 | 1516 | 1103 | 413 | 39 |
| 4 | 11336 | 5 | 6.38 | 1 | 4289 | 1747 | 1541 | 206 | 2542 |
| 3 | 11340 | 8 | 4.91 | 2 | 2254 | 2242 | 2061 | 181 | 12 |
| 3 | 11416 | 12 | 4.65 | 3 | 2393 | 875 | 588 | 287 | 1518 |
| | Average: | **11** | **4.84** | **3** | **3054** | **2666** | **2520** | **147** | **388** |
| | Median: | 9 | 5 | 2 | 2478 | 2242 | 2182 | 80 | 39 |
| | | | | **Sum** | **76353** | **66654** | **62991** | **3663** | **9699** |

# CHAPTER 5

# HIGH THROUGHPUT DETECTION OF SPECIES-SPECIFIC MALARIA INCLUDING SUBMICROSCOPIC INFECTIONS USING A MULTIPLEX REAL-TIME PHOTO-INDUCED ELECTRON TRANSFER (PET) PCR

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*Submitted* for internal review at the CDC

**Abstract**

Accurate diagnosis of malaria infection(s) remains challenging, especially in the identification of submicroscopic infections due to low parasite densities. New diagnostic tools that are inexpensive, sensitive enough to detect submicroscopic malaria infections, and suitable in a developing world laboratory setting are required for malaria control and elimination programs. In the present study, the contribution of a recently developed real-time photo-induced electron transfer (PET) PCR assay was investigated. We aimed to (i) test the use of this assay as a high-throughput screening method for both *P.falciparum* and *Plasmodium* species infections in a developing world laboratory setting, and (ii) determine the sensitivity and specificity of the assay in detecting submicroscopic infections in children and pregnant women. For the 1021 samples tested, we show that the multiplex PET-PCR assay has a 100% sensitivity ($CI_{0.95}$ = 100%) and 100% specificity ($CI_{0.95}$ = 100%) in the general population. Furthermore, the multiplex PET PCR assay is able to detect submicroscopic malaria infections at a sensitivity of 92.25% ($CI_{0.95}$ =96.69%) and specificity of 97.83% ($CI_{0.95}$ = 99.42%), with a positive predictive value of 96.76%, in children and pregnant women treated with artemesinin combination therapy (ACT) and sulfadoxine pyrimethamine (SP). Due to the self-quenching nature of the primers, the PET PCR assay is inexpensive and suitable as a high-throughput screening method for malaria, including sub-microscopic infections, in a developing world laboratory setting.

**Introduction**

Malaria is caused by protozoan parasites of the genus *Plasmodium* that infect humans through the bites of an infected female mosquito. *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* are the main species of malaria parasites, with the first two species causing most infections. Although progress has been made toward controlling malaria world-wide, it continues to be a major public health problem [2,3]. The most recent report by the World Health Organization (WHO) estimates that 216 million cases and 655,000 deaths occurred due to malaria in 2011[3]. This report indicates that 106 countries are malaria endemic and up to one-half of the worldwide population is at risk for infection, with the African region accounting for 81% of malaria cases and 91% of malaria-related deaths [3].

Global malaria elimination and control programs rely currently greatly on two diagnostic tools: immunochromatographic-antigen based rapid diagnostic tests (RDTs) and giemsa microscopy, with RDTs first pioneered in the 1980s and giemsa microscopy in the late 18$^{th}$ century. There is an urgent need for newer, more effective, practical, and inexpensive diagnostics if malaria control and elimination is to succeed—especially in light of the recent emergence of drug resistance to artemesinin-combination therapy (ACT). Microscopy is laborious and time consuming; moreover, diagnosis of sub-clinical infections is very challenging. Furthermore, there is a two to three-fold discrepancy in parasite quantification between technicians [4]. Yet microscopy is still the most widely used method, with 165 million smears performed during 2010 [3], and remains the gold standard for malaria diagnosis. The use of RDTs in the field seems effective, but new data show that caution needs to be taken with the positive predictive values of RDTs [5]. Furthermore, the test falls below the desired 95% sensitivity/specificity target in children under the age of five [5]. Overall, RDTs appear highly valuable in the management of febrile illness in remote regions [6,7] ; however,

some reports from remote malaria-endemic regions indicate a variation in sensitivity of RDTs [8,9]. At this time, RDTs must be used in combination with other methods to confirm the results and characterize the type of infection.

Accurate diagnosis followed by treatment is presently the main malaria control strategy besides preventive measures. A sensitive, high-throughput screening and diagnosis method for malaria infections is therefore critical for control and elimination programs. Today, with the advantage of molecular tools, we understand that successful malaria elimination will depend on our ability to identify persistent reservoir infections. The duration of a single infection in a naturally infected child can last up to nearly 800 days [10], as the parasite is able to persist by clonal antigenic variation in the context of sub-clinical infection. Consequently, accurate diagnostics must be able to identify sub-microscopic infections in order to verify lack of infection in high risk population(s).

Advancements in molecular technologies and methods such as real-time PCR provide a continually evolving system for high-throughput screening and diagnosis [11-13]. The present study aimed to test a recently developed photo-induced electron transfer (PET) multiplex real-time PCR assay to detect *P. falciparum* and *Plasmodium spp.* infections. We assessed (i) the use of PET-PCR as a high-throughput screening method for malaria infections in a developing country laboratory setting and (ii) its efficiency in detecting submicroscopic infections in populations that are at higher risk for contracting malaria: children and pregnant women.

## Results

### *Study Population*

A total of 1021 clinical samples were collected from three separate populations and tested for both *P. falciparum* and *Plasmodium spp.* infections using the multiplex PET PCR assay. The first

population consists of 303 samples collected from eight district hospitals of the provinces Dodoma and Iringa, Tanzania. The second population, which consists of a total of 622 clinical samples from a study conducted in the Miono District in Bagamoyo, Tanzania, includes children under the age of 5 years who were microscopy positive for malaria on day 0, consequently treated with Artemether /Lumefantrine (Coartem), and followed on days 3, 7, and 42. Both populations are shown in Figure 5.1 on the 2010 map of spatial distribution of *Plasmodium falciparum* malaria endemicity and entomological inoculation rate (EIR) [1]. The third population consists of 96 microscopy negative placental samples from Kisumu and Siaya, Kenya. A summary of each population, region(s), time during which the samples were collected, malaria prevalence, and EIR are shown in Table 5.2.

*Assessment of PET PCR as a screening method for species specific malaria at a local laboratory in Tanzania*

A total of 303 clinical samples were randomly and blindly selected from eight district regions in the province of Dodoma and Iringa in Tanzania (Figure 5.1). All samples were screened for malaria infection(s) at the Bagamoyo Health Institute in Tanzania using the multiplex PET-PCR assay. A total of 27 (8.9%) were found to be positive for *P.falciparum* infection. All samples were positive by both the *P.faciparum* and *P. spp* primers. Of the eight district hospitals, Tosamaganga, Mlowa, and Idodi accounted for 21 out of the 27 infections, a region with a *P.falciparum* entomological inoculation rate (*Pf*EIR) of < 1 and *P.falciparum* prevalence (*Pf*PREV) of < 5%. A summary of the total number of positive *P.falciparum* samples by district region is shown in Table 5.3. To determine the sensitivity and specificity of the multiplex PET PCR assay, a set of 117 samples (~15 from each district) were randomly selected including the 27 positive samples and tested against nested 18s rRNA PCR method [14]. Using the nested 18s rRNA PCR method as the gold

standard, we observed 100% sensitivity ($CI_{0.95}$ = 100%) and 100% specificity ($CI_{0.95}$ = 100%) with both the *P.falciparum* and *Plasmodium spp.* primers (Table 5.4).

*Evaluation of PET PCR to detect submicroscopic malaria infection in children under the age of 5 years post artemesinin-combination therapy (ACT)*

To investigate whether PET PCR could be used to detect submicroscopic malaria infections, we tested a total of 622 samples from children under the age of 5 years, who were treated for malaria on day(s) 0 with Artemether /Lumefantrine (Coartem), and followed on days 3, 7, and 42. All samples were microscopy positive for malaria infection on day 0 and microscopy negative for day(s) 3, 7, and 42 (shown in Figure 5.1). Of the 622 clinical samples tested, 128 samples were from day 0, 203 samples from day 3, 176 samples from day 7, and 115 samples from day 42. Among the 128 samples from day 0, 100% (128/128) and 99.2% (127/128) were positive for *P.falciparum* by nested 18s rRNA PCR and PET PCR, respectively. On day 3, 43.35% (88/203) were positive by nested 18s rRNA PCR, compared to 29.06% (59/203) by PET PCR. By day 7, 18.75% (33/176) and 11.93% (21/176) were positive by nested 18s rRNA PCR and PET PCR, respectively. Lastly, on day 42, 5.22% (6/115) were positive by nested 18s rRNA PCR and 4.35% (5/115) by PET PCR. A summary of the results is presented in Figure 5.2.

In contrast to microscopy, both the nested 18s rRNA PCR and PET PCR assay detected *P.falciparum* infections up to day 42. Detection by nested PCR was more sensitive compared to PET PCR on day 3, $p = 0.028$ (Figure 5.2). However, we assumed that on day 3, both assays were likely detecting high numbers of dead parasite DNA still present in the host's blood circulation. The ACT treatment regimen ended on day 2 for all patients. We considered patients to be truly sub-microscopic (i.e. have a persistent parasite population) for malaria infection beginning with day 7.

Toward this end, there was no statistical difference for the detection of sub-microscopic infections between nested 18s rRNA PCR and PET-PCR, $p = 0.350$ (day 7) and $p = 0.973$ (day 42) (Figure 5.2). The artemesinin-combination therapy (ACT) cleared the malaria infection on day 3 by 70.94%, 81.25% by day 7, and 95.65% by day 42, as detected by PET PCR. Using the nested PCR method as a gold standard, the sensitivity and specificity of PET PCR was calculated to be 92.20% ($CI_{0.95}$ = 95.59 %) and 97.42% ($CI_{0.95}$ = 98.94%), respectively, with a positive predictive value of 98.60% ($CI_{0.95}$ = 99.63%), shown in Table 5.5. All infections were positive by both *P.falciparum* and *Plasmodium spp.* primers.

*Evaluation of PET PCR to detect submicroscopic malaria infection(s) in pregnant women*

The efficacy of the PET PCR assay in detecting submicroscopic infections was further validated with 96 placental malaria samples from western Kenya. Among the 96 patients included in the study, the median gravidity and age were 3 (interquartile range [IQR], 1-9) and 25 years (IQR, 15-40), respectively. Seventy five out of the ninety six (77.08%) reported using sulfadoxine pyrimethamine (SP) treatment at some point during the course of their pregnancy.
Both thin and thick smears of maternal placental blood were malaria negative for all patients. Furthermore, no adverse maternal or fetal outcomes were observed.

We recently showed [15] the use of a multiplex single step-PCR method to be as sensitive as the two-step nested 18S rRNA PCR assay in the detection of malaria. To further validate this method in a group at high risk for malaria infection, we tested the 96 microscopy negative samples using the multiplex single step-PCR and PET PCR method(s). A total of 39/96 (40.62%) were positive for *P.falciparum* infection by the multiplex single step-PCR and 36/96 (37.50%) by PET PCR. Using the multiplex single step-PCR method as a gold standard for PET PCR, both the

*P.falciparum* and *P. spp.* primers showed a 92.30% ($CI_{0.95}$ = 97.99%) sensitivity and 98.24% ($CI_{0.95}$ = 99.90%) specificity, with a positive predictive value of 97.29% ($CI_{0.95}$ = 99.85%), as shown in Table 5.6.

**Discussion**

In the present study, we demonstrate that the multiplex real-time PET-PCR assay can be used as a high-throughput screening method for *Plasmodium falciparum* and *Plasmodium spp.* infections, including submicroscopic infections, in both the general population and those that are at considerably higher risk for contracting malaria—children and pregnant women. Submicroscopic infections in the general population usually result in fewer clinical consequences for the individual, but impose a higher transmission fitness for the parasite [16]. Furthermore, this study reveals a clear underestimation of malaria infection in pregnant women and children, even under treatment, when diagnosed by microscopy.

The detection of a vast number of submicroscopic infections reported here is comparable to previous reports [17-21]. Similar to another Kenyan study conducted in pregnant women [22], we observed a four-fold higher detection with the PET PCR assay 36/96 (37.50%) when compared to microscopy. The PET PCR method compared favorably to the more complicated nested 18 srRNA assay in detecting infections in children at day 42 and following ACT treatment. The multiplex PET PCR assay is highly specific and sensitive for both *P.falciparum* and *Plasmodium spp.* infections (Table 5.7), and offers several diagnostic advantages over standard microscopy. Collecting dried blood on filter paper is cheap, safe to transport, and an effective form of DNA storage. DNA extraction from dried blood spots is easy and can be automated to avoid risk of contamination.

Importantly, real-time PCR based assays demand less labor, obtain results in a relatively short time, and are well-suited for high-throughput screening [11].

The multiplex PET-PCR assay described here is useful for detecting species specific malaria infections from peripheral blood, placental blood or dried blood spots. The assay can be employed as a high-throughput screening method for malaria infection(s) in combination with RDTs to confirm the results and characterize the type of infection. Importantly, the multiplex PET PCR assay is able to detect submicroscopic malaria infections at a sensitivity of 92.25% ($CI_{0.95}$ =96.69%) and specificity of 97.83% ($CI_{0.95}$ = 99.42%), with a positive predictive value of 96.76%, in two high risk groups (children and pregnant women) (Table 5.7).

A recent review by Fried et. al [23], suggests that RDTs sensitivity and specificity for diagnosis of placental malaria is suboptimal when compared to PCR-based methods. Nonetheless, the PET PCR assay still needs to be compared to the sensitivity and specificity of RDTs. Additionally, unlike the nested 18 rRNA [14] or multiplex single step-PCR method [6], the PET PCR assay is less time consuming and labor intensive.

Microscopy and RDTs remain the most appropriate methods for detecting febrile uncomplicated malaria in a field setting; molecular diagnostic tools such as the multiplex real-time PET PCR described here offer a more reliable and high-throughput method for detecting malaria infections, especially at low levels. We show proof of the concept that this assay can be utilized in a developing country laboratory setting, and due to the self-quenching nature of the primers, instead of sensitive and expensive probes, is a practical and inexpensive assay.

## Conclusion

In conclusion, the PET PCR assay described here is the first of its kind: a self-quenching real-time PCR method developed for malaria diagnosis. It is practical enough to be used as a high-throughput screening method for detecting malaria, including submicroscopic infection(s), in a developing country laboratory setting.

## Material and Methods

### *Ethics Statement*

Samples used in this study were obtained from studies conducted in Tanzania and Kenya. This study was approved by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee, Tanzania Ifakara Health Institute (IHI), the University of Georgia Institutional Review Board and the CDC Institutional Review Board, and informed written consent forms were obtained from all subject(s).

### *Clinical Samples*

A total of 1021 clinical samples were collected and tested anonymously from studies conducted in Tanzania and Kenya during the years of 2010-2011 and 2002-2008, respectively. Of the 1021 clinical samples, 925 samples included the general population and children under the age of 5 years from Tanzania and 96 placental samples from Kenya. Recruitment of pregnant women and sample collection have been described previously [24,25] Placental blood was collected by the prick method [26] or placental perfusion [27]. The blood was centrifuged to remove plasma and the buffy coat and the red blood cell pellet frozen.

*DNA extraction*

For the Tanzanian samples, blood spots were cut from each filter paper using scissors and deposited into individual 1.5 mL tubes. Scissors were sterilized twice with 100% ethanol between each cut. The same protocol was performed for negative control spots using the same scissors to show lack of DNA carry over. Genomic DNA (gDNA) was extracted from the blood spots using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) protocol "Isolation of DNA from dried blood". Genomic DNA was eluted into 100 µl of elution buffer and stored at -20°C for use in PCR assays. For the Kenyan samples, DNA was isolated from placental blood using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) protocol "Isolation of DNA from whole blood" and genomic DNA was eluted into 200 µl of elution buffer, aliquoted into two separate tubes, and stored at -20°C for use in PCR assays.

*Photo-induced electron transfer (PET)-PCR Assay*

Briefly, PET PCR relies on the photo-electron transfer mechanism of quenching without a quenching moiety. This method utilizes unique primers, with a flurophore (FAM or HEX) on the 5' end that form a loop-like structure, which in the absence of amplification remains in a closed form. Upon amplification, the PET primer loop structure opens up and fluorescence increases due to a conformational change and dequenching effect. By multiplexing 2 primer sets this method is able to detect both *Plasmodium* genus and *P. falciparum* species in a single reaction as low as 10 parasites/uL.

Amplification of *P. falciparum* and *Plasmodium spp.* was performed in a 20µl reaction containing 2X TaqMan Environmental Master Mix 2.0 (Applied BioSystems), 125nM each forward and reverse primer, and 2µl of DNA template. The reactions were performed under the following cycling

parameters: initial hot-start at 95°C for 10 minutes, and then 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 40 seconds (for multiplex assays). The correct fluorescence channel was selected for each fluorescently labeled primer set and the cycle threshold (CT) values recorded at the end of annealing step. A cut-off CT value of 40 was used to indicate a positive result. Multiplex assays were performed for all the samples.

*PET-PCR primers*

The target for the *P.falciparum* specific primers was described previously [15]. The genus specific primers were designed to amplify the 18S ribosomal RNA gene of *Plasmodium* (GenBank Accession # GU815531). In both cases, the 5' end of the reverse primers were modified with the PET tag and labeled with FAM (genus) and HEX (*P. falciparum)*. The *Plasmodium* spp. and *P. falciparum* primers are shown in Table 5.1.

*Nested 18s rRNA PCR and multiplex single step PCR*

The nested 18s rRNA PCR method by Singh et al. [28] and multiplex single step PCR method by Demas et al. [15] were chosen as reference tests in calculating the sensitivity and specificity of the PET-PCR assay due to superior sensitivity/specificity over microscopy and other molecular methods. For the nested 18s rRNA PCR method by Singh et al. [28], the protocol was slightly modified: reactions were performed in 20 µl total volume containing 1X buffer, 2.5mM $MgCl_2$, 200 µM dNTPs, 200 nM primers, and 1.25 units of Taq Polymerase (New England Biolabs, Ipswich, MA). The multiplex single step PCR method was performed as described in Demas et al. [15]. The amplicon products were analyzed for the appropriate size on a 2% gel.

*Clinical Sensitivity/Specificity and Statistical Analysis*

For calculating sensitivity and specificity, including the positive predictive value(s) and negative predictive value(s) (*npv)*:

$$\text{Sensitivity } = \frac{\#\ of\ true\ positives}{\#\ of\ true\ positives + \#\ of\ false\ negatives}$$

$$\text{Specificity } = \frac{\#\ of\ true\ negatives}{\#\ of\ true\ negatives + \#\ of\ false\ positives}$$

$$\text{Positive predictive value } (ppv) = \frac{\#\ of\ true\ positives}{\#\ of\ true\ positives + \#\ of\ false\ positives}$$

$$\text{Negative predictive value } (npv) = \frac{\#\ of\ true\ negatives}{\#\ of\ true\ negatives + \#\ of\ false\ negatives}$$

PPV: is used as an indicator of the proportion of positive test results that are true positives (i.e. correct diagnosis).

NPV: is defined as the proportion of subjects with negative test results who are correctly diagnosed.

For calculating the significance of difference between two separate proportions:

We assume that the sampling distribution of $_{p1-p2}$ is approximately normal with the mean

$\mu = _{p1-p2}$

and variance (standard error)

$\sigma_{p1-p2} = \frac{p1(1-p1)}{n1} + \frac{p2(1-p2)}{n2}$

we calculate $z$, $z = \frac{\mu}{\sqrt{\sigma_{p1-p2}}}$

and by looking under the area of a standard normal curve, find the probability $p$ of the difference between the two proportion(s).

For calculating the 95% confidence interval(s) ($CI_{0.95}$):

We assume homogeneity of variance, the population is normally distributed and values were

sampled independently from each other value Lower limit $= \mu - z_{0.95}\sigma_{\mu}$

Upper limit = $\mu + z_{0.95}\sigma_{\mu}$

Where $z_{0.95}$ is the number of standard deviations extending from the mean of a normal distribution required to contain 0.95 of the area, and $\sigma_{\mu}$ is the standard error of the mean.

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**Table 5.1. Primer sequences used in the multiplex real-time PET PCR assay**

| Primer Name | Sequence 5' to 3' |
|---|---|
| ***Plasmodium spp.* (genus specific 18S ssrRNA)** | |
| Forward | GGCCTAACATGGCTATGACG |
| Reverse | FAM –aggcgcatagcgcctggCTGCCTTCCTTAGATGTGGTAGCT |
| ***Plasmodium falciparum**** | |
| Forward | ACCCCTCGCCTGGTGTTTTT |
| Reverse | HEX-aggcgcatagcgcctggTCGGGCCCCAAAAATAGGAA |

*Designed based on a previously described [15] *P.falciparum* target.

**Table 5.2. Summary of the year and number of samples collected from each region**

| Population | Samples | Year Collected | DNA source | *PfPRE/Yr | *PfEIR/Yr | Region | Country |
|---|---|---|---|---|---|---|---|
| 1- general | 303 | 2011 | Blood Spot | ≤ 5%/2010 | ≤ 1/2010 | Dodoma, Iringa | Tanzania |
| 2- children | 645 | 2010-11 | Blood Spot | ≤ 5%/2010 | ≤ 1/2010 | Bagamoyo | Tanzania |
| 3- pregnant | 96 | 2002-2008 | Placental blood | ≥5%/2009 | ≥ 1/2008 | Kisumu/Siaya | Kenya |
| **Total** | **1044** | | | | | | |

*_Plasmodium falciparum_ prevalence (PfPRE) [29] and _P.falciparum_ entomological inoculation rate (PfEIR) [28].

**Table 5.3. Number of positive samples detected both by nPCR and PET PCR by District Hospital in Tanzania**

| **District Hospital*** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **Total** |
|---|---|---|---|---|---|---|---|---|---|
| Total # of Samples | 51 | 33 | 36 | 31 | 46 | 23 | 59 | 24 | 303 |
| *P.falciparum* Positive | 7 | 7 | 4 | 0 | 7 | 0 | 1 | 1 | 27 |

*District hospitals: (A) Tosamaganga, (B) Mlowa, (C) Kimande, (D) Uso-kami, (E) Idodi, (F) Mafinga, (G) Kibao, (H) Kibao, and (I) Igomaa.

**Table 5.4. Sensitivity and Specificity of multiplex PET PCR assay in detecting malaria infection from eight different district hospitals**

| | **Gold standard:** | | |
|---|---|---|---|
| **Test result:** | nested PCR* | | |
| *Multiplex PET PCR | Present (+) | Absent (-) | |
| Test Positive (+) | 27 | 0 | 27 (18.70%) |
| Test Negative (-) | 0 | 117 | 117 (81.30%) |
| | 27 (18.70%) | 117 (81.30%) | **Total** |
| | | | 144 (100.00%) |

| | | **95 % Confidence Interval:** | |
|---|---|---|---|
| | | **Lower Limit** | **Upper Limit** |
| **Sensitivity** | 100.00% | 94.04% | 100.00% |
| **Specificity** | 100.00% | 96.10% | 100.00% |
| | | | |
| *PPV* | 100.00% | 100.00% | 100.00% |
| *NPV* | 100.00% | 100.00% | 100.00% |

*All samples were positive by both *P.falciparum* and *Plasmodium spp.* primers; nested PCR = nested 18s rRNA PCR [14]. *PPV* = positive predictive value and *NPV* = negative predictive value.

**Table 5.5. Sensitivity and Specificity of multiplex PET PCR assay in detecting submicroscopic infection in children under the age of 5 years treated with artemisinin combination therapy (ACT)**

| | **Gold standard:** | | |
|---|---|---|---|
| **Test result:** | nested PCR | | |
| *Multiplex PET PCR | Present (+) | Absent (-) | |
| Test Positive (+) | 153 | 6 | 159 (39.85%) |
| Test Negative (-) | 13 | 227 | 240 (60.15%) |
| | 233 (58.40%) | 166 (41.60%) | **Total** |
| | | | 399 (100.00%) |
| ***Submicroscopic Infection#*** | | | |
| | | **95 % Confidence Interval:** | |
| | | **Lower Limit** | **Upper Limit** |
| **Sensitivity** | 92.20% | 86.70% | 95.59% |
| **Specificity** | 97.42% | 94.21% | 98.94% |
| | | | |
| *PPV* | 96.22% | 91.60% | 98.45% |
| *NPV* | 94.58% | 90.70% | 96.96% |

*All samples were positive by both *P.falciparum* and *Plasmodium spp.* primers; nested PCR = nested 18s rRNA PCR [14]. *PPV* = positive predictive value and *NPV* = negative predictive value. #The ACT treatment regimen ended on day 2 for all patients. We considered patients to be truly sub-microscopic (i.e. have a persistent parasite population) beginning with day 7. Thus, day 3 samples were excluded from the analysis.

**Table 5.6. Sensitivity and Specificity of multiplex PET PCR assay in detecting submicroscopic infection in pregnant women**

| | **Gold standard:** | | |
|---|---|---|---|
| **Test result:** | nested PCR* | | |
| *Multiplex PET PCR | Present (+) | Absent (-) | |
| Test Positive (+) | 37 | 1 | 38 (39.58%) |
| Test Negative (-) | 2 | 56 | 58 (60.42%) |
| | 39 (40.62%) | 57 (59.38%) | **Total** |
| | | | 96 (100.00%) |

| | | **95 % Confidence Interval:** | |
|---|---|---|---|
| | | **Lower Limit** | **Upper Limit** |
| **Sensitivity** | 92.30% | 78.03% | 97.99% |
| **Specificity** | 98.24% | 89.36% | 99.90% |
| | | | |
| *PPV* | 97.29% | 84.19% | 99.85% |
| *NPV* | 94.91% | 84.94% | 98.67% |

*All samples were positive by both *P.falciparum* and *Plasmodium spp.* primers; nested PCR = multiplex single step-PCR [15]. *PPV* = positive predictive value and *NPV* = negative predictive value.

**Table 5.7. Summary Sensitivity and Specificity of the multiplex PET PCR assay in the general population, children under the age of 5 years years, and pregnant women**

| | **General Population** | **Children & Pregnant Women** | **$CI_{0.95}$*** |
|---|---|---|---|
| | | *(submicroscopic)* | |
| **Sensitivity** | 100.00% | 92.25% | 96.79% |
| **Specificity** | 100.00% | 97.83% | 99.42% |
| **PPV#** | | 96.76% | |

* $CI_{0.95}$ = 95% Confidence interval and PPV# = positive predictive value.

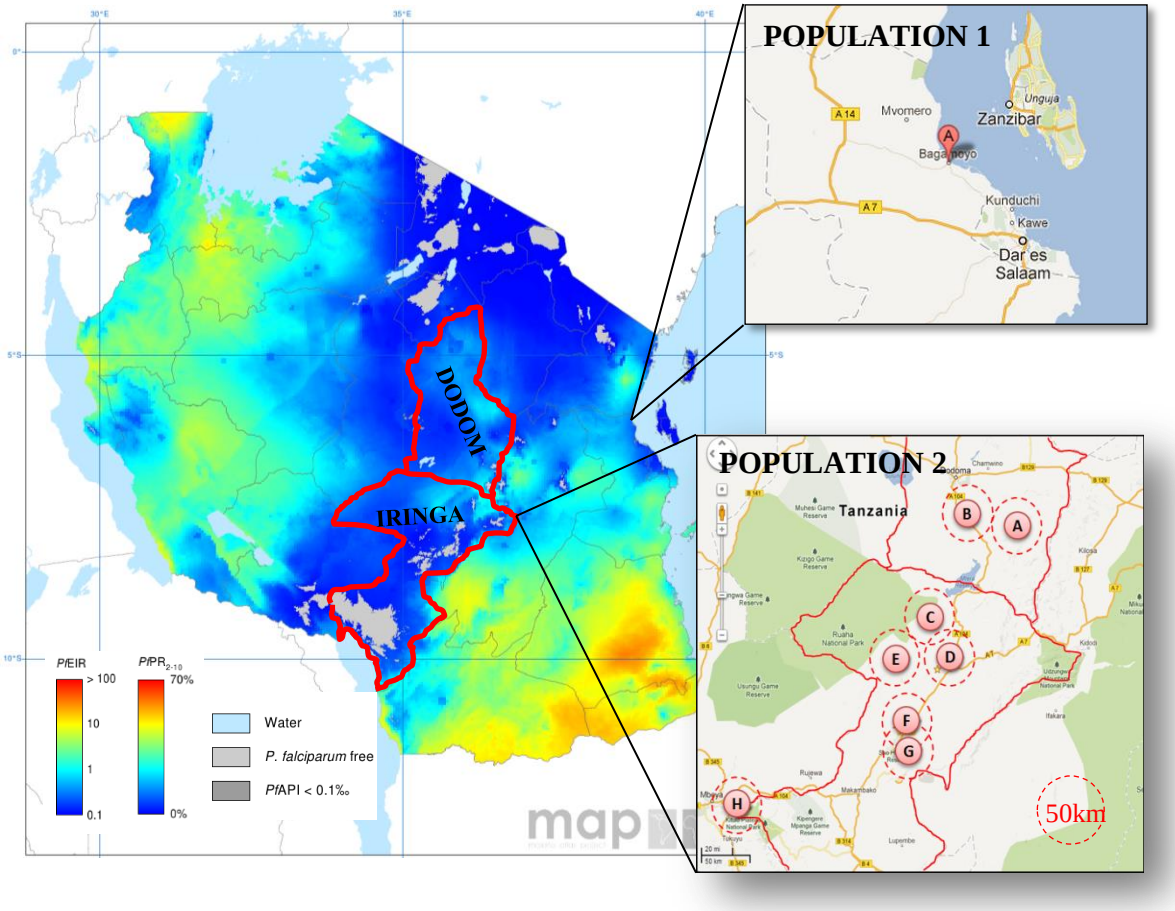


**Figure 5.1 Regions in Tanzania screened for species specific malaria infection using PET PCR**

For the first (**1**) population, a total of 303 samples from eight district hospitals (A-H) were screened via PET-PCR for malaria infection in the provinces of Dodoma and Iringa. The second (**2**) population (n=622), contains children under the age of 5 years from an artemesinin combination therapy (ACT) study conducted in the Miono District in Bagamoyo. *Plasmodium falciparum* prevalence (PfPR) and entomological inoculation rate (PfEIR) for selected region are shown, 0% > PfPR ≤ 5% and 0.1 > PfEIR ≤ 1, respectively. Shown is the 2010 map of spatial distribution of *Plasmodium falciparum* malaria endemicity and entomological inoculation rate (EIR) [1].

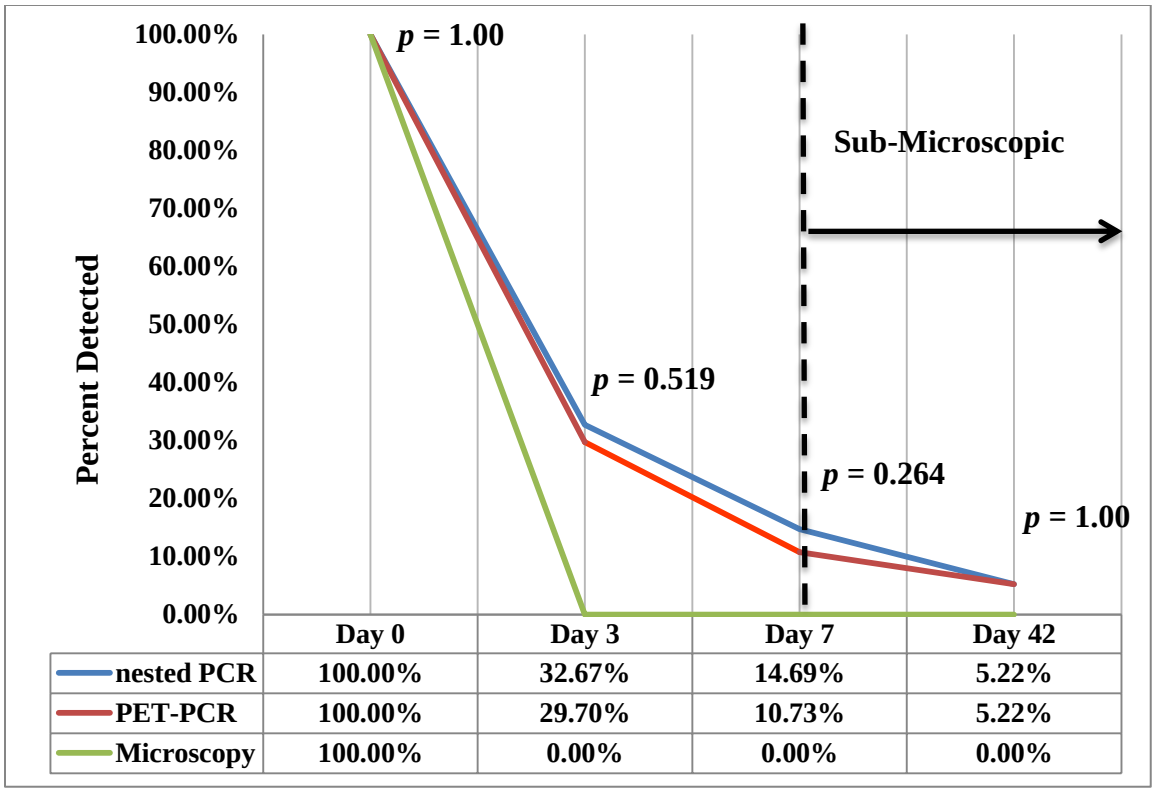


**Figure 5.2 Proportion of malaria positive samples detected by nPCR, PET-PCR and microscopy in children under the age of 5 years treated with artemisinin-combination therapy (ACT)**

A total of 622 samples were tested for *P.falciparum* infection from children under the age of 5 years, who were treated on day(s) 0-2 with Artemether /Lumefantrine (Coartem), and followed on days 3, 7, and 42. All samples were microscopy positive at day 0 and negative on days 3, 7, and 42. Compared to microscopy, both nested PCR and PET PCR detected *P.falciparum* infection after the completion of the ACT treatment regimen (i.e. day(s) 0 to 2). Patients were considered to be truly malaria sub-microscopic beginning on day 7. There was no statistical difference for the detection of infections between nested PCR and PET-PCR, $p = 1.00$ (day 0), $p = 0.519$ (day 3), $p = 0.264$ (day 7) and $p = 1.00$ (day 42), respectively.

# CHAPTER 7

# SUMMARY AND CONCLUSION(S)

Malaria is a disease that has co-evolved with humans for thousands of years. Today, half of the world population lives in areas at risk for malaria transmission. Every year, malaria takes more than half a million lives, of which most are children under the age of five years, pregnant women, and their fetuses.

In pregnant women, *Plasmodium falciparum* has evolved to take advantage of the placenta by expressing a unique antigen called VAR2CSA that allows it to bind to a low-sulfated chondroitin receptor. The evolutionary importance and ancient origin of this gene is indicated by its ortholog in the *P. reichenowi* genome and existence in almost all *P. falciparum* isolates. *Var2csa* is part of a family of ~60 *variant* genes, and the encoded protein, while still very polymorphic, is the most conserved *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1) identified to date, making it an important and intriguing virulence factor to study.

During pregnancy, *P. falciparum* infected erythrocytes sequester in the intervillous space of the placenta leading to pregnancy complications, which can result in maternal and neonatal death. Irrespective of previous immunity to malaria, women in their first and second pregnancies are at highest risk for developing placental malaria, as they lack a broad enough immune response to all the VAR2CSA variants. Interestingly, it only takes a few pregnancies for pregnant women to develop protective immunity primarily through inhibitory antibodies, suggesting that VAR2CSA variants have shared and/or conserved epitopes.

This led to the conjecture that a placental malaria vaccine is feasible, which led to numerous protein expression studies without careful analysis of *var2csa* genetic complexity. Nonetheless, the

information gained from these studies has been invaluable and helpful in identifying *var2csa* domain regions involved in binding to placental CSA. However, it has proven difficult to accurately assess binding to CSA as each study group used different expression systems (i.e. *E. coli*, baculovirus, or *Pichia pastoris*). The most promising data shows that the first three domains (e.g. NTS-DBL1X-DBL2X-IDS-DBL3X) likely form a specific CSA binding site, and the DBL3X domain principally induces inhibitory antibodies.

In order to design an efficacious PM vaccine candidate, detailed information on the genetic complexity of PM in terms of *var2csa* sequence types, gene copy numbers, and identification of specific immune-associated motifs will be required. The work herein provides such information on the antigenically critical DBL3X domain and supports the hypothesis that immune pressure drives selection of escape mutations in multigravid women, likely at the cost of decreased binding, that later revert back under selection in primigravid host. We provide evidence that this may be achieved through altering the size and composition of the loop region next to the binding pocket, varying *var2csa* copy numbers, and/or toggling critical amino acids involved in binding and immune evasion.

While finding a globally conserved epitope is unlikely, identifying gravidity-associated motifs and allelic variants should be the focus of future studies. The degree of antigenic variation between isolates and parity-specific motifs, as identified herein, still need to be determined at a global scale. This information will be critical in preventing vaccine-induced strain selection. Importantly, identifying and expressing one or two gravidity-associated recombinant protein variants that can induce a protective response may be beneficial to women in their first pregnancies, as it could help lower the parasite load.

Once parity-associated motifs or sequence types are identified, expression of high quality recombinant proteins will be required. The integration of genomic and proteomic data will be essential, especially in terms of how codon usage bias can regulate gene expression and the final protein product. The genetic code contains the basic instructions for efficient protein synthesis and

folding, with the latter being critical to accurately express natively recombinant proteins. Synonymous codons are not used at equal frequencies between different species and this may have evolved to preserve structural function of proteins across species.

Thus, future studies need to consider the host in which the *P. falciparum* protein is to be expressed and their codon usage bias. Codon harmonization, an algorithm for identifying regions of slowly translated mRNA, which is associated with linking segments and modulating heterologous expression, provides an ideal method for heterologous protein expression. This system has been tested in *E. coli* with numerous *P. falciparum* proteins and has yielded correctly folded proteins, as indicated by their reactiveness to a variety of functional-specific antibodies.

In order to produce an ideal PM vaccine candidate, further data is required on the genetic complexity of the immunogically critical *var2csa* domains and a consensus protein expression method and system, as suggested herein, will be needed. The goal of a PM vaccine should be to stimulate enough of a protective immune response to decrease the parasite burden, which will drastically enhance treatment effectiveness and, ultimately, save lives.