ANTIBODY RESPONSE TO VAR2CSA IN PREGNANT CAMEROONIAN WOMEN

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ANNA BABAKHANYAN

DISSERTATION COMMITTEE:

DIANE WALLACE TAYLOR, CHAIRPERSON
GEORGE HUI
ABBY COLLIER
WEI-KUNG WANG
JOHN CHEN
PAUL PATEK

DEDICATION

To my dear mother, *Naira Chovelidze*, who through many hardships and sacrifice of her own career, invested in my education and continually encouraged me to pursue further heights.

I am the first person in my family to receive a doctoral degree, but mom this is YOUR accomplishment!

გალაკტიონ ტაბიძე – მზეო თიბათვისა

მზეო თიბათვისა, მზეო თიბათვისა,
მიწად მუხლმოყრილი გრააღს შევედრები.
იგი ვინც მიყვარდა დიდი სიყვარულით,
ფრთებით დაიფარე – ამას გევედრები.

ტანჯვა-განსაცდელში თვალნი მიურიდენ,
სული მოუვლინე ისევ შენმიერი,
დილა გაუთენე ისევ ციურიდან,
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ხანმა უნდობარმა გზა რომ შეეღება,
უხვად მოიტანა სისხლი და ცხედრები,
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Galaktion Tabidze - Mzeo Tibatvisa

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"Have I not commanded you? Be strong and courageous. ***Do not be afraid****; do not be discouraged, for the Lord* ***your God*** *will be* ***with you wherever you go****." Joshua 1:9*

ABSTRACT

Women are highly susceptible to malaria during pregnancy, because parasites express the VAR2CSA-adhesin that facilitates the binding of infected erythrocytes to placental syncytotrophoblasts, resulting in a condition known as placental malaria. Currently, vaccines to prevent placental malaria are being developed; however, testing vaccines for pregnant women will be challenging in the absence of serological assays that predict protection. This study sought to identify correlates of protection for placental malaria. Plasma from women with ≥ 3 pregnancies were evaluated, allowing us to delineate fine immunologic differences between placental malaria-positive and -negative women. First, we evaluated naturally acquired immunity to a recombinant protein, ID1-ID2a that contains the minimal sequence of VAR2CSA required for binding to the placenta. Our results showed that antibodies to ID1-ID2a were not associated with protection from placental malaria. Next, we employed a multi-assay approach and compared immune responses to full length and different VAR2CSA domains in 24 assays. Data showed that women in their 3rd and 4th pregnancies residing in a low transmission areas were acquiring immunity, and had lower antibody levels to VAR2CSA, recognized fewer VAR2CSA domains, and had lower avidity antibodies to VAR2CSA; whereas, women with ≥5 pregnancies were likely to have malaria only if they had low avidity IgG to VAR2CSA. Multivariate regression models and recursive partitioning methods resulted in Younden index of 0.39 and 0.45, respectively. Since models with different combinations of assays resulted in similar predictive power, it is likely that more than one correlate of protection exists depending on a woman's age, gravidity, exposure to VAR2CSA and antibodies to other malarial antigens. Lastly, we measured how long immunity to VAR2CSA persists. Antibody half-life estimates ranged from 4 years to a lifetime in multigravidae; but, antibodies to VAR2CSA in primigravidae were short lived, averaging less than a year. Thus, a single pregnancy is not sufficient to generate long-lived memory B cells. Knowing what assays correlate with protection and how long after vaccination protective immunity persists is imperative for development of a vaccine.

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Ab	antibody/antibodies
CIDR	cysteine-rich inter-domain
CSA	chondroitin sulfate A
DBL	Duffy-Binding-Like
FV2	full length VAR2CSA
ID	interdomain region
IE	*P. falciparum*-infected erythrocytes
IFNγ	Interferon γ
IgG	immunoglobuling G
ITN	insecticide-treated bednets
IPT	Intermittent Preventive Treatment
MG	multigravidae
MΦ	macrophages
OR	Odds ratio
P. falciparum	*Plasmodium falciparum*
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PM	placental malaria
PG	primigravidae
Rifin	repetitive interspersed family
SP	Sulfadoxine and Pyrimethamine
Stevor	subtelopmeric open reading frame
TNFα	tumor necrosis factor α
VSA	variant surface antigen
YI	Younden index

CHAPTER 1

INTRODUCTION

1.1 Global burden of malaria during pregnancy

Shortly after the discovery of malaria parasites by Laveran in November 1880 [1], scientists observed that women living in areas where *Plasmodium falciparum* (*P. falciparum*) was endemic and presumably immune to malaria could harbor malarial parasites during pregnancy and have few signs of disease; however, the same infection pregnant women living in non-endemic areas developed severe consequences if infected during pregnancy [2]. Today, malaria in pregnancy is a major public health problem in *P. falciparum* endemic countries, as the disease influences the health of both the mother and the developing fetus.

Although people living in malaria endemic areas develop immunity to malaria by adulthood, pregnancy is a period of increased vulnerability to *P. falciparum* infections. Pregnant women are at higher risk of malaria infection and disease due to parasite evasion mechanisms, immunological and hormonal changes during pregnancy. Both frequency and levels of *P. falciparum* parasitemia are higher in pregnant women compared to non-pregnant women [3]. Globally, about 85.3 million pregnancies occur in areas where *P. falciparum* is transmitted [4], with 54.7 million pregnancies in stable transmission areas and 30 million (54.1% of 54.7 million) pregnancies at risk of *P. falciparum* infection in Africa. Malaria susceptibility among pregnant women is highest in those who are pregnant for the first time, i.e., primigravidae [5, 6] and those infected with HIV [7].

In malaria low transmission areas, such as South America, the Western Pacific and Asia, pregnant women have little or no acquired immunity to malaria. Therefore, placental malaria manifestations are usually very severe and often result in maternal and fetal death [8]. In 2007, 30.6 million pregnancies occurred in *P. falciparum* low transmission areas, with 80% of them occurring in the South East Asia and 5.2% in Americas [4].

Adverse outcomes associated with malaria during pregnancy include low birth weight (LBW) babies. LBW due to prematurity or intrauterine growth restriction of full-term newborns is a major risk factor for neonatal morbidity [9-11]. An estimated 3.3 million neonatal deaths occurred in 2009 in mothers exposed to malaria during pregnancy, of which one-third occurred in Africa [12]. Eisele et al. [13] estimated that malaria infection was responsible for 14% of all LBW infants worldwide. In sub-Saharan Africa, 11 % of LBW related infant mortality is due to malaria, one of the largest causes of adverse newborn outcomes in this region.

Malaria, including placental malaria remains one of the "big three" infectious diseases disproportionally affecting people living in developing countries. Placental malaria is the most common preventable cause of maternal and neonatal mortality and morbidity in sub-Saharan Africa. Since malaria has inexpensive cure, mortality and morbidity rates are devastating, because they are preventable. Poor education, limited access to health care, lack of drugs at local pharmacies, especially in small public clinics are among some of the reasons malaria still strikes millions of individuals every day.

1.2 Adverse outcomes of malaria during pregnancy: past and present

Malaria in non-pregnant individuals has many façades, but malaria during pregnancy is distinguished by tragic consequences in its two victims, i.e., the mother and the developing fetus.

1.2.1 Consequences for the mother

Early physicians reported that malaria was intensified during pregnancy, and noted that the "worst cases of malaria during the epidemic are in non-immune pregnant women" [3]. Disease presentation depended on the immune status of the woman. Early studies suggested that immune mothers, i.e., women living in malaria endemic areas, did not have many symptoms [2] and did not have serious complications other than fever [14]. These women, were more likely to have placental malaria if they had abortions or miscarriages [2]. However, the absence of serious symptoms was deceiving, because they masked chronic malaria infections in immune women that had adverse effects on the

fetus. In women in sub-Saharan Africa, malaria during pregnancy is a serious disease, characterized by maternal anemia and LBW infants, which predisposes to both the mother and the baby to death.

Some of the early researchers suggested that malaria could occur anytime during pregnancy, but chronic forms of malaria began at 4-5 months of pregnancy, with severe progressive forms occurring during the second half of pregnancy [15]. Greatest risk of severe disease in non-immune women was during last trimester [2, 16-20] and perhaps more fatal in the peripartum period [3]. In early reports from Mozambique, 15.5% maternal mortality was attributed to malaria [21]. Death occurred from cerebral malaria, hypoglycemia, pulmonary edema and adult respiratory distress syndrome and premature labor [22].

Historically, in the absence of definitive laboratory confirmatory tests, malaria has been often misdiagnosed as some other disorders in pregnant women. [17]. Malaria can mimic hyperemesis gravidarum, acute yellow atrophy of the liver, pre-eclampsia, eclampsia, uremia, pyelitis, nephritis, puereperal sepsis, pernicious anemia of pregnancy, heat exhaustion and advanced hookworm anemia [17]. Multiple symptoms are possible, since *P. falciparum* parasites can sequester in various tissues and elicit organ-specific symptoms.

In non-immune pregnant women and primigravidae in malaria endemic regions were described to present with severe consequences, including, acute/chronic, cause fevers, anemia, cachexia (weight loss), anasarca (extreme generalized edema), nephritis, coma, dysentery, seizures, precipitate abortions, stillbirths, fetal growth restriction and premature delivery [17]. Women with cachexia, also had intense anemia, anasarca, splenomegaly and often died during or shortly after delivery, while others died from cerebral malaria exacerbated by the strain of labor [17].

Today, placental malaria is known as a chronic disease, as pregnancy provides a way parasites can evade the immune system by adhering to the placenta. The major adverse outcomes of placental malaria for the mother are anemia, cerebral malaria [23], hypoglycemia [24], pulmonary edema [25], severe hemolytic anemia [23], premature

labor and in severe cases death [9, 23]. The population attributable risk of malarial anemia was estimated as 14.6% in holoendemic regions in Malawi [26]. Mechanisms of anemia are poorly understood and further confounded by factors such as concomitant folate deficiency [27], iron deficiency [26], HIV infection [28] vitamin A and B12 deficiency [29].

1.2.2 Consequences for the fetus

Malaria during pregnancy causes placental dysregulation, which leads to intra-uterine growth restriction, low birth weight babies, premature deliveries, and in severe cases, increases the risk of infant mortality [30].

Mortality

Interruption of pregnancy was reported by Hippocrates and Torti in 1700 [31]. Fetal death was postulated to be due to hyperpyrexia or from the direct effect of infection [16], which in turn caused uterine contractions especially during late gestational age and caused premature delivery. Chronic malaria predisposes to pregnancy interruption [15]. In 1911, study in Algeria showed that 8% women with placental malaria had abortions and 28% premature deliveries; however, term deliveries could be attained with antimalarial treatment [19]. Mortality among children born to mothers with chronic malaria without anemia was 10%, with anemia is 20%, marked anemia more than 75% [32]. Currently, researchers attribute 75,000 to 200,000 infant deaths each year to malaria infections during pregnancy [33].

Low birth weight

Placental malaria affects fetal development. Term-infants from malaria-infected women may weigh less than infants from healthy women (less by 1/5 of normal infant weight) and have higher mortality [16]. Both non-immune [15, 16, 34] and immune women [35-38] are at higher risk of delivering low birth weight babies if they have malaria during pregnancy. Low birth weight has a strong association with infant mortality in the first year of life.

Congenital malaria

Congenital malaria was initially ruled out [39-42]. However, in 1907 Economos documented parasites in the peripheral blood of newborns within 2 days of birth [43]. The low prevalence of congenital malaria in highly endemic areas is striking [44], demonstrating that the placenta is an effective barrier.

1.3 Pathophysiology: parasite sequestration in the placenta

In 1898, Bignami observed malaria parasites and hemozoin pigment in the placenta [39], followed by Sereni who also noted parasite pigment-lade leukocytes in malaria infected placentas [42]. In 1907, doctors described low peripheral parasitemia in pregnant women with heavy placental infections [45], assuming that parasites take refuge in the intervillous space of the placenta to multiply. Only *P. falciparum* parasites accumulate in the placenta in massive quantities and only late stages (trophozoites and schizonts) were found in placenta [2, 46, 47].

Pathogenesis of malaria during pregnancy is due to the sequestration of *P. falciparum* -infected erythrocytes (IE) in the intervillous space of the placenta. The resulting condition is known as placental malaria. As a part of the immune evasion mechanism, *P. falciparum* parasites remodel the surface of invaded erythrocyte by inserting malarial antigens into the erythrocyte membrane and are coded for by multi-copy gene families that produce variant surface antigens (VSA) [48]. In pregnant women, VAR2CSA malarial antigen is expressed on the surface of IE, which allows IE to bind to the chondroitin sulfate A chains (CSA) on the apical surface of syncytial trophoblasts lining the villi in the placenta. IE can also be found attached to fibrinoid masses or scattered throughout the placenta. CSA is a sulfated glycosaminooglycan present on syncytial trophoblasts and normally functions as a reversible immobilizer for cytokines, hormones, and other molecules [49].

Ismail et al. (2000) characterized histological changes in infected placentas and classified placental malaria into three categories: acute infection (presence of parasites, absent or minimal hemozoin), chronic infection (parasites present, significant pigment deposition in fibrin and immune cells), and past infection (pigment, no parasites) [50].

Peripheral and placental parasitemias generally correlate [51]. However, a number of studies have reported the presence of placental parasitemia and without peripheral parasitemias [2], in fact, up to 50% of women with placental malaria are blood-smear negative for malaria [50]. Whereas only 5% of women have parasites in the peripheral blood without parasites being detected in the placenta.

1.4 Inflammation and dysregulation of cytokine balance

1.4.1 Consequences for the mother

During chronic placental malaria, the accumulation of IE results in inflammation and immune cells that are recruited into the intervillous space of the placenta. The accumulation of monocytes and lymphocytes into the intervillous space was first described in 1930 [52, 53]. Placental syncytial trophoblasts, a single-layer barrier between maternal blood and fetal blood, are able to respond to the infetion. Lucci et al. (2011) reported peripheral mononuclear cells were recruited into the placenta in response to chemokines secreted by hemozoin-activated syncytial trophoblasts [54]. Parasite DNA bound to hemozoin pigment is also a potent proinflammatory agent; it activates TLR-9 endosomal receptors present on the syncytial trophoblasts [55]. In response to infection, placental TNFα levels rise [56]. All together, these changes in response to the placental malaria, result in dysregulation of placental functions: for example, soluble vascular endothelial growth factor receptor 1 levels, a preeclampsia biomarker, was found to be elevated in primigravidae with placental malaria [57].

1.4.2 Consequences for the fetus and the fetal response

Early on, solely based on microscopic findings, researchers implicated impaired oxygen/ nutrient exchange and malaria toxins as reasons for poor pregnancy outcomes and fetal death. Subsequently, studies have shown that malaria-induces placental inflammation and causes changes in syncytial trophoblast functions, i.e. dysregulation of cytokines, angiogenesis, placental hormones for fetal growth, and nutrient transport which leads to neonatal morbidity and mortality [54, 58-61]. Thus, changes in the placenta induced by malaria contribute to LBW infants.

Malaria induces a shift to type 1 cytokines in the intervillous space, with increases in placental TNFα and IFNγ and a decrease in IL10 in mothers who delivere low birth weight babies [56], which have 40-fold increased risk of dying during the first year of life. Placental malaria infections are associated with a decrease in soluble fms-like tyrosine kinase-1 and leptin and an increase in C-reactive protein [62] and dysregulation of the insulin-like growth factor axis that contributes to reduced fetal growth [63] and therefore neonatal morbidity. In addition, increased levels of maternal soluble endoglin are associated with placental malaria and fetal growth restriction in placental malaria - positive primigravidae [64]. Thus, alteration in cytokine balance in the placenta contributes to pre-term deliveries [65] and increasing parasite densities dysregulate production of growth hormones, leading to intrauterine growth restriction and prematurity [66].

Finally, malaria during pregnancy may lead to increased risk of congenital infections [67] and in-utero priming [68] or immune tolerance [69] to malarial antigens.

1.5 VAR2CSA: a key player in placental malaria

1.5.1 Variant surface antigens

P. falciparum has four large multi-copy gene families: *var* genes (*P. falciparum* erythrocyte membrane protein 1 or PfEMP1), stevor genes (subtelopmeric open reading frame), surf (SURFIN proteins) and rif (RIFIN protein) genes (repetitive interspersed family) [70]. PfEMP1 and RIFIN allow erythrocytes infected with asexual stage parasites to cytoadhere and aggregate in the microvasculature in different organs, thereby preventing them from being cleared in the spleen [71]. *P. falciparum* parasites expressing VAR2CSA are selected for in pregnant women. VAR2CSA is a 350 kDa (3,000 amino acid) transmembrane protein [72] composed of six Duffy-Binding-Like domains (DBL domains 1-6), interspersed by interdomain regions (ID) (Figure 1). Due to its large size, the crystal structure of VAR2CSA has not been resolved.

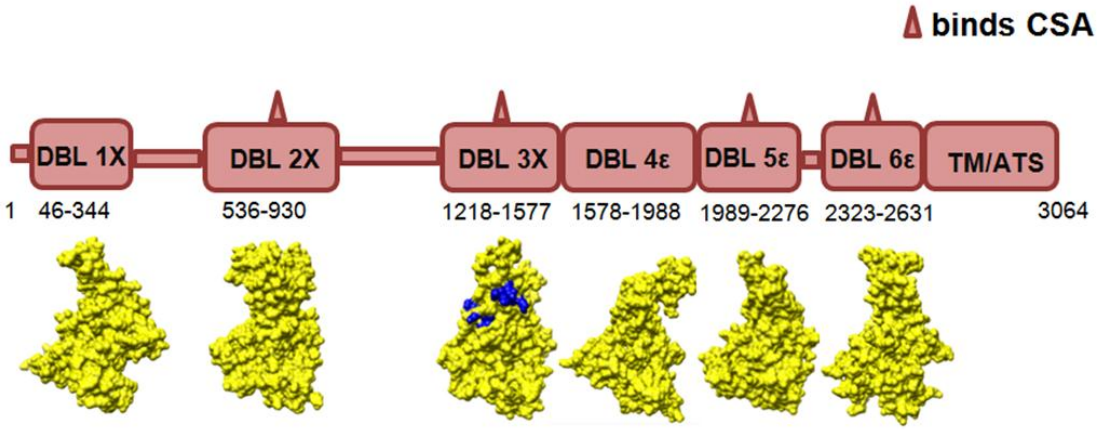

Figure 1. Domain diagram of A4 *var2csa* gene. Distribution of residues that form various DBL domains is shown. Modeled structures for the corresponding domains are given below each domain box. All models are shown in surface representation and colored yellow. The sulphate-binding residues on A4 DBL 3X crystal structure are colored blue. *Adapted from Gill J. et al. Malaria Journal 2009, 8:67.*

1.5.2 Polymorphisms

In general, antigenic variation of PfEMP1 genes is a fundamental mechanism exploited by *P. falciparum* for survival. Each parasite genome contains about 60 different *var* genes with high sequence diversity; however, at any given time, only one PfEMP1 gene is expressed within a single IE [70]. Exclusive expression of a single *var* gene is regulated at the level of transcription initiation [73-75]. Isolated placental *P. falciparum* parasites express only *var2csa*, whose structure is atypical of other var genes; *var2csa* has a unique domain architecture, an uncommon up-stream flanking region, and is remarkably conserved across different *P. falciparum* strains [76]. Sequence analysis showed that VAR2CSA proteins in parasites isolated from Asia, Africa and Central America have 78% amino acid homology. The 6 DBL domain varies in amino acid conservation (61%-88%), with DBL-6ε being the least conserved (61%), while DBL-4ε is the most conserved (88%) [77].

1.5.3 CSA-minimal binding site

Significant efforts have been made to discover the structure of VAR2CSA and identify regions on VAR2CSA that bind CSA. Andersen et al. (2008) have mapped S1and S2 surface exposed DBL subdomains and showed that these highly conserved parts of VAR2CSA protein are accessible for the binding of antibodies (Ab) [78]. The

same group also showed that immunization of laboratory animals with DBL1 and DBL3 resulted in the production of CSA-adhesion blocking Ab, indicating that the CSA-binding site on the VAR2CSA molecule is composed of regions from different domains [79]. Finally, full length VAR2CSA extracellular region (containing DBL1X to DBL6Ɛ) was expressed for the first time in 2010, and Ab to the extracellular region have been shown to inhibit IE binding to CSA [80].

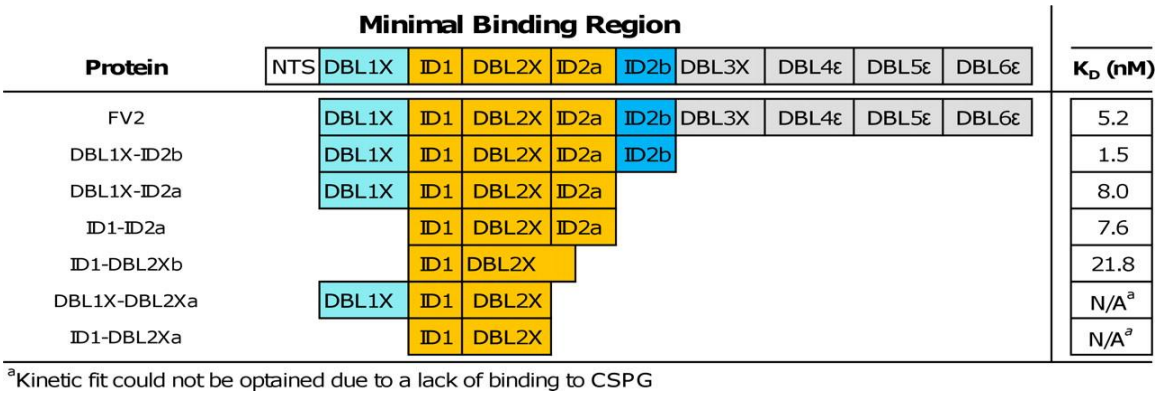

Figure 2. Schematic representation of produced VAR2CSA fragments. *Clausen T. M. et al. J. Biol. Chem. 2012; 287:23332-23345.*

Recent advances in the VAR2CSA research refute the original idea that the CSA minimal binding site is conformationally-created by multiple DBL domains [78, 81, 82]. Several groups have now shown that the minimal CSA binding site is located in the N-terminal DBL2X- CIDRPAM region [83]. The minimal sequence containing the binding site was recently identified as ID1-DBL2Xb (Figure 2), consisting of ID1, DBL2Xb and 93 amino acids from ID2a [84]. A slightly larger construct, termed ID1-ID2a, consisting of the ID1, DBL2Xb, plus the entire ID2a region, can be expressed in higher yields compared to ID1-DBL2Xb [84]. The ID1-ID2a is considered to be a strong vaccine candidate, because rat anti-ID1-ID2a Ab inhibit the binding of IE to CSA by nearly 100%. Furthermore, when Ab raised against full length VAR2CSA (FV2) in rats are affinity purified on recombinant ID1-ID2a, the purified Ab effectively inhibit IE binding to CSA by essentially 100% [84]. Thus, ID1-ID2a contains the minimal CSA binding site, induces inhibitory Ab in an animal model, contains major epitopes important in

inhibition of binding, and can be produced on a large scale. There is hope that this construct can use used as a malaria vaccine that will reduce placental malaria.

1.5.4 Other variant surface antigens

It is possible that receptors in addition to VAR2CSA might allow IE to sequester in the placenta. Several research groups speculate that additional DBL and cysteine-rich inter-domain region (CIDR) domains present in other PfEMP1 proteins may have some affinity to CSA [85-88]. However, the finding that VAR2CSA affinity for CSA is several orders of magnitudes higher compared to the other PfEMP1 domains suggests other ligands are of low importance [80, 89]. Finally, it was shown that selective knockout of VAR2CSA significantly decreased or abolished all together the ability of IE adhesion to CSA *in vitro* [90-92]. Therefore, it is now generally accepted that VAR2CSA, the primary ligand involved in IE binding to the placental CSA.

1.6 Maternal immune responses to VAR2CSA

1.6.1 Timing of antibody production

Malaria during pregnancy is a complex phenomenon, where host and pathogen interactions occur in the developing placenta. The placenta starts to develop upon implantation of the blastocyst, starting around week 5 and the intervillous space is fully developed by week 12-13 [93]. Therefore, *P. falciparum* sequestration does not take place until the end of the first trimester. During development, the placental vascularization, including number of the veins and their diameter, progressively increase throughout gestation. Simultaneous with the formation of the intervillous space by 8-10 weeks of pregnancy, *P. falciparum* parasites switch to VAR2CSA expression, up-regulate *var2csa* gene expression, and the selected parasite population that expresses VAR2CSA on the IE surface are selected for by adhesion to CSA [94]. Aitken et al. (2010) suggest that primigravidae obtain high levels of Ab to VAR2CSA as early as 17 weeks of gestation [95]. Thus, protective Ab are not produced until the second trimester, so primigravidae are usually at the highest risk of placental malaria.

1.6.2 Strain-transcending antibodies

Strain-transcending immunity to VAR2CSA exists. People who live in stable malaria transmission areas obtain clinical protection from malaria, as they have acquired immunity to multiple polymorphic malarial antigens in response to repeated *P. falciparum* infections during their lives [96]. Likewise, repeated exposure to placental malaria generates VAR2CSA Ab, results in the production of Ab to VAR2CSA that are correlated with the absence of placental malaria. Multigravidae have higher VAR2CSA Ab levels and more protection compared to primigravidae. Yet, a number of studies have shown that VAR2CSA-specific Ab recognize *P. falciparum* isolates outside their geographic region [56, 97-102]. Thus, limited antigenic diversity in CSA-binding *P. falciparum IE* explains why immunity to malaria in pregnancy can be achieved after only one pregnancy. Finally, based on the fact that VAR2CSA Ab block adhesion of IE to placental CSA, as well as opsonize IE for removal by Fcγ-mediated phagocytosis, VAR2CSA is a placental malaria vaccine candidate [77, 94]. It is likely a limited number of variants may be sufficient for a vaccine that will protect against the array of polymorphic variants circulating in nature [103]. Thus, information on which VAR2CSA domains/antigenic variants are important in protection is important for development of a vaccine to protect pregnant women.

1.7 Correlates of protection from malaria in pregnant women

In malaria endemic areas, pregnant women produce Ab to VAR2CSA that inhibit the binding of IE to CSA in vitro [89, 104], reduce maternal anemia [105], lower placental parasitemia at delivery [106, 107], increase the length of gestation [108], and improve infant birth weight [108]. Thus, Ab to VAR2CSA play an important role in protecting pregnant women from the severe effects of placental malaria. However, the protective effector mechanism of Ab to VAR2CSA remains unclear.

There are a number of ways VAR2CSA Ab might prevent/eliminate IE from the placenta, including, activation of the complement cascade, activation of natural killer cells, inhibition of binding, and opsonic phagocytosis. Since traditional vaccine development has not lead to efficacious malaria vaccine and production of full-length

VAR2CSA on large scale is difficult and expensive, understanding the mechanism by which VAR2CSA Ab prevent placental malaria could provide a novel approach for vaccine development, vaccine efficacy testing, as well as other interventions for pregnant women. Studies suggest that inhibition of IE binding to the CSA is an important effector mechanism. Alternatively, opsonic Ab that mediate phagocytosis constitutes another possible effector mechanism for clearing IE from the infected placenta. Future studies are needed to determine the precise role of Ab in reducing the severe effects of placental malaria.

Some of the impediments that inhibit research on placental malaria include, but are not limited to, the absence of good placental malaria animal model, unresolved crystal structure of VAR2CSA, difficulty producing recombinant full-length VAR2CSA, regulatory constraints related to research on pregnant women, and implementation of Intermittent Preventive Treatment (IPT) that changes immune response dynamics to VAR2CSA.

1.8.1 Maturity of response to VAR2CSA

Previous studies in our laboratory showed that the presence of Ab to full length VAR2CSA, as well as several domains of VAR2CSA (DBL 3 and 5) early in pregnancy correlate with the absence of placental malaria at delivery and improved pregnancy outcome [109, 110]. Moreover, women who have Ab to multiple VAR2CSA DBL domains are at a decreased risk of placental malaria [109]. In malaria high transmission areas, most multigravid women produce “protective Ab” responses and do not have placental malaria at delivery. However, some multigravid women fail to produce protective Ab and have placental malaria, even though they develop high Ab levels to full-length VAR2CSA. Also, high Ab levels at the time of delivery do not correlate with absence of placental malaria. At delivery both women with and without placental malaria have similar Ab levels to VAR2CSA, with difference seen only during 5-6 month of pregnancy [110]. Finally, there is an association between high Ab avidity at 3-4 months of pregnancy and absence of placental malaria [110], showing that Ab must be present for a significant period of time in order to eliminate IE from the placenta.

1.7.2 Inhibition of binding

The most accepted theory of protection from placental malaria is that Ab prevent or inhibit IE from binding to CSA, i.e., prevent adhesion and sequestration of IE in the IVS [111]. It has been reported that maternal anti-adhesion antibodies in multigravid women from Kenya, Malawi and Thailand reduce the prevalence and density of infection [111]. However, these Ab did not completely inhibit binding in an *in-vitro* assay, under a static condition; however, the number of IE, CSA concentration, and plasma dilution used may not reflect the physiological condition in the body. Theoretically, there are about 150, 000 to 200, 000 closely packed VAR2CSA molecules displayed on the surface of each IE that have a high density of knobs [112]. Based on our placental parasitemia for Yaounde, Camerooon (0.001% to 82.6%, with 65% of the pregnant women having parasitemias between 0.1 and 1.0 %), we estimate that there about $2x10^{10}$ VAR2CSA molecules in the microliter of blood of the infected pregnant woman. Hence, since inhibition of binding requires saturation of all CSA-binding epitopes on VAR2CSA to prevent adhesion to CSA, a lot of VAR2CSA Ab are needed to prevent binding of IE in the placenta. Therefore, since there is no definitive data in support of inhibition of binding as the sole mechanism of protection, inhibition of binding may be a characteristic of the protective Ab, but it may not represent the primary mechanism of clearance.

1.7.3 Phagocytosis

The other proposed mechanism is phagocytosis, for which saturation of all the epitopes on the IE would not be necessary. Recent studies showed that PfEMP1 is a major target of Ab that mediate opsonic phagocytosis [113]. Few opsonizing Ab on the surface of the IE would be sufficient for FcγR oligomerization signalling for macrophages to engulf an IE. Fcγ-mediated opsonic phagocytosis would ensure that the IE are removed from the placenta. In addition, hemozoin (Hz), a by-product of parasite digestion, activates already present placental mononuclear phagocytes and syncytial trophoblast to secrete cytokines and chemokines that attract new monocytes and macrophages to the intervillous space [60, 65, 114]. Finally, intervillous space fibrinoid material can increase phagocytosis by trapping IE and making it easier for macrophages to engulf the opsonized target. HIV positive women are twice as likely to have placental

malaria and have significantly lower plasma opsonizing activity than HIV-negative women [115]. Hence, it is possible that phagocytosis might be a more plausible protective mechanism for removal of IE from the intervillous space.

1.8 Intermittent Preventive Treatment (IPT) with sulfadoxine pyrimethamine and insecticide-treated bednets (ITN)

1.8.1 World Health Organization recommendations

To prevent maternal and neonatal morbidity and mortality, the World Health Organization recommends effective case management for malaria and anemia, and prevention of infection using intermittent preventive treatment (IPT) with sulfadoxine pyrimethamine and insecticide-treated bednets (ITN) as a part of Millennium Development Goals. IPT/ITN consists of oral administration of 2 doses of sulfadoxine-pyrimethamine during regularly scheduled antenatal visits after the first trimester and use of ITN during pregnancy [116]. It has been established that IPT-SP and ITN are efficient at decreasing placental parasitemia and significantly reduced malaria prevalence in pregnant women [117]. Therefore, IPT-SP and ITN are currently the standard of care in most African countries, although the coverage rates for intervention remain low. It was estimated that in 2007 of 47 sub-Saharan countries assessed, 39 had IPT-SP policies, but in 31 countries with the policy 19 million pregnancies remained unprotected [118].

Surprisingly, no pharmacokinetics and safety studies of sulfadoxine-pyrimethamine have been conducted in pregnant women. Despite the fact that most countries with IPT-SP/ITN policy implement 2 doses, some argued that according to the pharmacodynamics modeling, 3 doses for all women may be more efficacious, in areas where SP resistance is absent [116, 119]. In 2012, the policy was revised and now it is recommended that all women take 4 doses of SP during pregnancy [120].

1.8.2 IPT Influences on immunity

IPT-SP/ITN intervention markedly reduces malaria and improves pregnancy outcome, but it also reduces exposure to malarial Ag, including VAR2CSA. To date, there are only few studies that have looked at the effects of the chemoprophylaxis on the

naturally acquired VAR2CSA Ab in pregnant women. One of the earlier publications by Greenwood and colleagues (1994) looked at a cohort of 165 Gambian secundigravidae women, who were treated with Maloprim (dapsone-pyrimethamine) during their first pregnancy. The authors concluded that the prevalence of *P. falciparum* in peripheral blood and placenta was similar in women who have received Maloprim to those who were treated with placebo [121]. However, these findings were criticized by others due to the fact that primigravidae women were not treated in the Gambian study until the third trimester; allowing women to produce Ab to the pregnancy associated malarial antigens prior to the drug administration [122]. In addition, effect of the IPT-SP on acquisition of Ab to pregnancy-associated variant surface antigen (VSA) was studied in 281 Kenyan primigravidae women treated with IPT-SP (cross-sectional study). Based on the studies measuring Ab to the surface of CSA-binding IE in samples collected at during the third trimester, data showed that Kenyan primigravidae had lower immunoglobuling G (IgG) levels specific for *P. falciparum* VSA [122].

Likewise, Aitken et al (2010) studied malaria VSA-specific IgG levels in 549 Malawian women (primigravidae and multigravidae) using a longitudinal study design. They collected plasma samples at two time points during pregnancy: 14-26 & 28-34 gestational weeks, and at 3 time points postpartum: 1, 3, 6 months. Women received IPT-SP during the second and third trimesters . Once again, surface binding assays were performed. Results showed that IgG levels to pregnancy-associated *P. falciparum* VSA were decreased in women receiving IPT [95]. In addition, a placebo-controlled trial by Serra-Casas and colleagues (2010) using IPT-SP found that the in the IE surface binding assay using plasma collected at delivery showed that 88 HIV positive, but not 177 HIV negative women in Mozambique had reduced IgG levels to the VAR2CSA [123].

Finally, effects of IPT-SP chemoprophylaxis on Ab to VAR2CSA DBL5 were studied in 101 Senegalese women [124]. Once again, ELISA data show that VAR2CSA DBL5 IgG levels have decreased significantly between the time of enrollment and delivery in women who were treated with IPT-SP. However, in all these studies effects of the IPT-SP treatment were assessed only by looking at the Ab to the IE surface Ag in the CSA-binding *P. falciparum* cell lines or to the DBL5 domain by ELISA assay, using few

parasite strains. Furthermore, only the study by Aitken et al. had a longitudinal design (with only 2 time points during pregnancy). Thus, to date, there is no comprehensive study on the Ab levels to full length VAR2CSA or its individual domains and allelic variants throughout the entire course of pregnancy.

1.9 Advances in vaccine development for placental malaria

1.9.1 VAR2CSA: Leading vaccine candidate

Data support the feasibility of a VAR2CSA-based vaccine for protecting pregnant women. For a surface antigen, VAR2CSA is surprisingly well-conserved, with limited polymorphism and is the main target of protective immunity against placental malaria [97, 98, 103, 111]. The large size of the molecule makes it difficult to produce a vaccine using the entire molecule; accordingly, extensive efforts are being made to identify the region(s) within the molecule that binds CSA. The minimal binding site is a strong vaccine candidate based on the findings from animal studies. However, there is no data on immunity to ID1-ID2a in humans.

1.9.2 Information needed for vaccine development

Testing first-generation VAR2CSA-based vaccines in pregnant women is going to be a challenge. Thus, it is important to understand the characteristics of Ab that mediate protection and researchers need serological assays that accurately measure them. It is likely Ab mediate their effect by preventing the binding of IE to CSA, but this has been difficult to firmly establish in women with naturally acquired immunity. Therefore, identification of correlates of protection is needed. Approaches other than serology, such as functional assays (phagocytosis, inhibition of binding, etc.) are needed to identify an assay or combination of assays that assess the immune status of pregnant women for placental malaria.

In addition, there is limited information on how long Ab persist to VAR2CSA. Ab to common malarial adhesins appear to be short lived (ex. merozoite proteins 0.8-7.6 years) [125] compared to half-life of Ab to childhood viruses (ex. measles 457 years), requiring frequent exposure to antigens, in order to sustain protective serum IgG levels. Data from

study conducted by Fowkes et al. (2012) using samples from Thai pregnant women showed that Ab half-life to DBL5 is 36-157 years [125], in a low malaria transmission area. There are no Ab longevity data for high malaria transmission areas, e.g., those found throughout Africa . Knowing how long Ab to VAR2CSA persist is important for establishing continuous protective immunity against malaria in pregnant women.

1.10 Goals of the study

The goals of the current study were:

1. Estimate how long Ab to full length VAR2CSA and its 6 DBL domains persist in women who receive IPT, using a statistical modeling approach.
2. Determine if women produce Ab to ID1-ID2a during the course of natural infection and if Ab levels to ID1-ID2a correlate with protection from placental malaria.
3. Utilize a multi-assay approach to define correlates of protection from placental malaria.

CHAPTER 2

THE INTERMITTENT PREVENTIVE TREATMENT AND INSECTICIDE-TREATED BEDNETS REDUCE ANTIBODY LEVELS THAT CONTRIBUTE TO PROTECTION FROM PLACENTAL MALARIA IN PREGNANT CAMEROONIAN WOMEN

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Anna Babakhanyan[1], Yeung L. Tutterrow[1], Naveen Bobbili[1], Joseph D. Smith[2], Marion Avril[3], Ali Salanti[3], Josephine Fogako [4], Robert J. Leke [4], Freya Fowkes, Rose G.F. Leke[4], and Diane W. Taylor[1#]

1 University of Hawaii at Manoa, John A. Burns School of Medicine, Department of Tropical Medicine, Medical Microbiology and Pharmacology, Honolulu, Hawaii, U.S.A.

2 Seattle Biomedical Research Institute, Seattle, Washington, U.S.A.

3 Centre for Medical Parasitology, Department of International Health, Immunology and Microbiology, University of Copenhagen and Department of Infectious Diseases, Copenhagen University Hospital (Rigshopitalet), Copenhagen, Denmark.

4 The Biotechnology Center, Faculty of Medicine and Biomedical Research, University of Yaounde I, Yaounde, Cameroon

Corresponding author. Mailing address: Departments of Tropical Medicine, Medical Microbiology and Pharmacology, John A. Burns School of Medicine, University of Hawaii at Manoa. 651 Ilalo Street, BSB 320, Honolulu, HI 96813. Phone: 808-692- 1979. Email: dwtaylor@hawaii.edu.

2.1 Abstract

Humoral immunity to the malarial adhesin VAR2CSA plays a critical role in protection from placental malaria. We evaluated the influence of combined use of intermittent preventive treatment and insecticide-treated bednets (IPT/ITN) on the antibody response to VAR2CSA in pregnant women living in Cameroon, where malaria is holoendemic. Archival samples collected longitudinally from 91 pregnant women "Before IPT/ITN" implementation and 144 pregnant women "After IPT/ITN" implementation were screened using multianalyte platform assay. Antibody levels to full-length VAR2CSA, the 6 DBL domains and 15 strain variants (3D7, 7G8, FCR3, A4) were measured, as well as antibody avidity to full-length VAR2CSA. The longevity of antibodies to VAR2CSA and its variants were determined using a statistical modeling approach. Antibody levels to the full-length recombinant VAR2CSA (all gravidities $p=0.0001$) and its domains (DBL3, DBL4, DBL5 and DBL6: $p<0.05$), as well as prevalence of Cameroonian women with antibodies to VAR2CSA (full-length protein, DBL3, DBL5, DBL 6: $p<0.05$), were lower in women who received IPT/ITN compared to women who did not. These changes are more notable in primigravidae, whose Ab levels were lower the longer they received IPT/ITN. VAR2CSA antibodies were short-lived in primigravidae and long-lived in multigravidae. Finally, the quality of response to VAR2CSA was decreased in primigravidae and multigravidae who received IPT/ITN: average number of domains and variants recognized, and average avidity of antibodies to the full-length VAR2CSA (all gravidities $p<0.05$). Knowing how long antibodies to VAR2CSA persist is important for establishing continuous protective immunity against malaria in pregnant women.

2.2 Introduction

In sub-Saharan Africa, an estimated 32 million pregnant women at risk of *Plasmodium falciparum* malaria and its adverse consequences such as severe maternal anemia, spontaneous abortion, stillbirth and low birth weight babies [4]. Birth weight has a strong association with a child's survival during the first year of life, and malaria is implicated in 75,000-200,000 infant deaths every year [11]. HIV positive pregnant women are twice as likely to have malaria during pregnancy, due to reduced humoral responses and the fact that antimalarials are not efficacious because of drug interactions with HAART [30, 77].

Pathology associated with malaria in pregnant women, or placental malaria, is largely due to the sequestration of *P. falciparum*-infected erythrocytes (IE) in the intervillous space of the placenta that induce an inflammatory response [30,77]. Specialization and adaptation of *P. falciparum* to unique ecological niche of the human placenta is mediated by v*ar2csa* virulence gene. VAR2CSA is expressed on the surface of IE and binds to chondroitin sulfate A (CSA) on syncytial trophoblasts lining IVS [48, 71]. Unlike UpsA and Ups B/C PfEMP1 gene groups, v*ar2csa* genes exhibit unusual sequence conservation. Antibodies (Ab) to VAR2CSA reduce the binding of IE to trophoblasts at the maternal-fetal interface and improve pregnancy outcomes [105-107]. Over successive pregnancies, women produce Ab to VAR2CSA, which is a prime vaccine candidate for placental malaria [77,84].

In the absence of a placental malaria vaccine, the World Health Organization recommends the use of intermittent preventive treatment and long-lasting insecticide-treated bednets (IPT/ITN) to control malaria during pregnancy and to reduce fetal and maternal morbidity and mortality [116]. Several studies that have evaluated the influence of IPT/ITN on Ab to VAR2CSA, but they have reached opposing conclusions and recommendations [95,121, 123, 124]. Initially, it was suggested that only primigravidae (PG) should receive chemoprophylaxis [121]. However, Staalsoe et al. showed a decrease in Ab to the surface of CSA-adhering malarial parasites in PG receiving IPT/ITN, suggesting that these women should remain on IPT during subsequent pregnancies [122]. Later, Aitken et al. confirmed that IPT reduces Ab levels to

VAR2CSA in Malawian primigravidae and multigravidae [95]; however, Serra-Casas et al. did not find a decline in Ab in a cohort of women in Mozambique [123]. All the studies were conducted in malaria low or seasonal transmission areas and measured Ab that bind to the surface of CSA-binding IE [95, 122, 123] or to the Duffy binding-like (DBL) domain 5 (DBL 5) [124, 125], the most immunogenic DBL domain of VAR2CSA [124]. Only one of the studies was longitudinal which was community-based and did not obtain data on placental malaria status in individual women. Therefore, the impact of IPT/ITN is unclear. Thus, a basic question becomes, is the decrease in Ab to VAR2CSA significant enough to be of biological relevance? Will women who receive IPT/ITN be susceptible to placental malaria in subsequent pregnancies? Currently, there is no ethical study design that will allow these questions to be directly addressed.

Recently, Ab to multiple DBL domains of VAR2CSA and multiple allelic variants (i.e., a broad Ab repertoire) from early in pregnancy through term was found to correlate with absence of placental malaria at delivery [109]. In addition, high avidity Ab to full-length VAR2CSA (FV2) correlated with absence of placental malaria [110]. Women who had ≥35% of high avidity Ab had reduced risk of placental malaria at delivery [110]. Accordingly, in the longitudinal study reported herein, we sought to evaluate the combined effect of IPT/ITN on the Ab response to VAR2CSA in malaria high transmission area and compare the Ab responses to VAR2CSA in the results with women who did not receive IPT/ITN using samples collected prior to implementation of ITP/ITN.

This is a first comprehensive study on the influence of IPT/ITN on the natural acquisition of Ab to the full-length recombinant VAR2CSA and all of its domains and 16 strain variants in women living in a malaria high transmission area. Further, this study provides side by side comparison of immune profile of Ab to VAR2CSA in "Before IPT/ITN" and "After IPT/ITN" implementation in cohorts of women. In addition, this study is unique, since we assessed the influence of IPT/ITN on the known correlates of protection from placental malaria, as a way to help assess the quality of the Ab response. Finally, we estimated the longevity of Ab to VAR2CSA, which provides data on the level of protection women may have in subsequent pregnancies.

2.3 Materials and Methods

IRB Approvals. Archival "Before IPT" samples used in the current study were originally obtained with IRB approval from Georgetown University and the Ethics Committee, Ministry of Health Cameroon. The use of coded plasma sample and clinical data in the current study was exempt from human subject research by the Committee on Human Studies, University of Hawaii, Manoa (CHS#19912). The study assessing the influence of IPT/ITN on immunity to malaria as is being conducted with the approval of the National Ethics Committee, Cameroon and the IRB at the University of Hawaii (CHS#14880). All women and men participating in the studies gave written informed consent.

Study Population. The studies took place in the rural villages of Ngali II and Ntouessong, Cameroon, in which *P. falciparum* malaria is holoendemic and an entomological inoculation rate of ~1 infectious bite / person/ every other night throughout out the year [128]. Between 2001 and 2006 prior to implementation of IPR/ITN, a cohort of pregnant women were enrolled at antenatal clinics during the first trimester, followed monthly until delivery, and prescribed anti-malarial treatment when slide-positive, see Leke et al. for details. Archival samples of peripheral plasma from 91 of these women, "Before IPT/ITN" cohort (n= 41 followed throughout pregnancy, n=50 enrolled at delivery) were used in the current study. In 2007, a second prospective longitudinal study was initiated to evaluate the impact of IPT/ ITN on pregnancy-associated immunity. Women attending the same antenatal clinics were enrolled at various times during pregnancy, at which time they received 1500 mg Sulfadoxine and 75 mg Pyrimethamine (SP) treatment and long-lasting ITN. SP treatment was given every other month. Accordingly, women were protected from malaria for various periods of time based on when they initially enrolled in the study (range 4 to 23 weeks). Women were monitored for parasitemia monthly until delivery. Plasma samples from 144 of these women ("After IPT/ITN" cohort) were used in the current study. Table 1 provides demographic and clinical information on the women studied before and after implementation of IPT/ITN. Blood samples from age-matched males (n = 20) residing in Ngali II were used as negative controls in the serological assays.

Detection of P. falciparum infections by microscopy and PCR. *P. falciparum* infections were detected microscopy and PCR. Routine thick and thin blood smears were prepared from peripheral blood samples collected during pregnancy and at delivery. They were strained with DiffQuick (Stain Kit IMEB Inc. Catalog Number: K7128-3, San Marcos, CA) and examined by two microscopists for the presence of infected erythrocytes. PCR was conducted by isolating DNA from 100ul of erythrocyte pellets and amplification of the small subunit of ribosomal RNA according to Snounou et al. [109]. At delivery, a section of the placenta was collected and used to prepare impression smears and histological sections of placental tissue. A woman was considered to have placental malaria if IE were detected in either blood smears of intervillous space blood, impression smears of villous tissue, or histological sections of the placenta.

VAR2CSA antigens. The panel of recombinant proteins of different regions of VAR2CSA has been described previously [109], and included: full length (FV2), DBL1+2, DBL 2, DBL 3, DBL 4, DBL 5, DBL 6 of the FCR3 strains expressed in Baculovirus (BV) from A. Salanti (University of Copenhagen, Denmark); DBL-1, DBL3, DBL4, DBL5, and DBL6 for the 7G8 and 3D7 strains expressed in *Pichia pastoris* (PP) from J.D. Smith and colleagues (Seattle Biomedical Research Institute), and DBL 3 from A4 parasites expressed in *Escherichia coli* provided by Kavita Singh and Carole Long (NIAID, NIH, Bethesda, MD). In addition, three non-pregnancy-associated malarial antigens (AMA-1, MSP-1, MSP-2 of the 3D7 strain) were used to assess malarial immune status of the women.

Multianalyte Platform (MAP) assay. Details of the methodology have been published previously [109, 132]. Each recombinant protein was covalently coupled to 1 million SeroMap beads with different spectral addressed at optimal concentrations: 1 µg for DBL domains, 3 µg for FV2. Antigen-coupled beads were pooled and tested to verify that competition did not occur among the antigens. The MAP assay was performed as previously described [109, 110, 132]. Briefly, 50µl of antigen-coupled microspheres (2,000 microspheres/test) were incubated with 50µl of a 1:100 dilution of plasma in PBS-1% BSA (phosphate buffered saline containing 1% bovine serum albumin [BSA]) in pre-wetted wells of filter plates (96 well Multiscreen BV; Millipore, Billerica, MA) for 1 hr

at 25°C on a rotating shaker at 500 rpm (Microplate Shaker, Lab-line, Melrose Park, IL). Wells were washed twice with PBS-0.05% Tween20 and once with PBS-1% BSA. Then, 100µl of secondary Ab, R-phycoerythrin-conjugated, Affini Pure F(ab')2 fragment, Goat anti-human IgG Fc fragment specific, (Jackson Immunoresearch, West Grove, PA, Cat # 109-116-170) diluted to 2µg/ml in PBS-1% BSA was added to each well and incubated as above in the dark for 1hr. Microspheres were washed as described above, re-suspended in 100µl PBS-1% BSA, and 85 µl of the microsphere suspension was analyzed using a Liquichip M100 reader (Qiagen, Valencia, CA). The reader was programmed to analyze a minimum 100 beads per spectral address, DD Gate 7500-15000, and 35 sec timeout. The results are expressed as mean fluorescence intensity (MFI). Positive and negative controls were run on each plate that included 3 different pools of plasma from 8 Cameroonian multigravidae with high Ab levels to VAR2CSA and pools of plasma from 40 Americans (US control) who never travelled to malaria endemic areas.

***Determining the breadth of the VAR2CSA antibody response*.** The panel included 15 different recombinant variants from 3 different strains (i.e., 3D7, FCR3, 7G8) of VAR2CSA that represent the 6 DBL domains. A woman was considered to be Ab-positive for each of the 15 antigens if her plasma had a mean MFI plus 2 standard deviations greater than 20 males living in the same villages. Thus, in determining the breadth of the Ab response (repertoire) the total number of variants a women recognized was determine, giving a score of 0-15 variants (note: DBL1+2 was excluded from this analysis). A woman was considered Ab positive for a particular domain, if she had Ab to one or more variants of that domain, i.e., giving a breadth of DBL domains of 0-6. Mean Ab levels plus two standard deviations of US control was used as a cut-off for seropositivity to non-pregnancy specific malarial antigens.

***Avidity to the full-length VAR2CSA*.** Samples collected at delivery (or sample immediately prior to delivery if delivery sample was not available) were screened in the avidity assay for FV2-seropositive women. The avidity assay was performed as previously described [110]. Briefly, plasma was diluted 1:300, 1:1000 and 1:3000 in 1% BSA-PBS and 50µl of diluted plasma was added to six wells (each dilution in duplicate) containing 50µl of FV2-coupled microspheres (2,000 microspheres/test) and incubated

for 1 hr on a shaker. After incubation, 100µL of 3M NH4SCN in 1% BSA-PBS was added to half of the wells and 100µL of 1% BSA-PBS was added to the other half (matching control well for each dilution). After 30 minutes incubation, the wells were washed and incubated with secondary Ab, washed, and analyzed by MicroChip 100 as described in MAP assay. Avidity was determined for each dilution by the following formula: (MFI obtained from wells incubated with salt)/(MFI obtained from corresponding control wells) X 100, and average was taken across 3 dilutions. Positive and negative controls were included on each plate and no plate to plate variation was observed for positive controls, indicating that the assay is highly repeatable.

***Antibody half-life determination*.** Plasma samples (total of 498) obtained from IPT/ITN study were used to calculate Ab half-lives to the full-length VAR2CSA and its variants using previously published methods [125] and Simple STATA 12 software. In brief, antibody half-lives were estimated from the fixed-effects slope component of the linear mixed-effects repeat measure regression models, the 95% confidence intervals were calculated using SD of the slope. Models were adjusted for predefined confounders, such as gravidity, number of IPT doses received, *P. falciparum* infection (peripheral blood slide data). Model was initially adjusted for the ITN variable as well; however, since it did not have any significant influence on the model, ITN variable was eliminated from the model. Gravidity was included as an interaction term, since we assumed that the slope describing Ab decrease for PG and multigravidae (MG) is different. The 95% confidence intervals were derived from the estimate and between-woman standard deviation of the slope of the linear mixed-effects model.

Statistical analysis. Discrete variables in Table 1 and Table 3, such as mean age, gravidity, average number of domains, etc., were compared using an unpaired, two-tailed 2-sample t-test, with 95% CI. Prevalence data were compared using Fisher's exact t-test, 2 tailed (95% CI). In order to compare Ab levels between "Before IPT" and "After IPT" groups of women in Fig. 1A and Fig. 2A, data were analyzed using Mann-Whitney U-test. Prevalence data in Fig. 1B and Fig. 2B were analyzed using Fisher's exact test. Percent changes between 3 groups in Figure 3A and 3 groups in Figure 3B were

compared using Fisher's exact test. Statistical analysis was performed using GraphPad Prism 6.

2.4 Results

Description of study participants. A comparison of demographic and clinical characteristics of pregnant women enrolled "Before IPT/ITN" and "After IPT/ITN" are shown in Table 1. Women in the two cohorts were similar with respect to age (PG: p=0.64, MG: p=0.89), gravidity (MG: p=0.57), and prevalence of peripheral malaria at enrollment (PG: p=0.2; MG: p=0.48). Prior to implementation of IPT/ITN; however, none of the women took chemoprophylaxis or used ITN; whereas, after implementation all enrolled women received SP and 69% received ITN. The intervention had a significant impact on prevalence of malaria (Table 1). Prevalence of malaria was significantly reduced in women receiving IPT/ITN with respect to prevalence of malaria based on peripheral blood slides made during pregnancy, t delivery and of placental tissue.. Of importance, PM was reduced from 75% to 11% in PG (p=0.0045) and from 52% to 6.4% in MG (p<0.0001). Data in Table 1 confirm the major detectable difference between the two cohorts was prevalence of malaria due to use of IPT/ITN.

Influence of IPT/ITN on acquisition of Ab to VAR2CSA in primigravidae. To determine how IPT/ITN influences acquisition of IgG to VAR2CSA, IgG to individual domains/variants and full-length VAR2CSA and non-pregnancy associated malarial antigens (AMA-1, MSP-1 and MSP-2), Ab levels were measured in plasma samples from PG in the "Before" and "After" IPT/ITN cohorts at delivery. In addition, plasma samples from 20 Ngali II males were collected and screened for comparison Males do not have Ab to VAR2CSA because high numbers of CSA-binding IE are not selected for except in the placenta. . Mean IgG levels (Fig. 1A) and prevalence of women who had Ab (Fig. 1B) were calculated and compared to the Ab levels in "Before IPT/ITN" cohort. No significant differences in IgG levels to MSP-1 (p=0.25) and MSP-2 (p=0.16) were observed between "Before IPT/ITN" and "After IPT/ITN" cohorts, with slightly higher IgG levels to AMA-1 (p=0.0083) in the "After IPT/ITN" cohort. PG who received

IPT/ITN had some Ab to VAR2CSA, despite chemoprophylaxis during pregnancy (Fig. 1A); however, these PG had significantly lower IgG levels to FV2 (p=0.0001) and DBL3 (p=0.0037), DBL4 (p=0.0018), DBL5 (p=0.0003) and DBL6 (p=0.002) compared to PG who did not receive IPT/ITN (data for other variants in Fig. S1A). Further, prevalence of PG who had Ab to FV2 (p=0.006), DBL3 (p=0.04), DBL5 (0.01) was significantly decreased in the "After IPT/ITN" cohort (data for other variants in Fig. S2A). Thus, about half as many PG who received IPT/ITN acquired Ab to VAR2CSA and its domains than those who did not receive treatment. In addition, among the PG who did produce Ab, Ab levels were approximately 2-times lower in women who received IR/ITN compared to those who did not. No significant differences were observed in prevalence of Ab to non-pregnancy malarial antigens between the two studies, 95-100% PG in both studies had Ab to AMA-1, MSP-1 and MSP-2.

Influence of IPT/ITN on maintenance of Ab to VAR2CSA in multigravidae. MG were exposed to CSA-binding malaria parasites during previous pregnancies and are therefore likely to have VAR2CSA-specific IgG. We evaluated the influence of IPT/ITN on boosting and maintenance of Ab toVAR2CSA in MG and compared these data with "Before IPT/ITN" MG Ab profiles. MG in "After IPT/ITN" cohort had IgG to AMA-1 (p=0.06) and MSP-1 (p=0.5) at similar levels compared to "Before IPT" cohort, with lower Ab levels to MSP-2 (p<0.0001) (Fig. 2A). Results show that VAR2CSA-specific IgG to FV2 (p<0.0001), DBL3 (p=0.0015), DBL5 (p<0.0001) and DBL6 (p<0.0001) at delivery were significantly lower in MG receiving IPT/ITN compared to MG in "Before IPT/ITN" cohort (Fig. 2A; data for other variants in Fig. S1B). Marked decreases in Ab prevalence to FV2 (p=0.001), DBL 3 (p=0.01), DBL 5 (p=0.066), and DBL 6 (p<0.0001) were found in women who received IPT/ITN (Fig. 2B; data for other variants in Fig. S2B). Once again, no differences were found in prevalence of MG with Ab to AMA-1, MSP-1, and MSP-2.

Changes in antibody levels between enrollment and delivery in multigravidae receiving IPT/ITN. Since some MG in IPT/ITN study maintained their Ab levels to FV2 and its domains while on IPT/ITN and others did not, we determined the proportion of MG with a decrease in their Ab levels to FV2 (Fig. S3). MG (n=41) who received IPT/ITN for 2

trimesters (i.e., were on SP for 14-23 weeks) were stratified into two groups: those who were peripheral-smear positive at least once during pregnancy (Fig. 3A) and those who were not (Fig. 3B). Each group of women was further divided into 3 groups based on changes in Ab levels to various Ag between enrollment and delivery: increased, no change, and decreased. The proportion of MG in each group was calculated and plotted (Fig. 3). In most smear-positive and smear-negative MG there was no change in Ab levels between enrollment and delivery to AMA1; whereas, among smear-negative MG a higher proportion had a decrease in Ab levels to MSP-1 and MSP-2. The proportion of MG in each of 3 groups was variable to VAR2CSA domains and FV2, with 20-45% of women having a decrease in Ab levels at delivery compared to the levels at enrollment.

The trend was that if a woman had decreased IgG for one VAR2CSA variant/domain, she had decrease in IgG for all the other domains and variants as well. Overall, a higher proportion of MG who were smear-positive had a increase in Ab levels to FV2 and its domains compared to smear-negative MG. No statistically significant differences were observed between smear-positive and smear-negative MG, as assessed by Fisher's exact test, probably due to small sample size in each of the 3 groups of women. Thus, Ab boosting in some MG occurred despite IPT/ITN intervention. No significant difference was observed in number of IPT doses and bednet usage between women who were smear positive during pregnancy and those who were not.

Estimated antibody longevity in women receiving IPT/ITN. In the current study, we used plasma collected longitudinally from pregnant Cameroonian women receiving IPT and using ITN to help establish how long Ab to VAR2CSA persist and how Ab persistence to VAR2CSA compares with longevity of Ab to AMA1, MSP1 and MSP2. . On average, Ab levels to FV2 decreased in PG during the course of pregnancy; the longer PM received IPT the lower their Ab levels were (Fig. S4A). Ab half-life in PG was about 1 year for FV2 and 0.5-3 years for Ab to DBL1-6 (Table 2). Ab to FV2 in MG did not change during the course of pregnancy for most women, especially among low-responders (Fig. S4B). Ab half-life to FV2 in MG ranged from 4 years to infinity; Ab half-life to other domains ranged from 0.6 years-6 years (Table 2). Those PG and MG who had none/little Ab to FV2 before they started IPT, did not have any changes in their

immune response. Ab half-life to AMA-1 was about 11 years, and shorter for Ab to MSP-1 (2 years) and MSP-2 (1 years).

Influence of IPT/ITN on potential correlates of protection from PM. Since Ab levels alone do not correlate with protection from PM [22,23], we sought to determine the Ab quality of the immune response to VAR2CSA different between women who received IPT/ITN and those who did not. In the absence of well- established correlate of protection for PM, we assessed the influence of IPT/ITN on the potential correlates of protection identified previously [22,23], namely Ab to the FV2, breadth of VAR2CSA antibody repertoire, and avidity (Table 3 and Fig. S5). Prevalence of women with Ab to FV2 dramatically decreased in the IPT/ITN cohort of women (PG: p=0.006; MG: p=0.001). In addition, women on IPT/ITN produced Ab to fewer domains (PG: p=0.016; MG: p=0.024) and variants (PG: p=0.016) of VAR2CSA, , indicating that the breadth of response diminished. Further, on average, women in the IPT/ITN study had lower Ab avidity to FV2 (PG p=0.0001, MG p=0.021). Although not statistically significant, fewer women had high avidity Ab to FV2 (high avidity Ab are defined as >35%). Overall, these results indicate that women on IPT/ITN are likely to be less protected from PM based on these results. .

2.5 Discussion

This study evaluated the combined effects of IPT/ITN on the Ab response to VAR2CSA in women living in a high malaria transmission area and compared the data with Ab responses to VAR2CSA in the absence of these interventions. The principal findings were that Ab levels to full-length recombinant VAR2CSA and its domains, as well as the prevalence of Cameroonian women with Ab to VAR2CSA, are lower in women who received IPT/ITN compared to women who did not. These changes were more notable in PG, whose Ab levels continued to decrease the longer they were on IPT/ITN. VAR2CSA Ab were short-lived in PG and long-lived in MG. Finally, the quality of response to VAR2CSA, as assessed by currently available correlates of protection, were reduced in PG and MG who received IPT/ITN.

We established that the use of IPT/ITN during the second and third trimesters interfered with the natural acquisition of Ab to VAR2CSA in Cameroonian PG and maintenance of Ab in MG. IPT/ITN, which effectively reduce exposure to *P. falciparum* during pregnancy, resulted in lower prevalence of peripheral and placental malaria at delivery (Table 1, note that prevalence of peripheral malaria at enrollment in "Before IPT/ITN" and "After IPT/ITN" cohorts was the same). Our findings suggest that PG on IPT/ITN were less likely to make Ab to the full-length VAR2CSA and to fewer domains and variants (Fig. 1); and they produced Ab at a significantly lower levels than women who did not receive IPT/ITN. These results are in accord with data from Kenyan [122], Malawian [95] and Senegalese [124] women, but differ from results reported from Mozambique [123].

Interestingly, both PG and MG on IPT/ITN produce high levels of Ab to non-pregnancy specific antigens, with essentially all women having Ab to the MSP-1, MSP-2 and AMA-1 (Fig. 1 and Fig. 2). Women in the village are bitten by infectious mosquitos every other night; hence, they experience a chronic infection and high exposure to malarial antigens in the absence of IPT/ITN. This results in high levels of Ab to non-pregnancy associated malarial antigens by adulthood, but these Ab do not have high affinity. Women who used IPT/ITN have significantly reduced exposure to malarial antigens, including AMA-1, MSP-1 and MSP-2. Therefore, it is theoretically possible that lower antigen exposure leads to affinity maturation of plasma cells and increase in Ab titers.

Although some Cameroonian MG maintained their Ab levels to VAR2CSA, mean Ab levels and prevalence of MG with Ab to the FV2 and its immunogenic domains was lower in MG who received IPT/ITN. Further, MG who received IPT for 2 trimesters had variable immune responses to FV2 and its domains, based on exposure to CSA-expressing parasites despite presence of IPT/ITN. Some MG maintained Ab levels; whereas, a higher proportion of women who remained smear-during pregnancy had decreased Ab levels, and a higher proportion of women who were blood smear-during pregnancy had increased Ab levels (Fig. 3). The results in Figure 3 are in line with findings from the Malawian study [95], in which the investigators suggested that

submicroscopic infections, as detected by PCR, are likely sufficient to boost Ab to VAR2CSA in MG.

Ab to common malarial adhesins appear to be short lived (MSP-1, MSP-2) in Cameroonian women, requiring exposure to antigens, in order to sustain serum IgG levels (Table 2). These results agree with findings in Thai pregnant women [125]. Ab to VAR2CSA and its domains in Cameroonian MG persisted post-partum, as was suggested before [95, 125], and half-life estimates for MG women ranged from 4 years to infinity (Table 2). Longer half-life of Ab to VAR2CSA could be partially explained by the relatively conserved nature of VAR2CSA compared to other PfEMP1 and malarial antigens. Thus, MG may have long-lived B cells that were generated during previous pregnancies. Ab to FV2 are short-lived in Cameroonian PG (less than a year), possibly indicating that plasmablasts did not mature into functional long-lived plasmablasts during a single pregnancy. The fact that VAR2CSA Ab decay rate is different in PG and MG is due to the fact that PG develop a primary immune response, while MG experience a secondary immune response. A longitudinal study with several post-partum time points is necessary to precisely to delineate VAR2CSA Ab longevity in MG. Knowing how long Ab to VAR2CSA persist is important for establishing continuous protective immunity against malaria in pregnant women.

Nevertheless, Ab levels to VAR2CSA alone do not correlate with protection from PM [110]. Thus, we examined the quality of the immune response to VAR2CSA at delivery in women who received IPT/ITN using previously established correlates with absence of PM [109, 110]. Prevalence of PG ($p=0.006$) and MG ($p=0.001$) who had Ab to the FV2 were significantly lower in women who received IPT/ITN (Table 3). Both PG and MG in IPT cohort had Ab to fewer VAR2CSA domains (PG: $p=0.016$; MG $p=0.02$) and variants (PG: $p=0.016$), and on average, had significantly lower Ab avidity to FV2 (PG: $p=0.0001$; MG $p=0.02$). A lower, but not statistically significant, prevalence of women with high avidity Ab to FV2 was observed in IPT/ITN cohort. Thus, protective immunity to PM, as assessed by currently available putative correlates of protection, was lower in women receiving IPT/ITN and therefore may not be sufficient to provide protection during subsequent pregnancies. Aitken et al. suggested that PG obtain high

levels of Ab to VAR2CSA as early as 17 weeks of gestation [95]; however, it is still to be determined when PG develop protective immunity to PM, since Ab levels alone do not correlate with protection [109,110].

Since, the new recommended IPT regimen sates that women should receive four IPT doses of PS [120], fewer PG and MG will be exposed to malaria during pregnancy and consequently to VAR2CSA. Also, since number of MG who received IPT at least for 1-2 pregnancies is increasing each year, many MG women have an immunological fingerprint similar to that of PG. Thus, during their next pregnancy (especially, if it does not happen within a year, i.e., VAR2CSA Ab half-life in PG), PG women are at risk of PM and will need to use IPT/ITN to prevent infection [122]. Furthermore, MG with high quality protective immunity to PM will lose some of their protection if they receive four doses of SP, preventing natural maturation and boosting of the Ab response to VAR2CSA.

We acknowledge several shortcomings in this study. First, we were not able to analyze the data by stratifying women based on their HIV status, since these data were not available for many study participants. However, from the WHO 2008 epidemiological data, HIV rates in Cameroon are 5-10% (WHO factsheet 2008). Thus, the decrease in Ab levels and prevalence of women with Ab to the FV2 and its variants in this study were not caused by HIV as reported in Mozambique, i.e., the decline in our study was due largely to the use of SP/ITN. Because all women who received IPT were issued ITN, it was not possible to separate the effect of SP usage and use of ITN on the decline of immunity. Next, some women withdrew from the study during pregnancy or delivered at a different clinic; therefore, limiting the number of samples available for longitudinal analysis. Finally, clinical information on the date of last pregnancy and for how many previous pregnancies a woman received IPT/ITN was not available.

The golden standard for controlling an infectious disease is a vaccine. However, it is difficult to make a vaccine for PM due to the complexity of the parasite and its life cycle, antigenic variation, difficulty in manufacturing recombinant FV2 (large-size), inadequate knowledge of host immune system interactions with the pathogen, and absence of mechanistic correlate(s) of protection from PM. Thus, IPT/ITN preventive

treatment is the only approach to protect pregnant women from PM. However, due to the fact that women obtain Ab to VAR2CSA only after the first pregnancy and the fact that IPT/ITN usage decreases overall maturation of the protective response to PM, women receiving IPT/ITN will become vulnerable to PM and need to continue receiving IPT/ITN to be protected. In addition, women who have lost their immunity will develop PM if they become infected with a SP-resistance stain, even if they receive IPT. SP-resistant parasites have already emerged and have currently reached 50% or higher in East African countries: Malawi, Uganda, western Kenya, Ethiopia, Rwanda, Tanzania, Zambia, Mozambique and throughout Asia (Thailand). Therefore, until malaria vaccine is developmed, SP resistance should be closely monitored and new effective antimalarial drugs that are safe for pregnant women are urgently needed. Studies are needed to assess VAR2CSA Ab longevity over several pregnancies in women who received IPT/ITN since their first pregnancy.

Acknowledgments

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Table 2.1. Characteristics of women

		Before IPTp and ITN	After IPTp and ITN	p-value
All women				
	Number of women	91	144	n/a
	Average number of weeks receiving SP (±SD)	0	13.5 ± 5.9	n/a
	Prevalence of women who used bed nets (%)	0	69%	n/a
Primigravidae				
	Number	18	35	n/a
	Average age (±SD)	18.1 ± 2.3	18.9 ± 6.6	0.64
	Average number of weeks enrolled in the study (±SD)	19.6 ± 6.2 (n=13) §	17.1 ± 7.2 (n=27)	0.7
	Prevalence of women who used bed nets (%)	0	66%	n/a
	Average number of weeks receiving SP (±SD)	0	13.3 ± 6.2 (n=27)	n/a
	Average number of SP doses (±SD)	0	1.9 ± 0.8 (n=27)	n/a
	Prevalence of peripheral malaria at enrollment†	69.2% (n=13)	45.7% (n=35)	0.2
	Prevalence of women with peripheral malaria at least once during the course of study	77% (n=13)	60% (n=35)	0.32
	Prevalence of women with peripheral malaria more than once during the course of study	69% (n=13)	25.7% (n=35)	**0.0085** **
	Prevalence of peripheral malaria at delivery	41% (n=17)	18.5 (n=27)	0.16
	Prevalence of placental malaria ‡	75% (n=17)	11% (n=27)	**0.0045** **
Multigravidae				
	Number	73	109	n/a
	Average age (±SD)	27.2 ± 6.4	27.1 ± 6.6	0.89
	Average gravidity (±SD)	3.8 ± 2.4	3.6 ± 2.3	0.57
	Average number of weeks enrolled in the study (±SD)	22.2 ± 6.7 (n=29)	19.2 ± 6.9 (n=93)	0.62
	Prevalence of women who used bed nets (%)	0	69%	n/a
	Average number of weeks receiving SP (±SD)	0	13.2 ± 6.2 (n=93)	n/a
	Average number of SP doses (±SD)	0	1.8 ± 0.7 (n=93)	n/a
	Prevalence of peripheral malaria at enrollment†	31% (n=29)	24.7% (n=105)	0.48
	Prevalence of women with peripheral malaria at least once during the course of study	68% (n=28)	36.2% (n=105)	**0.0046** **
	Prevalence of women with peripheral malaria more than once during the course of study	39% (n=28)	8.6% (n=105)	**0.0003** ***
	Prevalence of peripheral malaria at delivery	21% (n=73)	6.4 (n=94)	**0.0088** **
	Prevalence of placental malaria ‡	52% (n=73)	6.4% (n=94)	**<0.0001** ****

§ Numbers in parentheses represent number of women whose demographic and clinical data were available for analysis.
† Peripheral malaria by smear, numbers represent prevalence of slide-positive women.
‡ See placental malaria definition in materials and methods.

Table 2.2. Longevity of antibodies to VAR2CSA

	Primigravidae	Multigravidae
Non-pregnancy specific antigens		
AMA-1 3D7	11.4 (4.3-∞)	
MSP-1 3D7	1.9 (0.9-∞)	
MSP-2 3D7	1.0 (0.6-1.8)	
Pregnancy-specific antigens		
D1 3D7	0.5 (0.2-∞)	∞ (0.7-∞)
D1 7G8	1.4 (0.4-∞)	∞ (0.8-∞)
D1+2	1.4 (0.3-∞)	∞ (0.9-∞)
D2	0.7 (0.2-∞)	∞ (2.1-∞)
D3 7G8	1.1 (0.3-∞)	∞ (1.0-∞)
D3	1.7 (0.3-∞)	∞ (0.9-∞)
D4 7G8	∞ (0.3-∞)	∞ (0.7-∞)
D4	2.2 (0.4-∞)	∞ (1.5-∞)
D4 IT4	∞ (0.4-∞)	∞ (0.6-∞)
D5 3D7	1.5 (0.4-∞)	∞ (1.3-∞)
D5 7G8	1.5 (0.4-∞)	∞ (1.3-∞)
D5	2.9 (0.4-∞)	∞ (1.2-∞)
D6 7G8	0.7 (0.3-10.7)	∞ (6.0-∞)
D6	0.5 (0.2-∞)	∞ (3.7-∞)
D6 IT4	0.9 (0.3-∞)	∞ (1.6-∞)
FV2	0.8 (0.3-∞)	∞ (4.3-∞)

† Values represent the estimated mean antibody response half-life in years with the 95% confidence intervals in parentheses. The predicted mean half-life was calculated from the mixed-effects repeat measure regression models with covariates set to the mean. The models were adjusted for number of IPT doses, gravidity and *P. falciparum* infection (slide data). The 95% confidence intervals were derived from the estimate and between-woman standard deviation of the slope of the linear mixed-effects model.

§ All the variants of VAR2CSA were from FCR3 strain of parasites, unless specified otherwise.

Table 2.3. Influence of IPT on putative correlates of protection

	Before IPT	After IPT	p-value
Prevalence of women with antibodies to the FL at delivery (%)			
Primigravidae	72.2†	28.6	**0.006**
Multigravidae	94.5	75.3	**0.001**
Average number of domains recognized at delivery (mean ± SD) §			
Primigravidae	2.67 ± 1.37††	1.64 ± 1.34	**0.016**
Multigravidae	3.62 ± 1.16	3.09 ± 1.67	**0.024**
Average number of variants recognized at delivery (mean ± SD) §			
Primigravidae	6.05±3.39	3.42±3.55	**0.0165**
Multigravidae	8.45±2.6	7.34±4.2	0.0528
Average antibody avidity at delivery (%) (mean ± SD) §			
Primigravidae	25.35±10.25§	4.9±10.6	**0.0001**
Multigravidae	38.5±13.9	31.96±20.27	**0.021**
Prevalence of women with high avidity antibody at delivery (%)			
Primigravidae	14.3	3.7	0.26
Multigravidae	65.8	60.2	0.51

† Prevalence data for women "Before IPT" and "After IPT" groups were compared using Fisher's exact t-test.
†† Discrete variables between "Before IPT" and "After IPT" groups were compared using two-sided unpaired t-test.
§ Large confidence intervals reflect women that did not have antibodies to VAR2CSA. Avidity for these women was set to 0.

Figure 2.1.

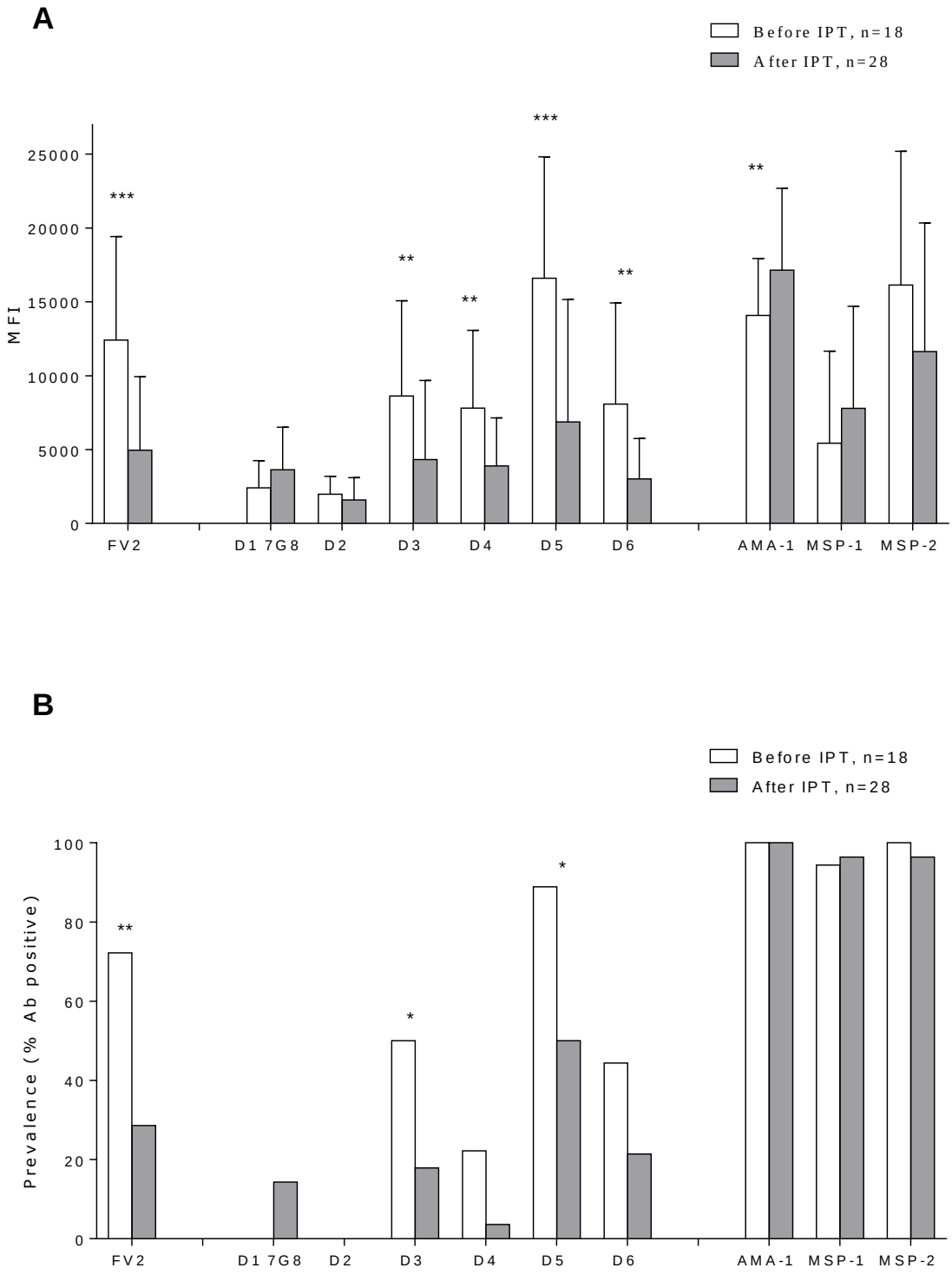


Figure 2.1. Influence of IPTp+ITN on acquisition of antibodies to VAR2CSA in primigravidae. Antibody levels at delivery (or last visit during the III trimester) were measured in Cameroonian primigravidae who received IPTp+ITN (n=28) and compared

to antibody levels in primgiravidae who did not receive IPTp+ITN (n=18). A. Mean antibody levels to VAR2CSA (+SD) and B. prevalence of women with antibodies to VAR2CSA are plotted. Antibody levels were measured to the full-length VAR2CSA and its 6 DBL domains and 15 strain variants. Note, data in the graph is presented only for FCR3 strain (besides DBL1 7G8), all the other strains had a similar pattern and are presented in Supplemental Fig. 1A and 2A. Non-pregnancy associated malarial antigens were included for comparison (AMA-1, MSP-1, MSP-2). Statistics was calculating using A. Mann-Whitney U- test, B. Fisher's exact t-test.

Figure 2.2.

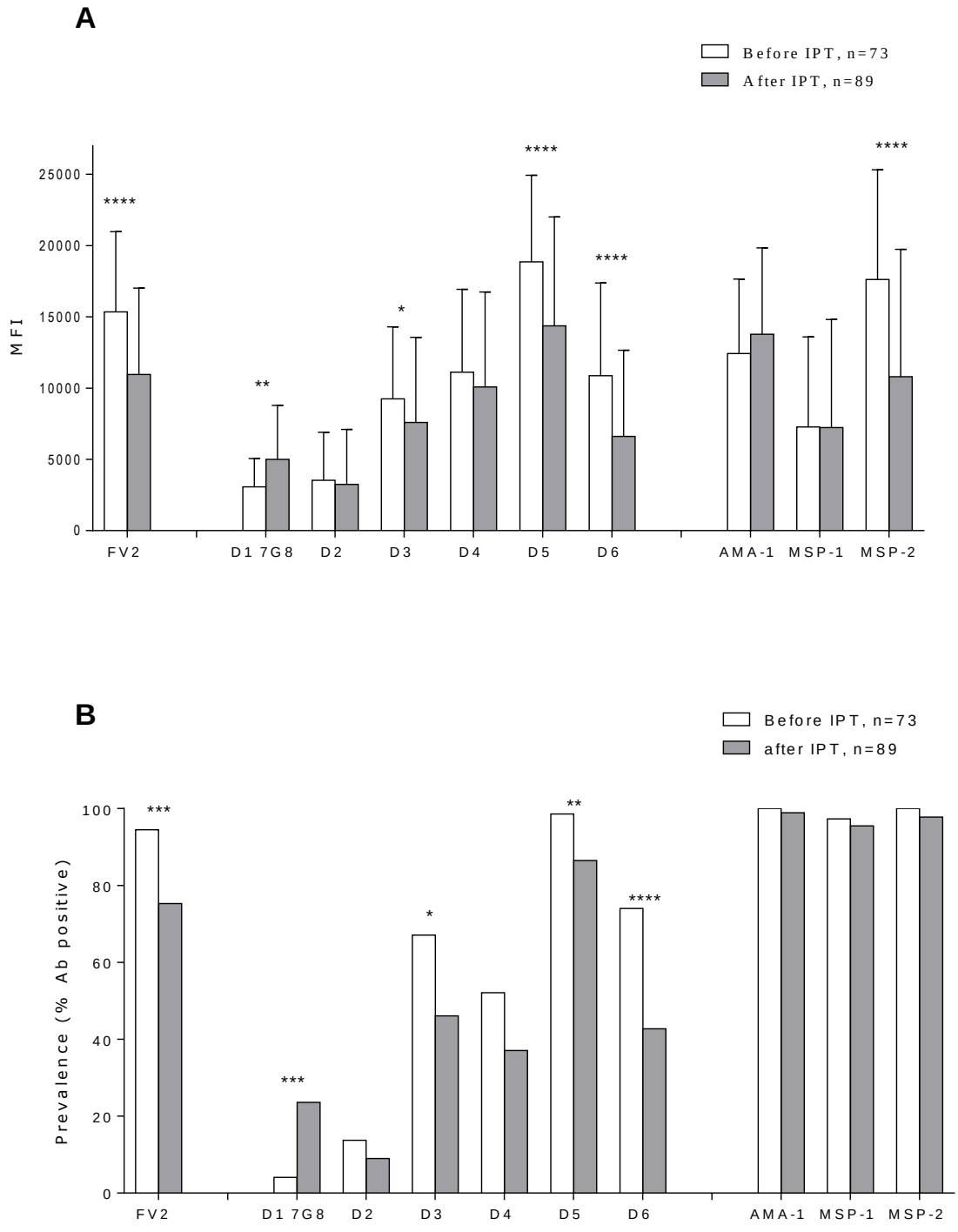
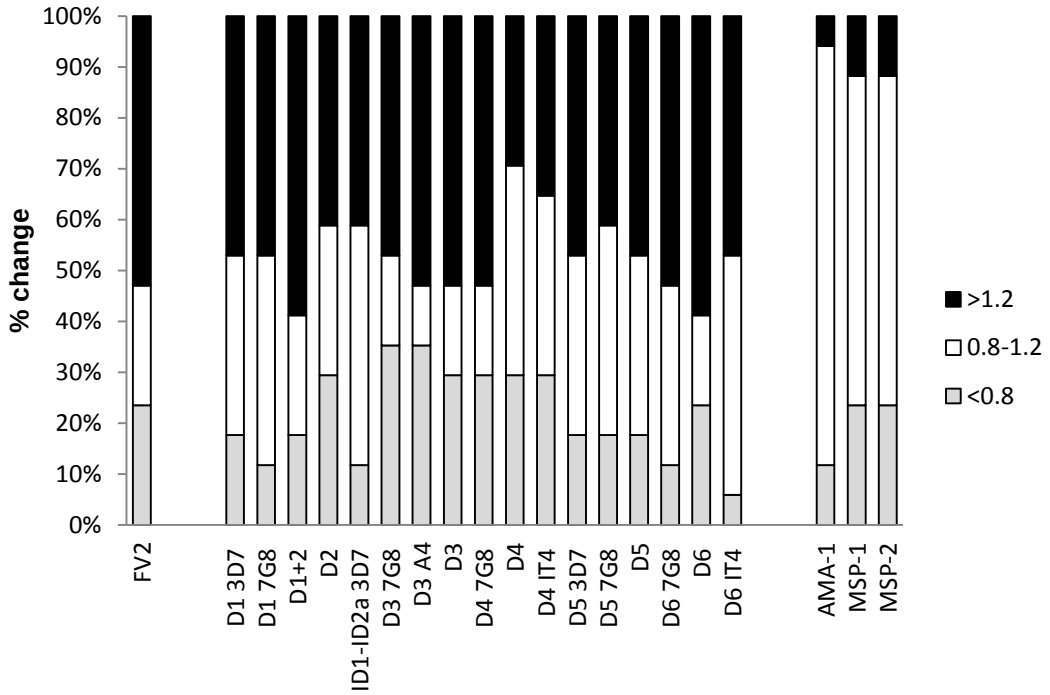


Figure 2.2. Influence of IPTp+ITN on maintenance of antibodies to VAR2CSA in multigravidae. Antibody levels at delivery (or last visit during the III trimester) were

measured in Cameroonian multigravidae who received IPTp+ITN (n=89) and compared to antibody levels in primgiravidae who did not receive IPTp+ITN (n=73). **A.** Mean antibody levels to VAR2CSA (+SD) and **B.** prevalence of women with antibodies to VAR2CSA are plotted. Antibody levels were measured to the full-length VAR2CSA and its 6 DBL domains and 15 strain variants. Note, data in the graph are presented only for FCR3 strain (besides DBL1 7G8), all the other strains had a similar pattern and are presented in Supplemental Fig. 1B and 2B. Non-pregnancy associated malarial antigens were included for comparison (AMA-1, MSP-1, MSP-2). Statistics was calculating using A. Mann-Whitney U-test, B. Fisher's exact t-test.

Figure 2.3.

A.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FV2
D1 3D7
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ID1-ID2a 3D7
D3 7G8
D3 A4
D3
D4 7G8
D4
D4 IT4
D5 3D7
D5 7G8
D5
D6 7G8
D6
D6 IT4
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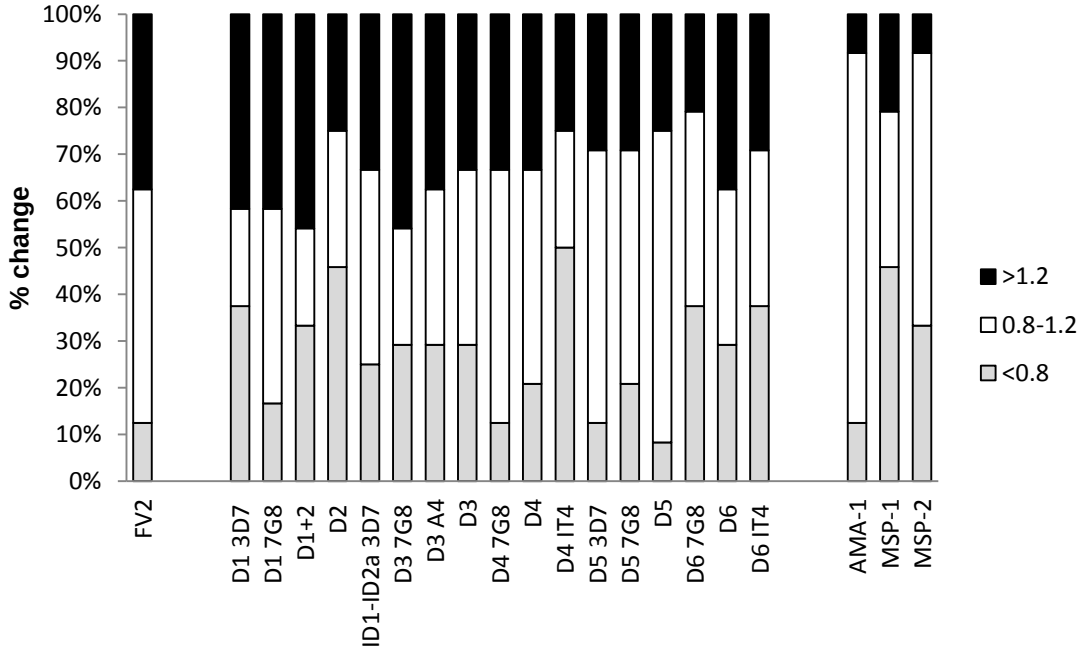
MSP-1
MSP-2
>1.2
0.8-1.2
<0.8

B.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90%
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D3 7G8
D3 A4
D3
D4 7G8
D4
D4 IT4
D5 3D7
D5 7G8
D5
D6 7G8
D6
D6 IT4
AMA-1
MSP-1
MSP-2
>1.2
0.8-1.2
<0.8

Figure 2.3. Influence of IPTp+ITN on changes in VAR2CSA levels between enrollment and delivery. Antibody levels at enrollment and delivery were measured in Cameroonian multigravidae who received IPTp+ITN for about 2 trimesters (14-23 weeks) to full-length VAR2CSA and its 6 DBL domains and 15 strain variants. Delivery/enrollment ratios were calculated for women who A. were slide positive at least once during pregnancy (n=17) and B. those who were not (n=24). Delivery/enrollment ratio of ≤ 0.8 were categorized into a "decreased" group, 0.9-1.1 were categorized into a "no change" group and those who had ratios of ≥ 1.2 were categorized into an "increased" group. Prevalence for each antigen for each of the 3 groups was calculated and plotted as % change.

Figure S2.1.

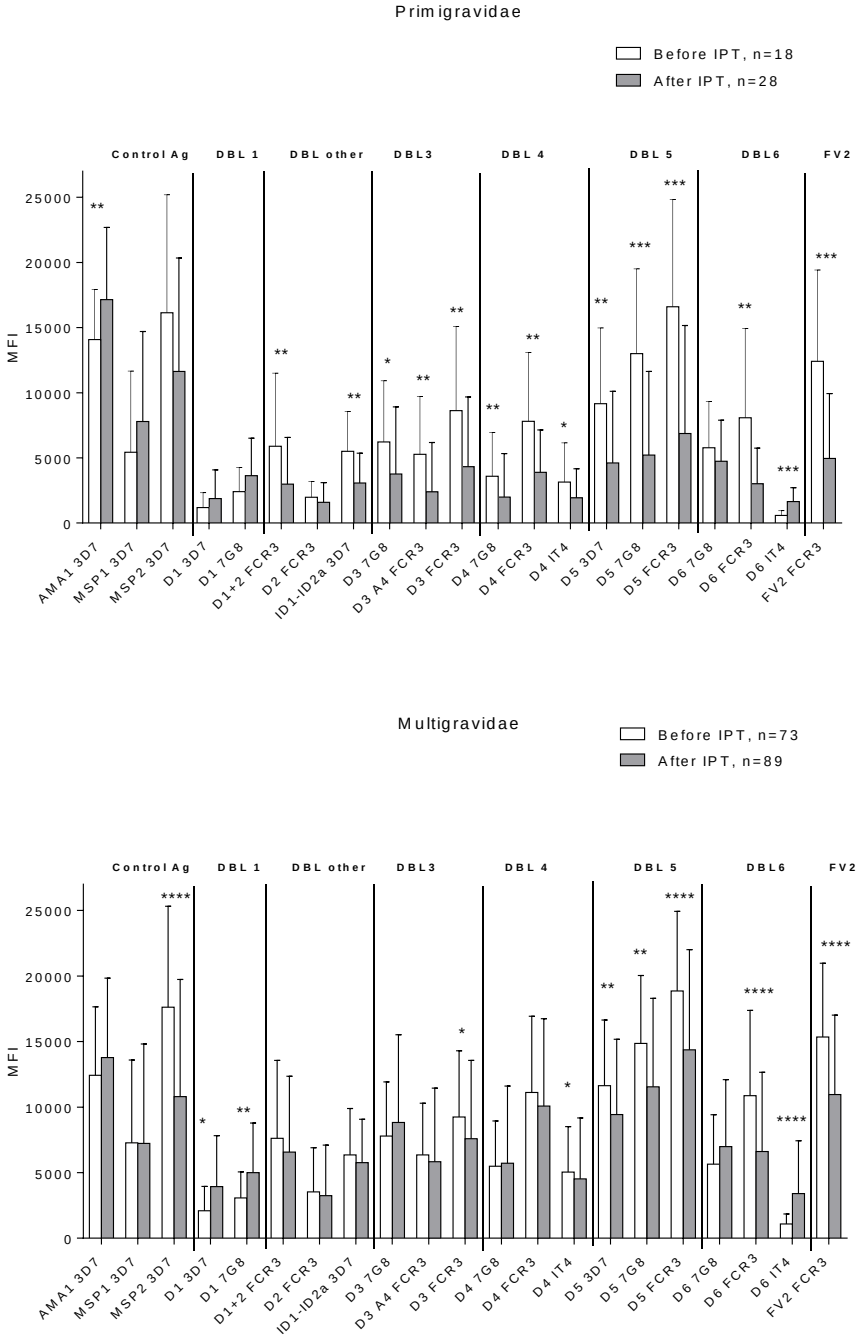


Figure S2.1. Influence of IPTp+ITN on antibody levels to VAR2CSA in Cameroonian women at delivery. Antibody levels at delivery (or last visit during the III trimester) were measured in Cameroonian **A.** primigravidae and **B.** multigravidae, who received IPTp+ITN (PG n=28, MG n=89) and compared to antibody levels in women who did not receive IPTp+ITN (PG n=18, MG n=73). Mean (+SD) antibody levels to the

full-length VAR2CSA and its 6 DBL domains and 15 strain variants are plotted. Note, ID1-ID2a 3D7 is the CSA-minimal binding domain. Non-pregnancy associated malarial antigens were included for comparison (AMA-1, MSP-1, MSP-2). Statistics was calculating using Mann-Whitney U-test.

Figure S2.2.

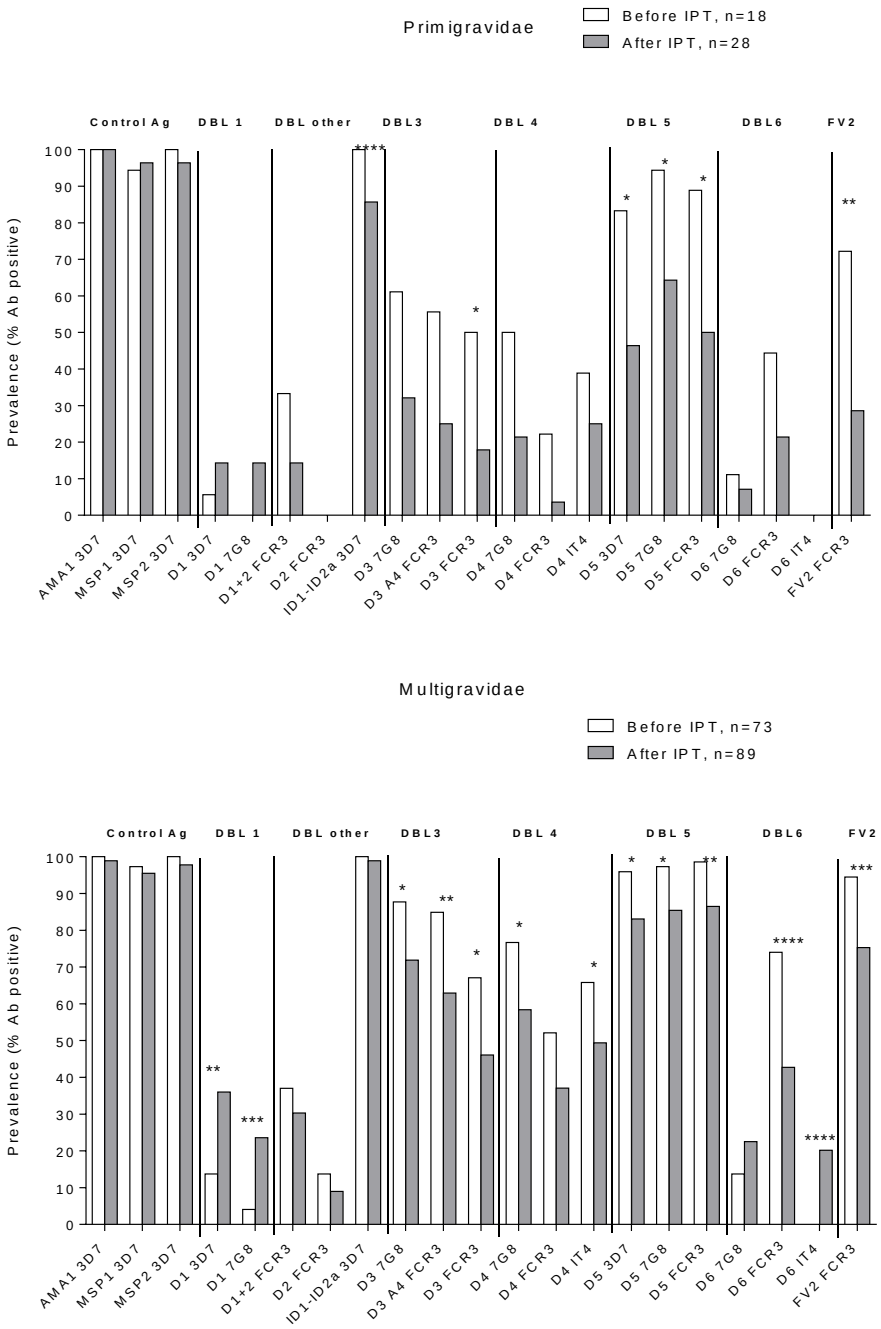


Figure S2.2. Influence of IPTp+ITN on maintenance of antibodies to VAR2CSA in multigravidae. Antibody levels at delivery (or last visit during the III trimester) were measured in Cameroonian **A.** primigravidae and **B.** multigravidae, who received IPTp+ITN (PG n=28, MG n=89) and compared to antibody levels in women who did not receive IPTp+ITN (PG n=18, MG n=73). Prevalence of women with antibodies to the

full-length VAR2CSA and its 6 DBL domains and 15 strain variants are plotted. Antibody levels were measured to the full-length VAR2CSA and its 6 DBL domains and 15 strain variants. Non-pregnancy associated malarial antigens were included for comparison (AMA-1, MSP-1, MSP-2). Statistics was calculating using Fisher's exact t-test.

Figure S2.3.

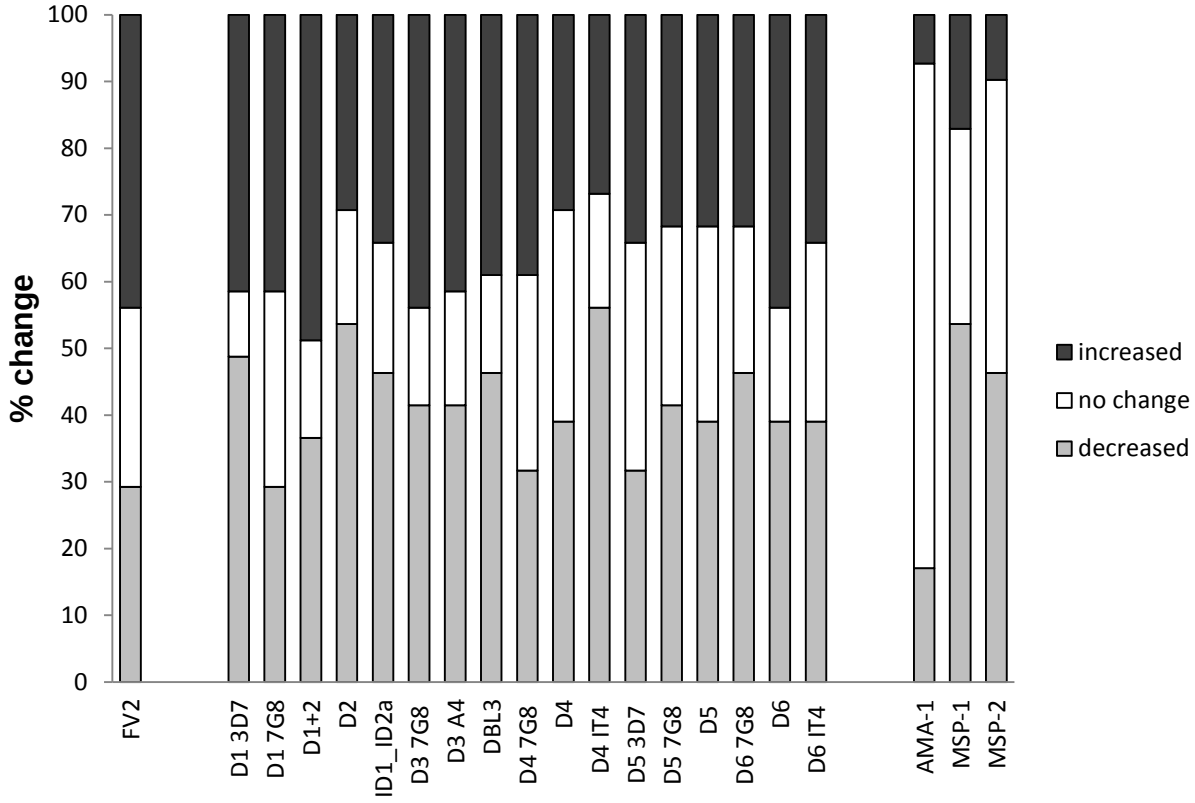
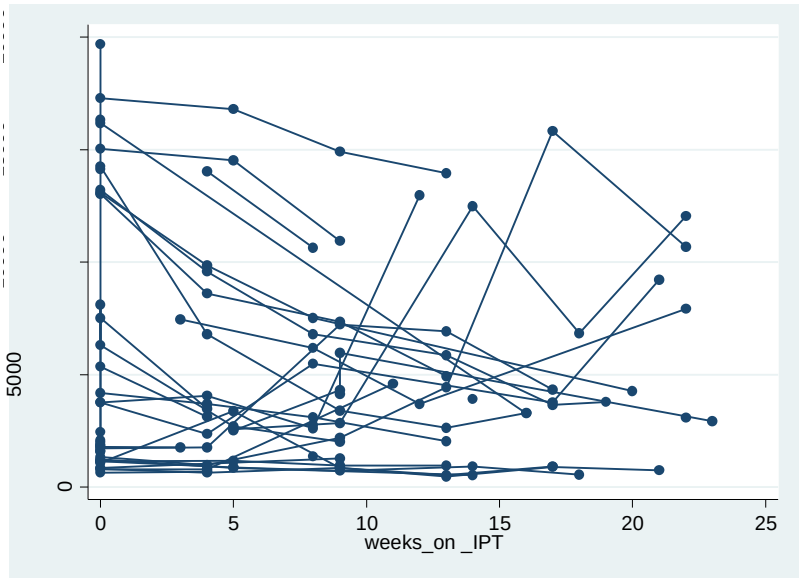


Figure S2.3. Influence of IPTp+ITN on changes in VAR2CSA levels between enrollment and delivery. Antibody levels at enrollment and delivery were measured in Cameroonian multigravidae (n=41) who received IPTp+ITN for about 2 trimesters (14-23 weeks) to full-length VAR2CSA and its 6 DBL domains and 15 strain variants. Delivery/enrollment ratios were calculated. Delivery/enrollment ratio of ≤ 0.8 were categorized into a "decreased" group, 0.9-1.1 were categorized into a "no change" group and those who had ratios of ≥ 1.2 were categorized into an "increased" group. Prevalence for each antigen for each of the 3 groups was calculated and plotted as % change.

Figure S2.4.

A



B

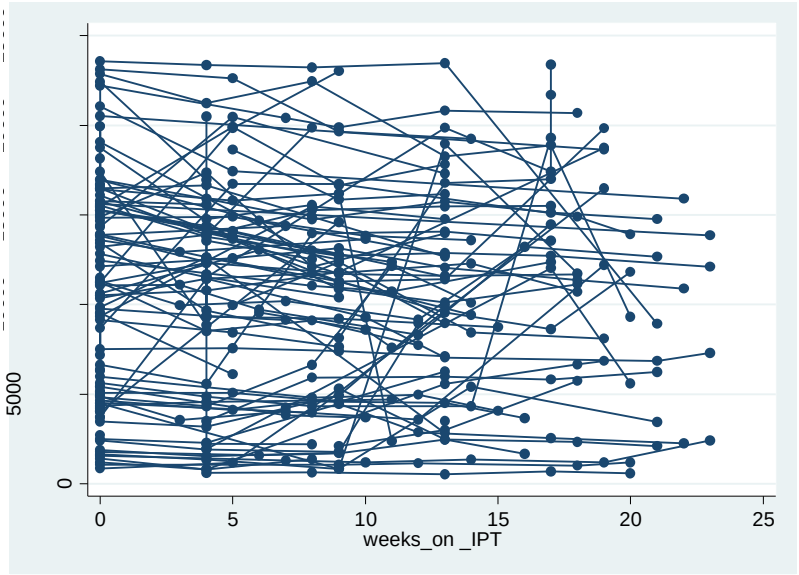


Figure S2.4. Antibody decay in Cameroonian women who received IPTp+ITN. Antibody levels to the full-length VAR2CSA were measured in Cameroonian A. primigravidae (n=35) and B. multigravidae (n=109), who received IPTp+ITN during pregnancy. Scatterplots showing raw data of antibody levels to the VAR2CSA over weeks-on-IPT are presented.

Figure S2.5.

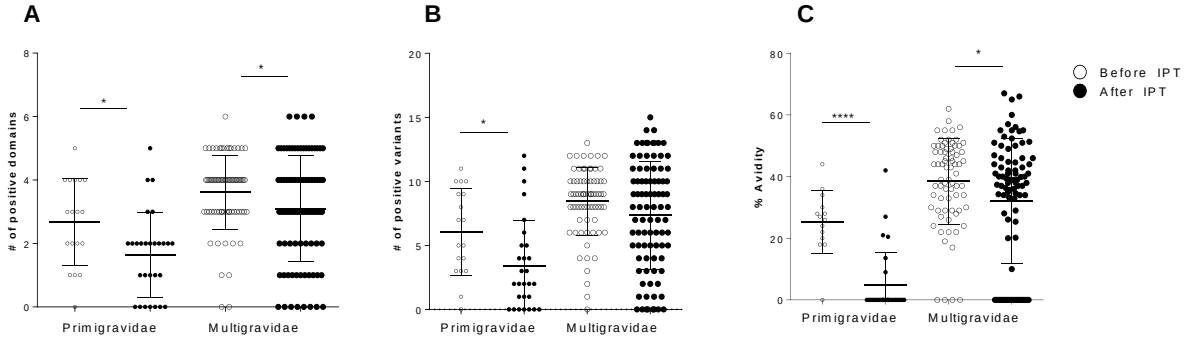


Figure S2.5. Influence of IPTp+ITN on putative correlates of protection from placental malaria. Plasma collected from women at delivery (or last visit during the III trimester) were used in assays to measure A. number of VAR2CSA DBL domains women recognized, B. number of VAR2CSA strain variants women recognized, and C. avidity of antibodies to the full-length VAR2CSA. Statistics was calculating using unpaired t-test with 95% CI.

CHAPTER 3

SHORT TITLE: **DO WOMEN NATURALLY PRODUCE ANTIBODIES TO THE CSA-BINDING SITE OF VAR2CSA?**

LONG TITLE: **THE ANTIBODY RESPONSE OF PREGNANT CAMEROONIAN WOMEN TO VAR2CSA ID1-ID2A, A SMALL RECOMBINANT PROTEIN CONTAINING THE CSA-BINDING SITE**

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Anna Babakhanyan[1], Naveen Bobbili[1], Ali Salanti[2], John J. Chen[1], Rose G.F. Leke[3], Philomina Gwanmesia[3], Robert J.I. Leke.[3], and Diane Wallace Taylor[1*]

[1] Department of Tropical Medicine, Medical Microbiology and Pharmacology, University of Hawaii at Manoa, John A. Burns School of Medicine, Honolulu, Hawaii, 96813, U.S.A. Email: A. Babakhanyan: annab8@hawaii.edu; N. Bobbili, bobbili@hawaii.edu; J.J. Chen, jjchen@hawaii.edu; D.W. Taylor: dwtaylor@hawaii.edu

[2] Centre for Medical Parasitology, Department of International Health, Immunology and Microbiology, University of Copenhagen and Department of Infectious Diseases, Copenhagen University Hospital (Rigshopitalet), Copenhagen, Denmark. Email: A. Salanti salanti@cmp.dk

[3]The Biotechnology Center, Faculty of Medicine and Biomedical Research, University of Yaounde 1, Yaounde, Cameroon. Email: R.G.F. Leke, roseleke@yahoo.com; P. Gwanmesia, gwanp2003@yahoo.com; R.J.I. Leke, robertjleker@yahoo.fr

*Corresponding author: Mailing address: 651 Ilalo Street, BSB 320, Department of Tropical Medicine, Medical Microbiology and Pharmacology, University of Hawaii at Manoa, Honolulu, HI 96813, USA. Phone: (808) 692-1608. E-mail: dwtaylor@hawaii.edu

3.1 Abstract Background: Antibodies to VAR2CSA expressed on *Plasmodium falciparum*-infected erythrocytes that blocks binding to chondroitin sulfate A in the placenta, should confer protection from placental malaria. This study sought to determine if pregnant Cameroonian women naturally acquire antibodies to ID1-ID2a and if antibodies to ID1-ID2a correlate with absence of placental malaria at delivery.

Methods and Findings: Antibody levels to full-length VAR2CSA and ID1-ID2a were measured in plasma samples from 745 Cameroonian women, 144 Cameroonian men, and 67 US controls (42 pregnant US women and 25 US adults). The prevalence of IgM and antibody avidity to ID1-ID2a was also determined. As expected, antibodies to ID1-ID2a were absent in US controls. Despite the fact that women were infected with CSA-binding parasites during pregnancy, as established by increasing levels of IgG to full-length VAR2CSA, no increase in IgM or IgG to ID1-ID2a was observed during pregnancy. Surprisingly, no statistically significant difference in antibody levels to ID1-ID2a was detected between Cameroonian men and pregnant women. In rural villages, 88%-97% of males were seropositive for ID1-ID2a, while only 2-7% had antibodies to full-length VAR2CSA. No differences in IgG avidity or proportion of high avidity IgG to ID1-ID2a were found between Cameroonian men and pregnant women. There was also no correlation between antibody levels to ID1-ID2a at delivery and absence of placental malaria. Thus, the response to ID1-ID2a is not pregnancy specific, but due to cross-reactivity with other antigens.

Conclusions: Since synthetic ID1-ID2a binds to the CSA with the same affinity as the full-length molecule and elicits binding-inhibitory antibodies in rats, it is a great vaccine candidate. Surprisingly, the cross-reactive response found both in pregnant Cameroonian women and sympatric men contrasts with the protective response elicited in rats. Thus, our results suggest that pregnant women do not naturally produce pregnancy-associated antibodies to the minimal binding site on the native VAR2CSA protein.

3.2 Introduction

In pregnant women, *P. falciparum*-infected erythrocytes (IE) express the adhesion ligand VAR2CSA that binds to chondroitin sulfate A (CSA) on syncytiotrophoblasts lining the intervillous space (IVS) of the placenta [48, 71, 80, 126]. As a result, IE accumulate at the maternal-fetal interface causing placental malaria (PM). Pathology resulting from PM increases the risk of maternal anemia and poor pregnancy outcomes, including low birth weight babies due to prematurity and intrauterine growth restriction [30, 77]. In malaria endemic areas, pregnant women produce antibodies (Ab) to VAR2CSA that inhibit the binding of IE to CSA in vitro [89, 104], reduce maternal anemia [105], lower placental parasitemia at delivery [106, 107], increase the length of gestation [108], and improve infant birth weight [108]. Thus, Ab to VAR2CSA play an important role in protecting pregnant women from the severe effects of PM.

VAR2CSA is a 350kDa transmembrane protein with 6 Duffy-Binding-Like (DBL) domains, a cysteine-rich inter-domain region (termed $CIDR_{PAM}$) between DBL2X and DBL3X, and a number of inter-domains [78, 81, 127]. Data support the feasibility of a VAR2CSA-based vaccine for protecting pregnant women. For a surface antigen, VAR2CSA is surprisingly well-conserved, with limited polymorphism and is the main target of protective immunity against PM [97, 98, 103, 111]. The large size of the molecule makes it difficult to produce a vaccine using the entire molecule; accordingly, extensive efforts are being made to identify the region(s) within the molecule that binds CSA.

Originally it was thought that the CSA binding site was conformationally-created by multiple DBL domains [78, 81, 82]; however, several groups recently showed that it is located in the N-terminal DBL2X- $CIDR_{PAM}$ region [83]. The minimal sequence containing the binding site was recently identified as ID1-DBL2Xb, consisting of ID1, DBL2Xb and 93 amino acids from ID2a [84]. A slightly larger construct, termed ID1-ID2a, consisting of the ID1, DBL2Xb, plus the entire ID2a region, can be expressed in higher yields compared to ID1-DBL2Xb [82]. The ID1-ID2a is considered to be a strong vaccine candidate, because antisera raised against it in rats inhibits the binding of IE to CSA by nearly 100% [84]. Furthermore, when Ab raised against full length VAR2CSA

(FV2) in rats are affinity purified on recombinant ID1-ID2a, the purified Ab effectively inhibit IE binding to CSA by essentially 100% [84]. Thus, ID1-ID2a contains the minimal CSA binding site, induces inhibitory Ab in an animal model, contains major epitopes important in inhibition of binding, and can be produced on a large scale.

The natural acquisition of Ab to ID1-ID12a in pregnant women has not been evaluated. Recently, we demonstrated that Cameroonian women, who were PM negative (PM-) at delivery, had significantly higher Ab levels to FV2 throughout pregnancy [109], and that women with a high proportion of high avidity Ab to FV2 during the second trimester were at reduced risk of having PM at delivery [110]. Using plasma samples from the same group, we found that women with Ab to more DBL domains and allelic variants were also more likely to be PM- at delivery than those with a smaller antigenic repertoire [109]. Accordingly, plasma from the above women, as well as samples from other cross-sectional studies, were used to determine if pregnant Cameroonian women naturally acquire Ab to ID1-ID2a and if Ab to ID1-ID2a correlated with absence of PM at delivery. If so, then measuring Ab to recombinant ID1-ID2a could be used to determine if pregnant women have sufficient immunity to be protected from PM. Our results indicate that the recombinant ID1-ID2a constructs used in this study contain epitopes not exposed in the full-length molecule that are detected by IgG antibodies commonly found in Cameroonians. Thus, levels of Ab to ID1-ID2a cannot be used to determine if women are protected from PM.

3.3 Methods

IRB approvals. The archival coded samples used in the current study were exempt from human subject research by the Committee on Human Studies, University of Hawaii, Manoa. The original studies were conducted according to the Helsinki Declaration principles and approved by the National Ethics Committee, Cameroon, and the Institutional Review Board at Georgetown University. All women participating in the study gave written informed consent, including the use of their blood samples to measure Ab to malaria.

Plasma samples. Archival plasma samples were collected in studies in 3 different malaria endemic settings. The study sites included Ngali II and Simbok where *P. falciparum* transmission is high, with an entomological inoculation rates of 0.7 infectious bites / person/ night and 1.2-1.9 infectious bites/person/night, respectively [65, 128]; and Yaounde, the capitol of Cameroon, where individuals receive 0.1 to 1.1 infectious bites/person/night [129]. Archival plasma samples from US adults (n=25) and US pregnant women (n=42) were used as negative controls.

The first set of archival plasma samples used were from a longitudinal study conducted between 2001 and 2005 in the rural village of Ngali II and the city of Yaounde, Cameroon, in which pregnant women were recruited early in pregnancy and followed throughout their pregnancy [109, 128]. Three plasma samples per pregnant woman collected during the first, second and third trimesters were used (n=83 Ngali II village, n=96 Yaounde). Further, samples from males (n=58 Ngali II village, n=35 Yaounde) were included for comparison. The second set of archival samples was from a cross-sectional study conducted between 1994 to1997 in Simbok, a peri-urban village [130]. All peripheral plasma samples available from males (n=51) and females (n=102) of reproductive age (18-35 years old) were used. The gravidity status was not known, but the women were not pregnant at the time the samples were collected. The third set of plasma samples was from a large cross-sectional study conducted in Yaounde from 1996 to 2001 [65, 131] and consisted of 1,944 peripheral blood samples collected at delivery. All samples from placental malaria positive women (n=116) and 348 randomly selected samples from PM- women (i.e. ratio of 1:3 for PM+ to PM-) were selected from women who had ≥3 pregnancies, ≥20 years of age, and had term or premature deliveries. All PM- women had been exposed to malaria since they had Ab to DBL5 (FCR3 strain).

Diagnosis of placental malaria. Blood smears of maternal peripheral and IVS blood, and impression smears of placental biopsies, were made. Slides were stained with Diff-Quick Stain Kit (IMEB Inc., San Marcos, CA. Catalog Number: K7128-3) and read by two microscopists to determine parasitemia. Placental biopsies were fixed in buffered formalin, embedded, stained with hemotoxylin-eosin and examined for parasites. A

woman was considered to have PM if IE were detected in blood smears of IVS blood, impression smears of villous tissue, or histological sections of the placenta [109].

Recombinant Protein Expression. Recombinant proteins used in this study included ID1-ID2a, DBL1+2, DBL2, DBL5 and FV2 produced in Sf9 insect cells using the Baculovirus vector, as described previously [109]; and DBL1 (3D7 and 7G8 strains) produced in *Pichia pastoris.* Sequence information and detailed protocol have been published previously [84, 109, 110]. Briefly, full length VAR2CSA [FCR3 strain, GenBank: GU 249598] and ID1-ID2a [3D7 strain, GenBank: JQ247428 and FCR3 strain, GenBank: GU 249598, sequence provided in the supplemental information section] were inserted into Baculovirus transfer vector pAcGP67-A (BD Biosciences) with a histidine tag on the C-terminal end. Linearized Bakpak6 Baculovirus DNA was co-transfected with pAcGP67-A plasmids into Sf9 insect cells for generation of recombinant virus particles. Then, 10 ml of second amplification was used to infect High-Five cells in 400 ml of serum-free medium (10486, Invitrogen) at 1million cells/ml. Secreted recombinant protein was harvested from the supernatant and purified using the AKTA-express purification system (GE Health Care).

Coupling of recombinant proteins for use in the Multianalyte platform (MAP) assay. The MAP assay was previously optimized for quantification of Ab to multiple VAR2CSA sequences [132]. To determine the optimal concentration of ID1-ID2a, different concentrations of the 3D7 (1.6, 5, and 10µg) and FCR3 (1, 5, 10, and 15 µg) strains were coupled to 1 million microspheres (SeroMAP beads, Luminex Corp., Austin, TX). ID1-ID2 (3D7) at 1.6µg/million microspheres and ID1-ID2 (FCR3) at 5µg/million microspheres were found to be optimal. DBL1 (3D7 and 7G8), DBL1+2 FCR3, DBL2 FCR3, DBL3 (FCR3) and DBL5 (FCR3) were coupled at 1µg/million microspheres; FV2 was coupled at 3µg protein per one million microspheres based on previous optimization studies.

***Optimization of the MAP assay*.** The MAP assay measures Ab to multiple antigens simultaneously, creating the potential that Ab might compete for the antigens. To assess this possibility, microspheres coupled with ID1-ID2a (3D7) and ID1-ID2a (FCR3) were tested either alone or pooled with FV2 (FCR3) for reactivity with plasma from

multigravid Cameroonian women (positive control) and pooled US plasma (negative control) (Figure S1 presents data for positive control). Data showed that Ab levels to both ID1-ID2a constructs were the same when used singly or multiplexed with the other ID1-ID2a construct and FV2.

Measuring IgG and IgM using MAP assay. MAP assay was performed as previously described [109, 110, 132]. Briefly, 50µl of antigen-coupled microspheres (2,000 microspheres/test) were incubated with 50µl of a 1:100 dilution of plasma in PBS-1% BSA (phosphate buffered saline containing 1% bovine serum albumin [BSA]) in pre-wetted wells of filter plates (96 well Multiscreen BV; Millipore, Billerica, MA), for 1hr at 25°C on a rotating shaker at 500 rpm (Microplate Shaker, Lab-line, Melrose Park, IL). Microspheres were washed twice with PBS-0.05% Tween20 and once with PBS-1% BSA. Then, 100µl of secondary Ab (R-phycoerythrin-conjugated, Affini Pure F(ab')$_2$ fragment, Goat anti-human IgG Fc fragment specific from Jackson Immunoresearch, West Grove, PA, Cat # 109-116-170) diluted to 2µg/ml in PBS-1% BSA was added to each well and incubated as above in the dark for 1 hr. Wells were washed as described above, microspheres were re-suspended in 100µl PBS-1%BSA, and 85µl of the microsphere suspension was analyzed using a Liquichip M100 reader (Qiagen, Valencia, CA). The reader was programmed to read a minimum 100 beads per spectral address, DD Gate 7500-15000 and 35sec timeout. The results were expressed as mean fluorescence intensity (MFI). Positive and negative controls were run on each plate that included 3 different pools of plasma from 8 Cameroonian multigravidae with high Ab levels to VAR2CSA and pools of plasma from 40 Americans who never travelled to malaria endemic areas. To measure IgM R-PE-AffiniPure F(ab')$_2$ fragment anti-human IgM Fc secondary Ab was used (Jackson Immunoresearch, West Grove, PA, Cat # 709-116-073) at 1:250 dilution in PBS-1% BSA.

Avidity to the FV2. Samples collected at delivery (or last sample in the third trimester/ >27 weeks) were used in the avidity assay for women who were seropositive for FV2. The avidity assay was performed as previously described [110]. Briefly, plasma was diluted 1:300, 1:1,000 and 1:3,000 in 1% BSA-PBS and 50µl of diluted plasma was added to six wells (each dilution in duplicate) containing 50µl of FV2-coupled

microspheres (2,000 microspheres/test) and incubated for 1 hr on a shaker. After incubation, 100µL of 3M NH_4SCN in 1% BSA-PBS was added to half of the wells and 100µL of 1% BSA-PBS was added to the other half. After 30 minutes of incubation, the wells were washed and incubated with secondary Ab, wash and analyzed by MicroChip 100 as described above. Avidity was determined for each dilution by the following formula: (MFI obtained from wells incubated with salt)/(MFI obtained from corresponding control wells) x100 for each dilution. Then, the average avidity for the 3 dilutions was determined. Positive and negative (same as in MAP assay) controls were included on each plate.

Statistical Analysis. Antibody prevalence to ID1-ID2a in Cameroonian males and pregnant women was calculated using a cut-off value equals to the mean + 2 standard deviations for US controls (67 adults and pregnant women). Cut-off value for seropositivity to FV2 FCR3 was calculated by using the mean + 2 standard deviations for Cameroonian males. For each continuous variable, such as Ab levels or average avidity, individual values, their median, lower and upper quartiles were presented for the study sample as a whole and for relevant subgroups. Two-sample t-tests (or Mann-Whitney tests when data was not normally distributed) were used to compare between Cameroonian males and pregnant women, and between PM+ and PM- samples. Paired t-test was used to compare IgM levels to FV2 FCR3 and ID1-ID2a of the same individuals. For repeated measures data, e.g., IgG levels during pregnancy, non-parametric Freedman test was implemented. For the comparison of Ab prevalence and prevalence of high avidity Ab between Cameroonian males and females, two-tailed Fisher's exact tests were performed. Finally, to evaluate the association between the reactivities of Ab to ID1-ID2a and to FV2 FCR3, correlation analysis was utilized and Pearson's correlation coefficient (r) was calculated. Two-tailed $p<0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 6.0 and SmallStata 12.

3.4 Results

IgG levels to ID1-ID2a in Cameroonian women during pregnancy. In this initial study, longitudinally-collected plasma samples were used from a cohort of women in whom we previously found a correlation between high Ab levels to multiple VAR2CSA domains and high avidity Ab to FV2 and absence of PM. We sought to determine whether these women naturally produced Ab to ID1-ID2a and if Ab to ID1-ID2a also correlated with protection. IgG to ID1-D2a (3D7 and FCR3 strains), as well as FV2 were measured in samples collected from women living in city of Yaounde (Fig 1. A-C) and in Ngali II village (Fig. 1 D-F). We confirmed our previous findings that Ab levels to FV2 are pregnancy-associated (city: all $p<0.001$, village all $p<0.0001$, , for Cameroonian women when compared with males). IgG to FV2 FCR3 increased during the course of pregnancy in the Ngali II village, as women were infected (PG $p=0.008$ and MG $p=0.001$, Freedman's test). In contrast, Ab levels to ID1-ID2a (both constructs) did not change during pregnancy (all $p>0.05$), in either the city of Yaounde or Ngali II village. Moreover, Cameroonian women in both locations had similar Ab levels to ID1-ID2a compared to sympatric males (all $p > 0.05$). In the Ngali II village, MG had significantly higher IgG levels to ID1-ID2a 3D7 construct compared to males ($p<0.05$). Next, data were stratified by PM status; no significant differences (all $p>0.05$) were found in Ab levels to ID1-ID2a 3D7/FCR3 between PM+ and PM- samples obtained at delivery, both in the city of Yaounde and the Ngali II village. Results indicate that Ab to ID1-ID2a are not pregnancy specific.

IgG levels to FV2 and ID1-ID2a in Cameroonian women at delivery. Since the above results were unexpected, additional plasma samples were tested for IgG to ID1-ID2a, including samples from US adults and US pregnant women, delivery samples from city of Yaounde and Ngali II village women, Simbok village males and females of reproductive age, and larger cohort of PM+ and PM- city of Yaounde MG delivery samples. Ab to ID1-ID2a are absent in US adults and pregnant women (Fig. 2A and B, Table 1) and found at equal levels in Cameroonian males, PG and MG living both in the city of Yaounde and Ngali II village. No significant differences in IgG to ID1-ID2a were observed between Cameroonian males and females living in the city and villages (all

p>0.05). As an exception, Ngali II MG, but not PG, had higher Ab levels to ID1-ID2a 3D7 (but not FCR3 strain) compared to males (p<0.0001). Further, no significant differences were observed in IgG to ID1-ID2a FCR3 between PM+ and PM- MG from the larger cohort (p=0.46) of women living in the city of Yaounde. Prevalence of Ab-positivity (Table 1) to the 3D7 and FCR3 strains of ID1-ID2a did not differ significantly among the Cameroonian males, PG and MG (3D7 city: p= 0.11, Ngali II village p=0.0013; FCR3city: p=0.21, Ngali II village: p=0.21). Both in the city (55%) and village (88-97%) males were seropositive for at least one of the recombinant variants of ID1-ID2a; while none of the city of Yaounde males and only 2-7% of village males were seropositive to FV2 FCR3 (Table 1). IgG to FV2 FCR3 were pregnancy specific (Fig. 1 and Fig. 2), as previously reported [109, 110], PG and MG had significantly higher IgG levels compared to males both in the city of Yaounde and Ngali II village (all p<0.05). The prevalence of Ab to FV2 was consistent with previous reports; i.e., Ab to FV2 FCR3 absent in males and present in high proportion of PG (city of Yaounde: 39%, Ngali II village: 73%) and MG (city of Yaounde: 38%, Ngali II village: 93%) (Table 1). Thus, Ab response to ID1-ID2a was ubiquitous and not pregnancy associated. Since, no consistent pattern was observed in Ab response to ID1-ID2a 3D7 between city of Yaounde and Ngali II and Simbok villages (Fig. 1, 2 and Table 1), 3 approaches were employed to further investigate these results: 1) assess if ID1-ID2a antigen is recognized, but isotype switching to IgG does not occur, 2) measure Ab avidity to ID1-ID2a, 3) evaluate Ab response to other N-terminal domains.

IgM levels to FV2 and ID1-ID2a. It is possible that ID1-ID2a is poorly immunogenic and/or women produce IgM and not IgG. It is also possible that malaria parasites use "IgM masking" [133] to evade immune response to the functionally important part of the VAR2CSA. Hence, IgM to ID1-ID2a was measured to determine if ID1-ID2a epitopes are recognized. IgM was measured in first blood-smear positive plasma sample and plasma sample collected during the following visit from PG and MG living in city of Yaounde and Ngali II village (longitudinal study). No statistically significant differences were observed between PG and MG IgM levels to any of the antigens in the city (Fig. 3A.) and village (Fig. 3B.), all p values > 0.05. Since beads were run at saturation, it appears that city women produced significantly higher IgM levels to FV2 FCR3

compared to ID1-ID2a 3D7and ID1-ID2a FCR3 (Figure 3A: all $p<0.05$). Interestingly, in the Ngali II village, both PG and MG produced significantly higher IgM to FV2 FCR3 compared to ID1-ID2a 3D7, but not ID1-ID2a FCR3. The immune response to ID1-ID2a is generally weak compared to FV2 FCR3. However, there is no evidence that women had strong IgM and not IgG response to ID1-ID2a. This suggests that low levels of IgG to ID1-ID2a are due to lack of immunogenicity, not due to failure to isotype switch.

Avidity of Ab to ID1-ID2a. We sought to determine if the high levels of male Ab recognition to ID1-ID2a was due to cross-reactivity. Since cross-reactive Ab exhibit low avidity, we expected to find low avidity Ab to ID1-ID2a in males and high avidity Ab to ID1-ID2a in females. Subset of plasma samples from city and Ngali II village (longitudinal study) with high Ab levels to ID1-ID2a FCR3 (cut-off for city of Yaounde women and men: > 5,000 MFI; Ngali II village women and men: > 6,000 MFI) were used to measure avidity of Ab to ID1-ID2a recombinant proteins. Avidity to ID1-ID2a 3D7 and ID1-ID2a FCR3 (Fig. 4B and 4C respectively) did not differ between males and females living in the city or Ngali II village (all $p>0.05$). In contrast, Ab avidity FV2 FCR3 (Fig. 4A) were higher in Ngali II village women compared to males ($p=0.0003$) and higher but not statistically significant in the city. Finally, we determined the proportion of individuals with high avidity Ab to ID1-ID2a (≥35% avidity [110]). In the village, significantly higher proportion of women had high avidity Ab to FV2 FCR3 compared to males (Table 1S, $p<0.0001$), with similar trend in the city. Proportions of men and women with high avidity Ab to both recombinant ID1-ID2a domains was similar, both in the city and the village. Interestingly, in the Ngali II village about 40-50% of males had high avidity Ab to ID1-ID2a, whereas only11% of males had high avidity Ab to FV2 FCR3, suggesting that Ab to FV2 FCR3 produced by males are weakly binding, while Ab to ID1-ID2a have high avidity. All together, these results show that binding interactions between the recombinant ID1-ID2a epitopes and corresponding Ab in Cameroonian women are weak, suggesting cross-reactive nature of these Ab in both males and females.

Comparison of Ab levels to ID1-ID2a and FV2. Since Ab to FV2 FCR3 is induced only in pregnant women, we examined correlation between the IgG to ID1-ID2a FCR3and

IgG to FV2 FCR3. For comparison correlation analysis was also performed between FV2 FCR3 and immunogenic domains of VAR2CSA: DBL3 and DBL5. Data suggests that Ab to DBL3 FCR3 and DBL5 FCR3 (Fig. 5A and 5B) are highly correlated with Ab to FV2 FCR3 (DBL3 r=0.78. DBL 5 r=0.86). However, only a moderate positive correlation was found between Ab to FV2 FCR3 and ID1-ID2a FCR3 (R^2=0.55) (Fig. 5C) and this pattern was similar of that found in males living in the city and villages (all r=0.60) (Fig. 5 C-E). Thus, Ab to ID1-ID2a FCR3 in males and females appear to be due to cross-reactive epitopes.

IgG levels to the N-terminal domains and DBL5. Since we observed low levels of Ab to ID1-ID2a compared to FV2, and similar to other N-terminal domains reported previously [109], we evaluated IgG response to other N-terminal domains of VAR2CSA and compared this response to that of DBL5 FCR3 (immunogenic domain). IgG levels to DBL1 3D7, DBL1 7G8, DBL2 1+2 7G8, DBL2 FCR3 and DBL5 FCR3 were measured in 116 PM+ and 348 PM- MG living in the city of Yaounde. The results were consistent with previous findings, showing that Ab response to the N-terminal domain is relatively low, with median Ab levels to the N-terminal domains ranging from 1,335-5,566 MFI (Fig. 6). This is in contrast to Ab response to DBL5 FCR3, with median Ab levels of 15,938 MFI in PM+ and 15,996 MFI in PM-. In PM+ and PM- pregnant women Ab response to DBL1-DBL2 are lower than to DBL5 (all $p<0.0001$). Thus, it is not surprising that Ab response to ID1-ID2a is low. No statistically significant difference in Ab levels to any of the N-terminal domains and DBL5 was observed between PM+ and PM- MG (all $p>0.05$).

3.5 Discussion

This study evaluated the potential of naturally acquired Ab to ID1-ID2a, a small protein that contains the minimal CSA-binding site, as correlates of protection from PM in a cohort of Cameroonian women with previously defined correlates of protection. We found that Ab to ID1-ID2a did not change during pregnancy and were at equal levels (and prevalence) in Cameroonian males, primigravidae and multigravidae. Ab levels to

ID1-ID2a were generally low compared to Ab to FV2; however, there was no evidence that women failed to produce IgM to ID1-ID2a or that IgM did not class-switched to IgG. No differences in Ab avidity to ID1-ID2a and prevalence of high avidity Ab was found between Cameroonian males and females. Our data demonstrates that Ab to ID1-ID2a are not pregnancy specific and are not associated with absence of PM.

These results were surprising, since ID1-ID2a is a strong vaccine candidate: it binds to CSA with the same affinity as the full-length antigen and induces Ab that are highly efficient in blocking the binding of IE to CSA in laboratory animals [84]. We expected Ab to ID1-ID2a to be lower, but highly pregnancy-specific and correlate with absence of PM (similar to Ab response to FV2 FCR3) [109]. Thus, when in the first longitudinal screen we did not observe difference in levels of Ab to ID1-ID2a between men and women and no association with absence of PM, we sought to study Ab responses to ID1-ID2a in larger cohort and different transmission sites. Data show that Ab to ID1-ID2a are absent in US adults and US pregnant women; however, are present at equal levels in Cameroonian primigravidae, multigravidae and males living both in malaria low transmission (city of Yaounde) and high transmission (Simbok and Ngali II villages) areas. This suggests that Ab to ID1-ID2a are cross-reactive and elicited due endemic pathogen found in Africa (other PfEMP1, malarial antigen or different pathogen) and not due to antigens commonly used for childhood vaccinations (measles, rubella, diphtheria, poliovirus, etc.) in the US.

To rule out possibility that ID1-ID2a is poorly immunogenic or "IgM masks protective IgG epitopes" [133] to evade immune response, we measured IgM in a longitudinally collected samples. Results demonstrate that women produce IgM to ID1-ID2a and there was no evidence that isotype switching was impaired. Next, we measured Ab avidity to ID1-ID2a to determine if low levels of "true" Ab to ID1-ID2a could be detected, as chaotropic salt would strip any non-specifically bound Ab. However, Ab avidity to both ID1-ID2a recombinant constructs was same in males and pregnant females. Ab levels to ID1-ID2a were similar to those found to other N-terminal domains, i.e. generally low. Finally, low positive correlation was found between Ab levels to ID1-ID2a and full-length VAR2CSA, while high positive correlation was found between Ab

levels to DBL3/DBL5 FCR3 and FV2 FCR3. Of note, our results show that Ab to DBL5 FCR3 alone do not correlate with absence of PM at delivery is consistent with previous findings [109]. Recent study showed that Ab to DBL5 elicit high levels of inhibition of binding to CSA [134], suggesting that Ab function and not Ab levels may be important in protection from placental malaria.

Thus, there is no evidence that Cameroonian women produce Ab to minimal CSA-binding site on the native VAR2CSA protein as a result of natural infection. The cross-reactive response found both in pregnant Cameroonian women and sympatric males contrasted the protective response elicited in rats using synthetic peptide. Animal studies were conducted in malaria-naïve rats with synthetic peptide, which apparently exposed CSA-binding epitopes of FV2 FCR3 [84]. Results suggest that in plasma from pregnant women, recombinant ID1-ID2a proteins bind to Ab generated to cross-reactive B cell epitopes normally hidden in the full-length conformation. Currently it is held that CSA-binding sites are cryptic, which could constitute a parasite immune evasion mechanism. Thus, it is conceivable that minimal binding site is cryptic in the native VAR2CSA and covered with cross-reactive immunodominant epitopes that lead to misdirected immunogenicity, an immune evasion mechanism used by *Trypanasome brucei* (VSG), *Neiserria meningitis* [135], and *Plasmodium* (CSP).

Main limitation of our serological MAP assay was inability to dissect the "true" immune response to ID1-ID2a from cross-reactive responses. Although, we attempted to define "true" response to ID1-ID2a using avidity assay, another approach would be to affinity purify women's plasma to FV2 FCR3 and measure Ab levels to ID1-ID2a using these purified plasma, which is currently used by our collaborators in Copenhagen. However, since only 2- 7% of Cameroonian males (Table 1) have Ab to FV2 FCR3, by definition, only 7% of males were exposed to VAR2CSA in non-pregnancy specific manner and therefore it is expected that only 7% of males should have Ab to the ID1-ID2a part of the molecule. Since, we observed 50 % (city of Yaounde) and 88/ 97% (Ngali II /Simbok village) of Cameroonian males with Ab to ID1-ID2a, this suggests that ID1-ID2a Ab are not elicited by exposure to FV2 FCR3. Similar findings were observed in FV2 FCR3 seronegative females in Simbok village (Table 1), where we show that 82%

of seronegative Simbok females had Ab to one of the ID1-ID2a constructs. Further, our Ab avidity study demonstrates that there is no significant difference in Ab binding to the recombinant proteins between males and females. Finally, it would be informative to measure functional characteristics of "true" Ab to ID1-ID2a, such as their ability to in-vitro inhibit binding of IE to CSA.

Clearly, significant progress has been made toward the development of a vaccine that may protect an estimated 85 million women world-wide, who are exposed to *P. falciparum* during pregnancy [4]. The discovery that IE bind to CSA in the placenta provided a key explanation as to why *P. falciparum*-infected erythrocytes accumulate at the feto-maternal interface, causing PM [111]. The subsequent identification of VAR2CSA as the parasite antigen responsible for the binding provided a critical step toward the development of a vaccine for prevention of PM [126]. Since VAR2CSA is a large molecule, determining the region responsible for binding to CSA was of high importance [82, 83, 136]. Our results suggest that Ab to ID1-ID2a are naturally not produced and clinical trials are necessary in order to determine efficacy of ID1-ID2a as a vaccine candidate.

However, testing first-generation VAR2CSA-based vaccines in pregnant women is going to be a challenge. Thus, it is important to understand the characteristics of Ab that mediate protection and have serological assays that accurately measure them. It is likely Ab mediate their effect by preventing the binding of IE to CSA, but this has been difficult to firmly establish in women with naturally acquired immunity. Therefore, identification of correlates of protection is needed. We demonstrated that Ab levels to these recombinant ID1-ID2a proteins cannot be used as correlates of protection to assess naturally-acquired immunity to PM. Approaches other than serology, such as functional assays (phagocytosis, inhibition of binding, etc.) are needed to identify an assay or combination of assays that assess the immune status of pregnant women for PM.

Acknowledgements

We wish to acknowledge the support of the entire Malaria Research Team at the Biotechnology Center, University of Yaoundé I, Cameroon, for their outstanding work, and the women and their families who participated in the studies. We also acknowledge Dr. Quakyi for providing serum samples from Simbok village and Dr. Smith for providing recombinant proteins expressed in 7G8 and 3D7 strains and Alex Kayatani for establishing the avidity assay. Finally, we thank RMATRIX biorepository for providing plasma samples from US pregnant women. The work was supported by grants from NIAID, NIH, UO1AI43888 and RO1AI071160 (DWT, RGFL), FP7/2007-2013 grant agreement #200889 (STOPPAM) (AS), and NIMHD U54MD007584 (JJC) and NIH G12MD007601 (JJC). We acknowledge the support of T. Dye, University of Hawai'i Biorepository (HiBR) for plasma from the US pregnant women, and John Chen, Biostatistics Core, for statistical analysis (support provided by NIMHD U54MD007584 and NIH G12MD007601).

Table 3.1. Prevalence of individuals with IgG to ID1-ID2a and FV2 at delivery

		Prevalence (%)			
	Number of individuals	**FV2 FCR3**[ʃ]	**ID1-ID2a 3D7**[§]	**ID1-ID2a FCR3**[§]	**ID1-ID2a FCR3 or 3D7**
US non-pregnant	24	0	0	8	8
US pregnant	42	0	9.5	2.4	9.5
Yaounde City - longitudinal					
Males	33	0	15	55	55
Primigravidae	33	39	39	36	46
Multigravidae	63	38	33	40	44
Yaounde City - cross-sectional					
All Multigravidae	464	91	-	50	50
PM+ Multigravidae	116	84	-	50	50
PM- Multigravidae	348	94[†]	-	51	51
Ngali Village - longitudinal					
Males	57	7	60	88	88
Primigravidae	15	73	67	93	93
Multigravidae	68	93	85	94	96
Simbok Village - cross-sectional					
Males	46	2	72	97	97
Females	102	51	64	85	87
FV2-seropositive	52	100	43	92	92
FV2-seronegative	50	0	42	78	82

ʃ Cameroonian city and village males (outliers excluded) were used to establish cut-off for FV2 seropositivity for females living in the city and village respectively.
§ US pregnant and non-pregnant controls were used to establish a cut-off for determination of seropositivity to ID1-ID2a, since IgG to ID1-ID2a were not pregnancy-associated.
† 6% of women that were not seropositive for FV2 were seropositive for DBL5 FCR3, indicating that they were exposed to CSA-binding *P. falciparum*

Table 3.1S. Prevalence of individuals with high avidity antibodies† to ID1-ID2a and FV2 at delivery

		Prevalence (%)		
	Number of individuals	**FV2 FCR3**	**ID1-ID2a 3D7**	**ID1-ID2a FCR3**
Yaounde City - longitudinal				
males	6	0	33	0
females	18	44	11	22
p value ‡		0.066	0.25	0.54
Ngali Village - longitudinal				
males	9	11	56	33
females	20	90	45	40
p value ‡		**<0.0001**	0.69	1.0

† High avidity Ab are defined as ≥35% avidity

‡ p value calculated using Fisher's exact test

Figure 3.1.

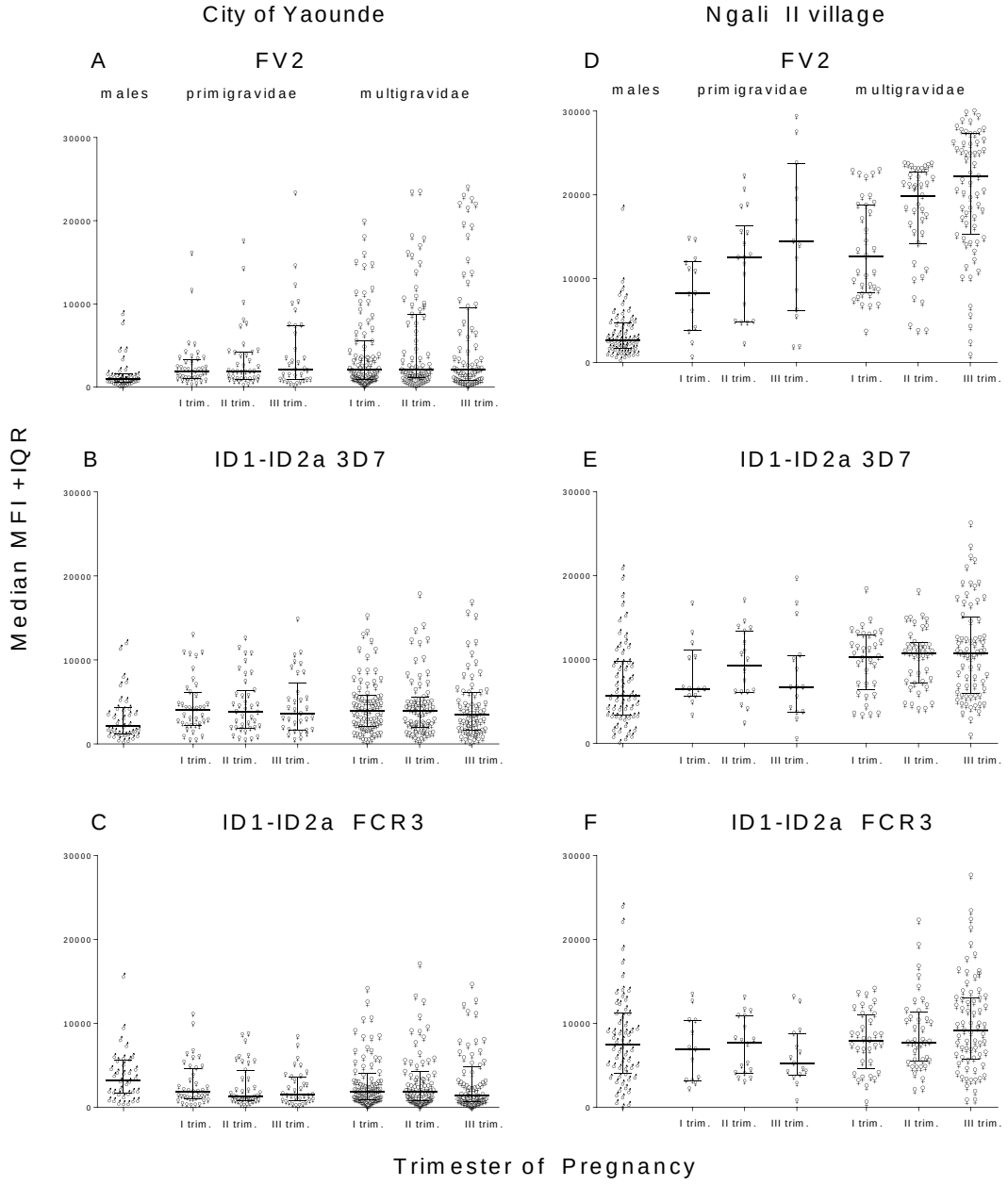
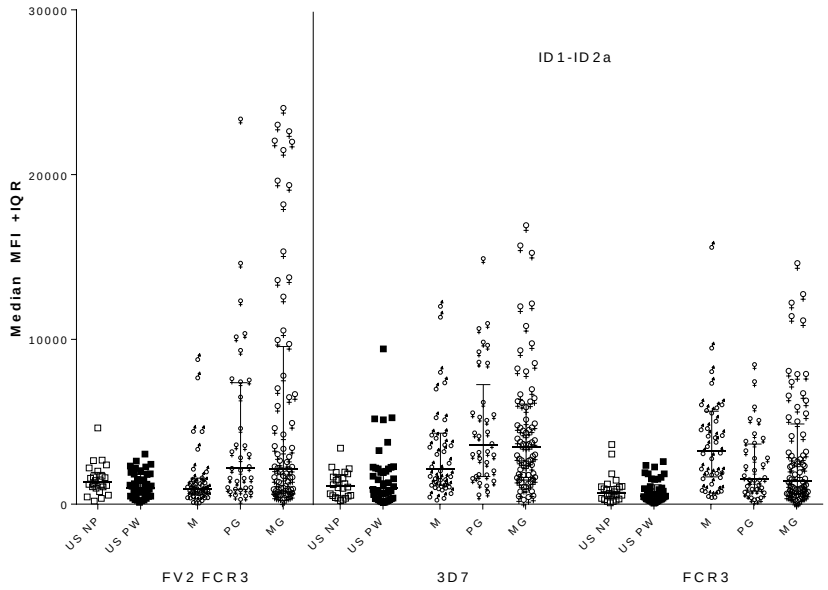


Figure 3.1. IgG levels to ID1-ID2a in Cameroonian women during pregnancy. Ab levels to FV2 FCR3 (A and D), ID1-ID2a 3D7 (B and E) and ID1-ID2a FCR3 (C and F) were measured in plasma samples from Cameroonian women living in the city (**A-C**) and Ngali II village (**D-F**). Plasma samples were randomly selected such that no one woman had more than one datapoint per trimester. Number of samples per trimester ranged **A-C**: PG 33-39 women, MG 63-74 women; **D-F**: PG 13-15 women, MG 33-68 women. Using datapoints for women who had samples in each of the 3 trimesters (City: PG n=32, MG

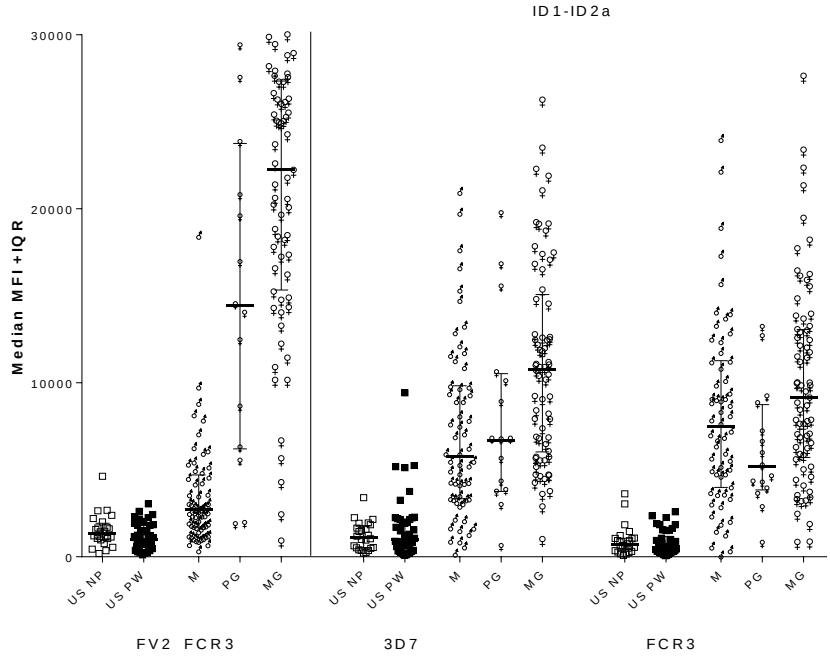
n=53; village: PG village n=7, MG n=17), Friedman's test was performed to identify whether Ab levels increase during pregnancy (Fig. S2 and S3). Ab levels to ID1-ID2a (both strains) did not increase during pregnancy in either location ($p>0.05$). Ab levels to FV2 FCR3 increased during pregnancy in the village (PG: $p=0.008$, MG $p=0.001$), but not the city. In both locations no significant differences were found in Ab levels to ID1-ID2a 3D7/FCR3 between males and females (all p values > 0.05). Slightly higher Ab levels to ID1-ID2a 3D7 were found in MG living in the village compared to males (all $p<0.05$). Statistically higher Ab levels to FV2 FCR3were observed in all PG and MG compared to males (city: all $p<0.001$, village all $p<0.0001$). ♂- males, small ♀- PG, big ♀- MG.

Figure 3.2.

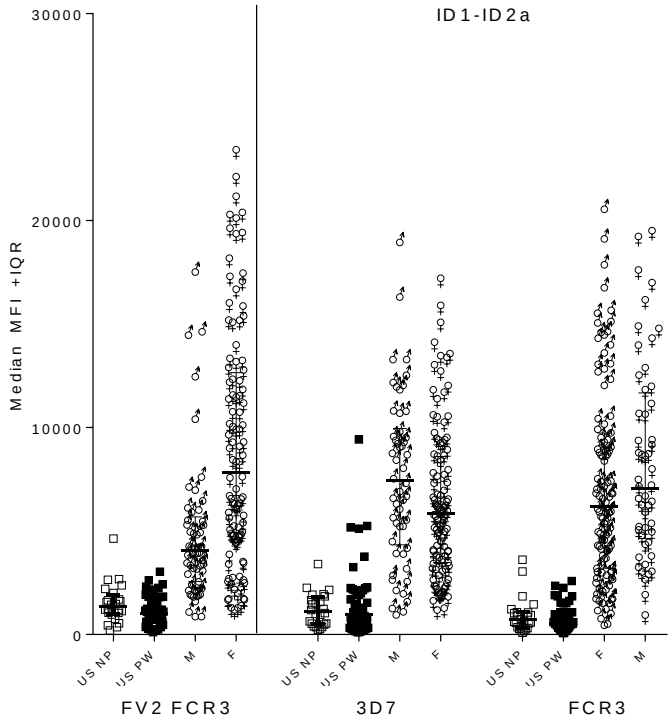
A.


ID1-ID2a
Median MFI +IQR
30000
20000
10000
0
US NP
US PW
M
PG
MG
FV2 FCR3
3D7
FCR3

B.


ID1-ID2a
Median MFI +IQR
30000
20000
10000
0
US NP
US PW
M
PG
MG
FV2 FCR3
3D7
FCR3

C.


ID1-ID2a
Median MFI +IQR
30000
20000
10000
0
US NP
US PW
M
F
FV2 FCR3
3D7
FCR3

D.

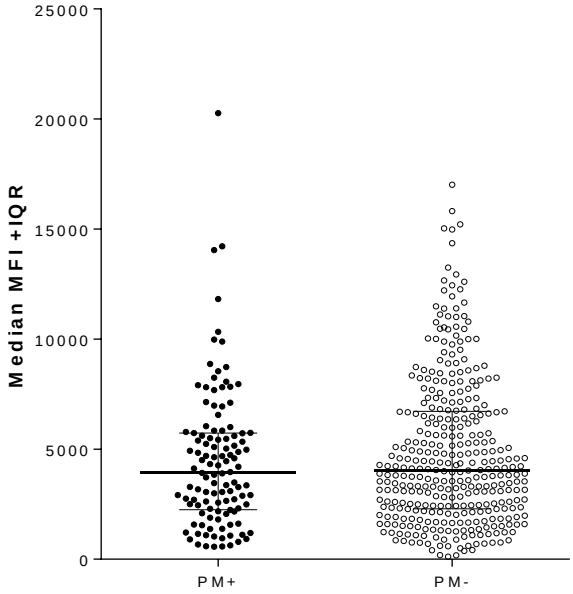

Median MFI +IQR
25000
20000
15000
10000
5000
0
PM+
PM-

Figure 3.2. Association of IgG to ID1-ID2a with absence of PM at delivery. IgG levels to FV2 FCR3 and ID1-ID2a 3D7/FCR3 were measured in delivery plasma collected from pregnant Cameroonian women living in **A)** Yaounde city (n=35 males, n=33 PG, n=63 MG), **B)** Ngali II village (n=58 males, n=15PG, n=68 MG), **C)** Simbok village (n=51 males, n=102 women; these samples were from cross-sectional study), **D)** MG from Yaounde city (PM+ n=116, PM- n=348), as well as US pregnant (n=42) and non-pregnant controls (n=24) and Cameroonian males (City: n=35 males; Villages: n=58 Ngali males, n=51 Simbok males). Significantly higher Ab levels to ID1-ID2a (both strains) were observed in Cameroonian males (city and village) compared to US pregnant women ($p<0.001$). No significant differences in IgG to FV2 were observed between US pregnant and non-pregnant controls and Cameroonian city males; higher IgG levels to FV2 observed in Cameroonian males living in the village ($p<0.0001$). No significant differences in IgG to ID1-ID2a were observed between Cameroonian males and females living in the city and village (both strains $p>0.05$). Few exceptions were found in the villages: Ngali II MG ($p<0.0001$) and Simbok females ($p=0.04$, was not confirmed when assessed in only FV2-seropositive women Fig. S4) had higher IgG to ID1-ID2a 3D7 compared to males. No significant differences were observed in IgG to ID1-ID2a FCR3 between PM+ and PM- MG in the city (D: $p=0.46$). Horizontal bar represent median and whiskers represent Inter-Quartile Range, IQR. □- US non-pregnant (US NP), ■- US pregnant (US PW), ♂- Cameroonian males (M), small ♀- Cameroonian PG, big ♀- Cameroonian MG.

Figure 3.3

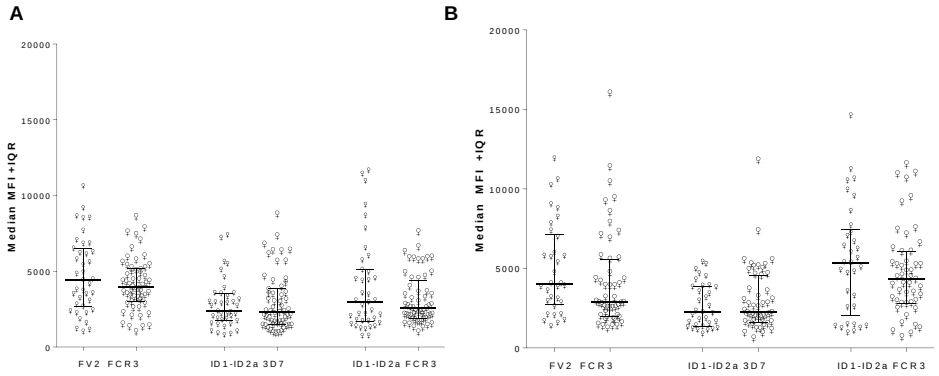


Figure 3.3. IgM levels to FV2 and ID1-ID2a in Cameroonian women. IgM levels to FV2 FCR3 and ID1-ID2a (3D7 and FCR3) were measured in plasma collected at first smear positive visit and the following visit from women living in **A)** city (PG n=17, MG n=26) and **B)** Ngali II village (PG n=21, MG n=33). No statistically significant differences in IgM levels to either ID1-ID2a or FV2 FCR3 were observed between PG and MG in both locations (all $p>0.05$). Similar results were observed when only second visit IgM levels were analyzed (Figure S5). Since beads were run at saturation, it appears that both PG and MG produced higher IgM levels to FV2 FCR3as compared to ID1-ID2a 3D7 (both locations $p<0.001$). City PG and MG produced higher IgM levels to FV2 FCR3compared to ID1-ID2a FCR3 ($p<0.05$). Small ♀- PG, big ♀- MG.

Figure 3.4

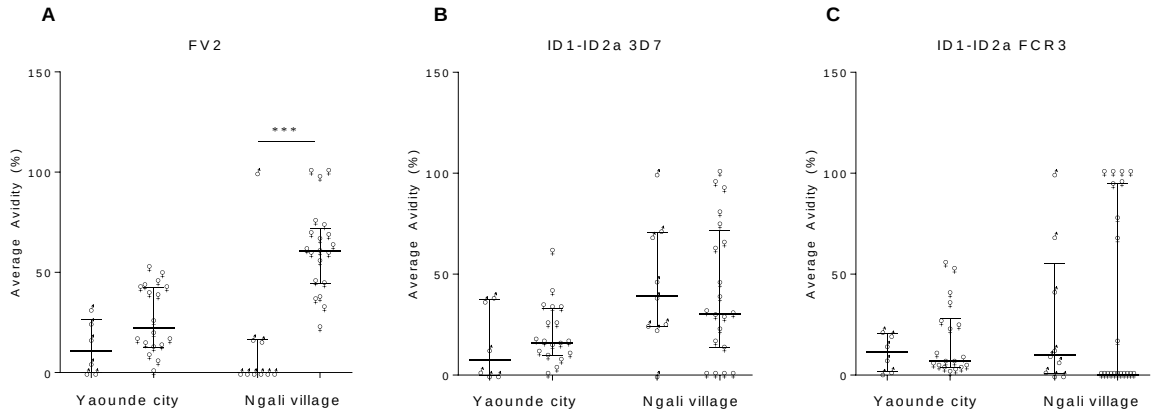


Figure 3.4. Avidity of IgG to FV2 and ID1-ID2a in males and females with high IgG levels to ID1-ID2a. A subset of city and Ngali II village plasma samples with high IgG to ID1-ID2a were tested in avidity assay. Avidity of IgG to **A)** FV2 FCR3, **B)** ID1-ID2a 3D7, **C)** ID1-ID2a FCR3 was measured in the city (n=6 males, n=18 women) and Ngali II village (n=9 males, n=20 women) . Avidity to FV2 FCR3was significantly higher in Ngali II females compared to males (p=0.0003). No statistically significant differences were found in Ab avidity to ID1-ID2a (3D7 and FCR3) between males and females, both in the city and village (all p>0.05). ♂- males, ♀- females.

Figure 3.5

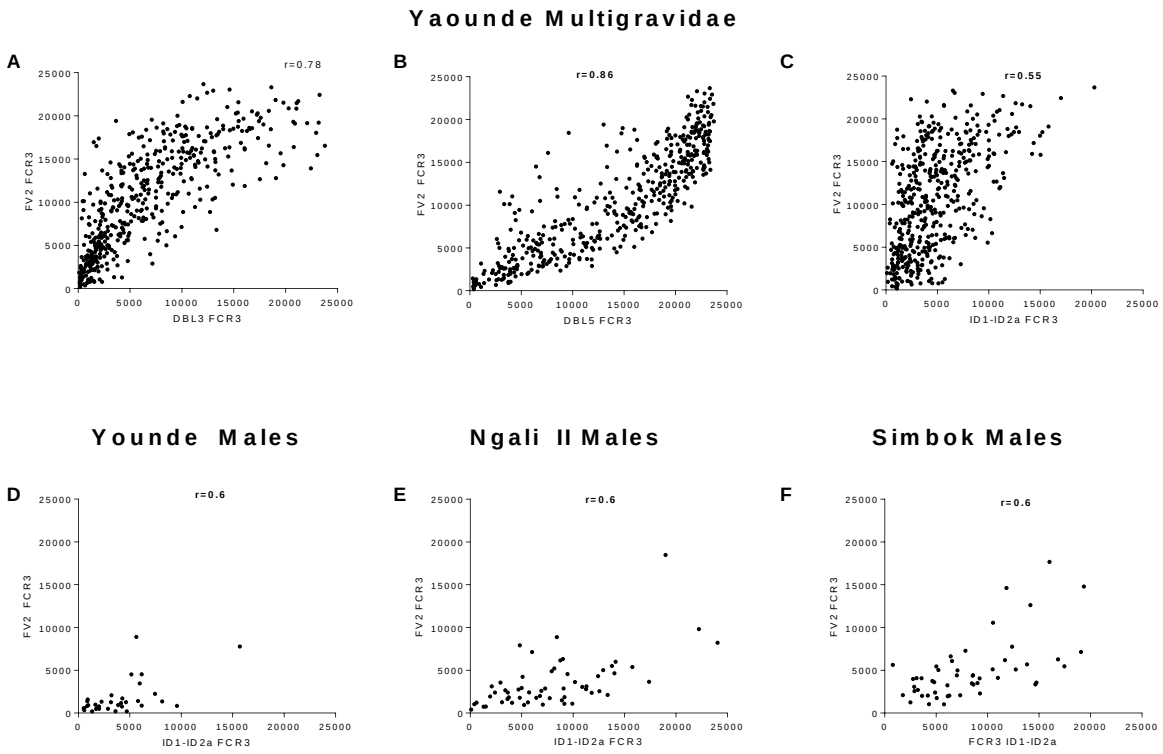


Figure 3.5. Comparison of Ab levels between ID1-ID2a and FV2. Ab levels to FV2 FCR3 and ID1-ID2a FCR3 were measured in plasma of **A-C)** 116 PM+ and 348PM- pregnant women living in Yaounde, **D)** Yaounde males, **E)** Ngali males, **F)** Simbok males. Reactivity between FV2 FCR3and DBL3 FCR3 (A) and DBL5 FCR3 (B) and ID1-ID2a FCR3 (C-F) were compared. Positive correlations were found for all the comparisons; Pearson's correlation coefficients (r) are reported.

Figure 3.6

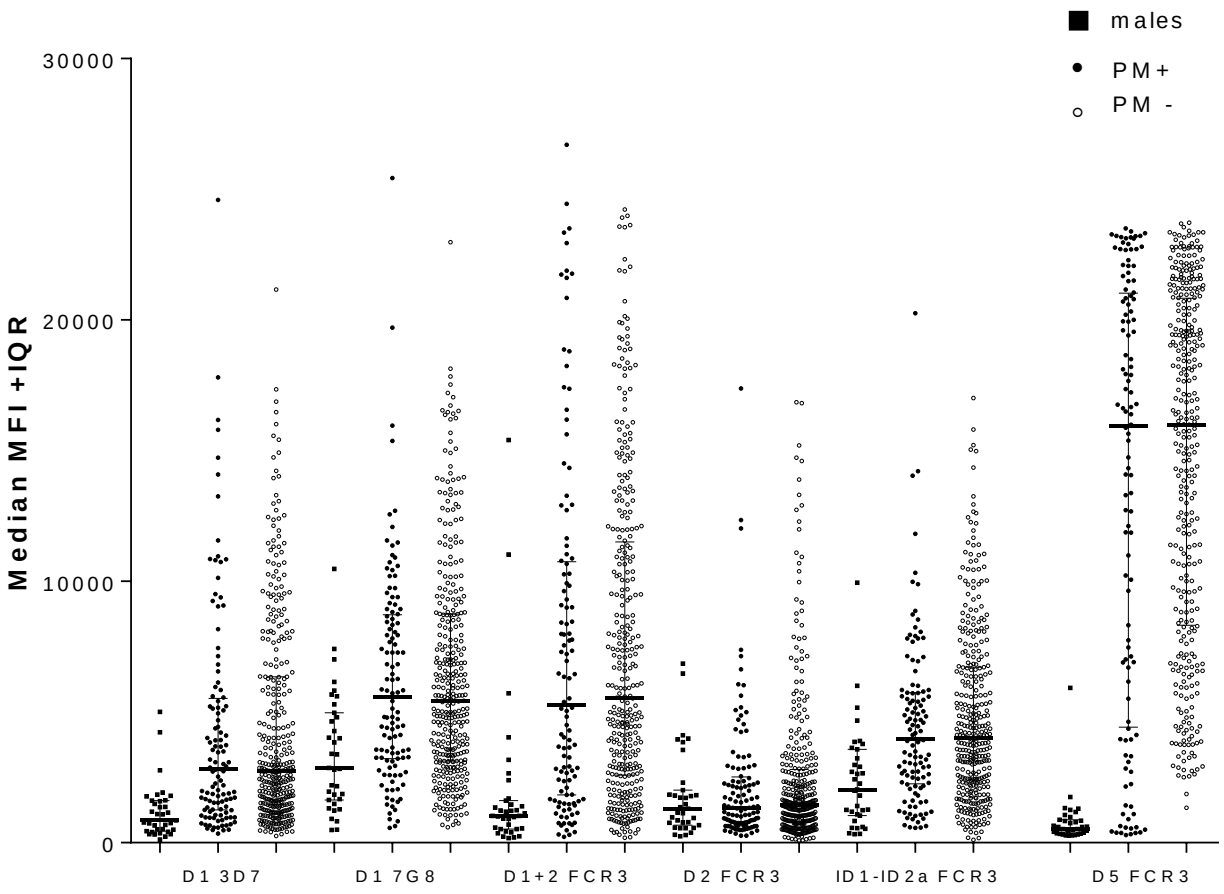


Figure 3.6. IgG levels to N-terminal domains of FV2 and DBL5. Ab levels to DBL1 (3D7 and 7G8), DBL1+2 (7G8), DBL2 FCR3, ID1-ID2a FCR3 and DBL5 FCR3 in plasma of 116 PM+ and 348PM multigravidae women living in the city were measured at delivery. Both PM+ and PM- women had significantly higher levels of Ab to DBL5 compared to Ab to DBL1, DBL 1+2, DBL 2 ID1-ID2a (all $p<0.0001$). No statistical differences were found in Ab levels to any of the domains between PM+ and PM- women (all $p>0.05$). Open circles represent PM- and black circles represent PM+.

Figure S3.1

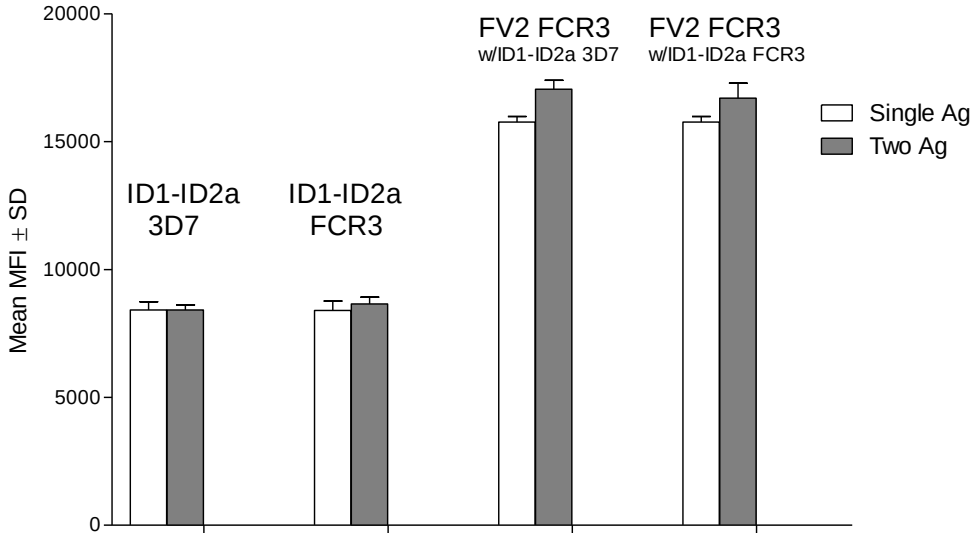


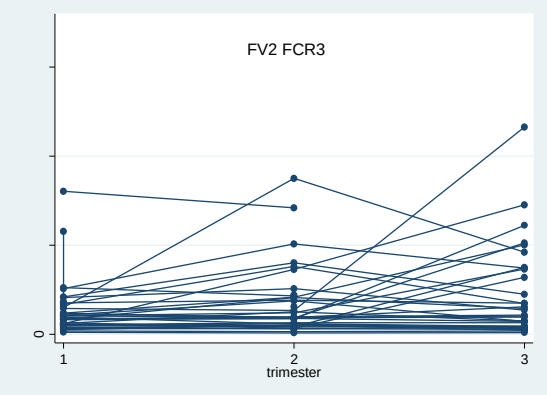
Figure S3.1. ID1-ID2a and FV2 used alone or combined in the MAP assay. Microspheres coupled with ID1-ID2a (3D7 and FCR3) were tested alone and combined with FV2 FCR3 in the MAP assay using a pool of plasma from pregnant women with high Ab titers to VAR2CSA. Mean MFI ± SD are shown. No significant differences were observed between Ab levels when the antigens were used alone or multiplexed, except a minor increase in Ab levels to FV2 FCR3 when it was multiplexed with ID1-ID2a 3D7 (p=0.032).

Figure S3.2

City of Yaounde

Ngali Village

A



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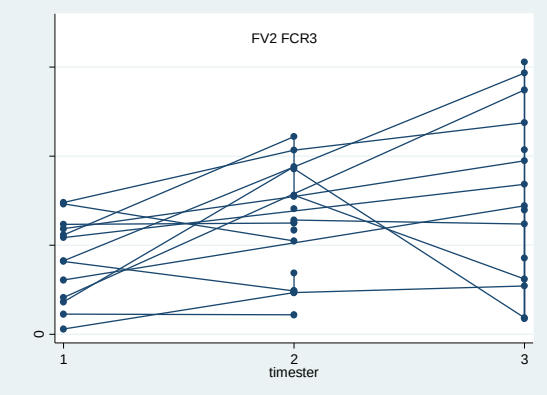

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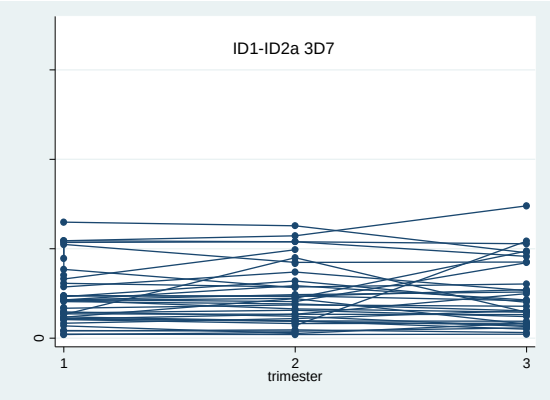

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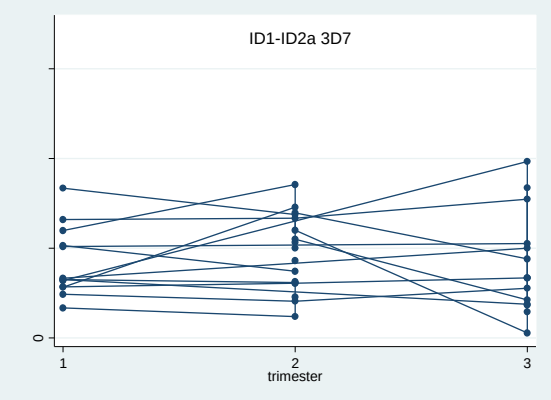

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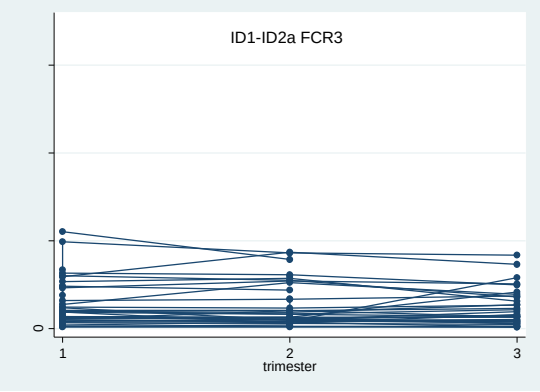

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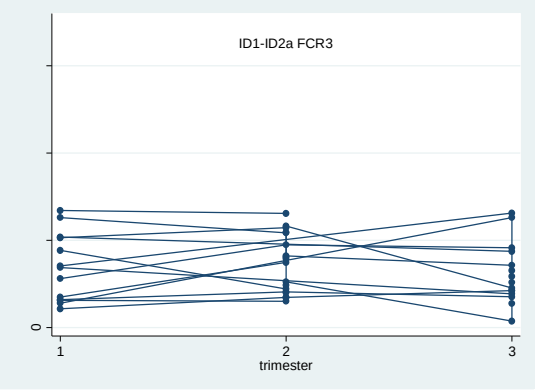

Figure S3.2. IgG to FV2 and ID1-ID2a in Cameroonian primigravidae during pregnancy. IgG levels to FV2FCR3 (**A,D**), ID1-ID2a 3D7 (**B, E**) and ID1-ID2a FCR3

(**C, F**) were measured in Cameroonian primigravidae living in the city (A-C, n=32) and the village (D-F, n=7) who had datapoints for each trimester. Ab levels to ID1-ID2a (both strains) did not increase during pregnancy in either location ($p>0.05$). Ab levels to FV2 FCR3 increased during pregnancy in the village (PG: $p=0.008$), but not the city.

Figure S3.3

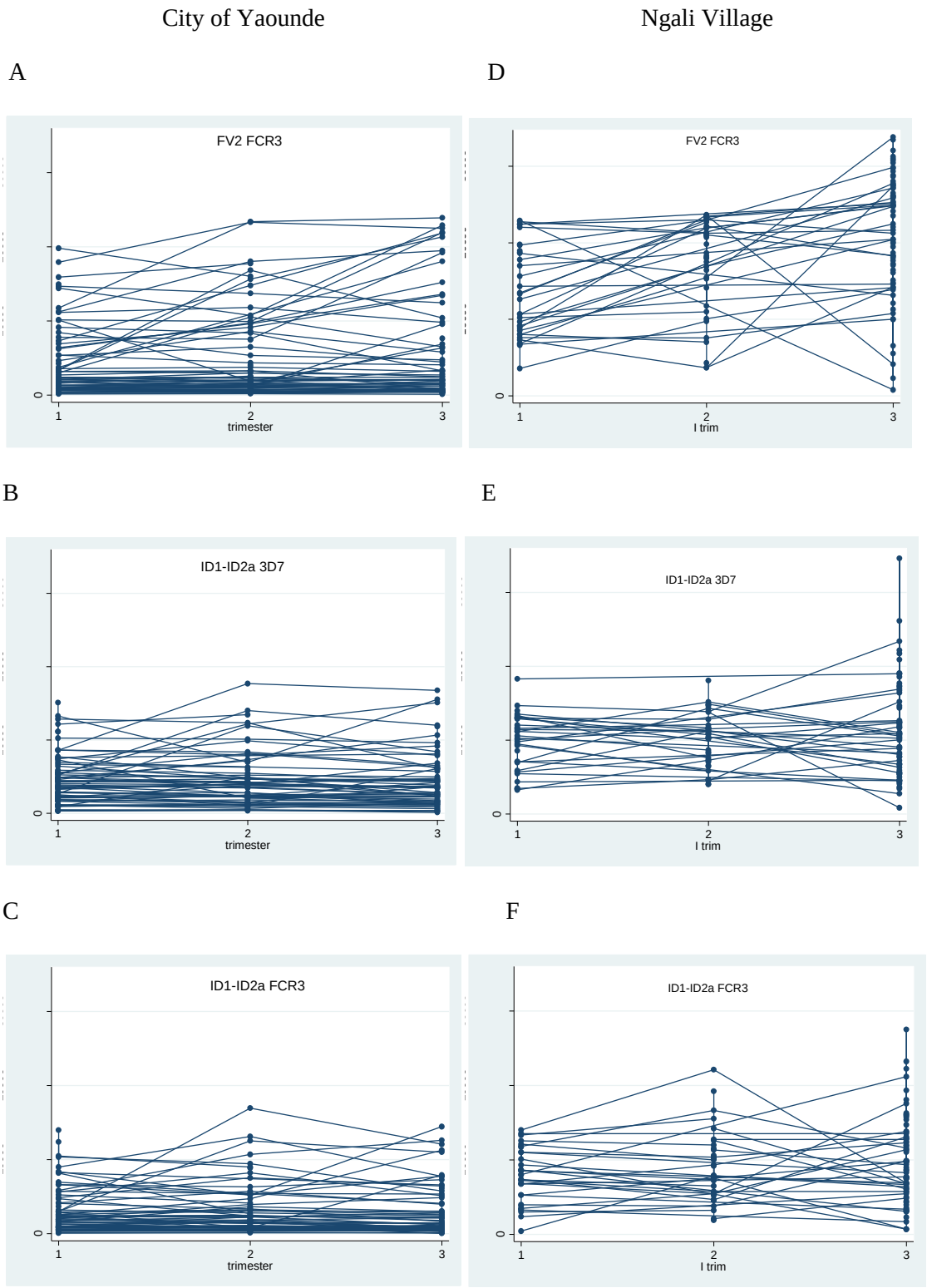

Figure S3.3. IgG to FV2 and ID1-ID2a in Cameroonian multigravidae during pregnancy. IgG levels to FV2FCR3 (**A,D**), ID1-ID2a 3D7 (**B, E**) and ID1-ID2a FCR3

(**C, F**) were measured in Cameroonian primigravidae living in the city (A-C, n=53) and the village (D-F, n=17) who had datapoints for each trimester. Ab levels to ID1-ID2a (both strains) did not increase during pregnancy in either location ($p>0.05$). Ab levels to FV2 FCR3 increased during pregnancy in the village (MG $p=0.001$), but not the city.

Figure S3.4

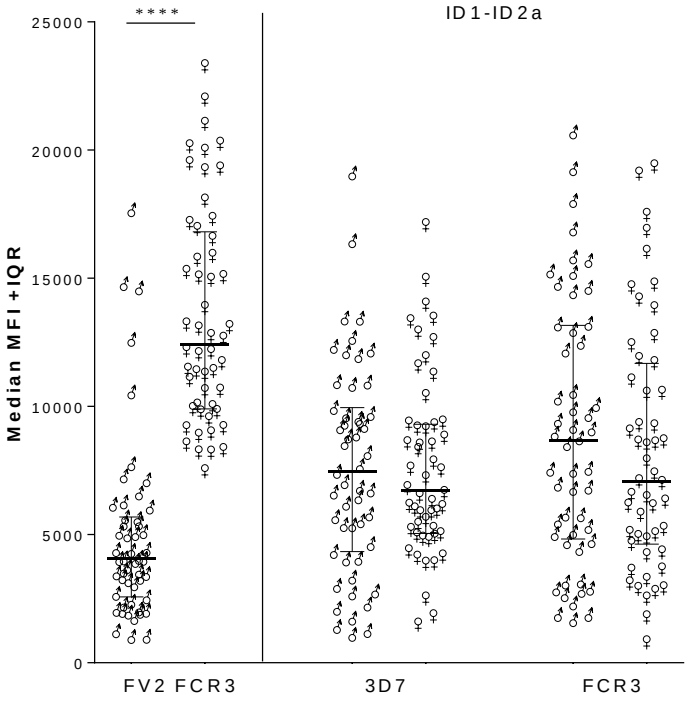

Figure S3.4. IgG to FV2 and ID1-ID2a in women and men living in Simbok village. IgG levels to FV2 FCR3, ID1-ID2a 3D7 and ID1-ID2a FCR3 were measured in plasma from women and men living in Simbok village (n=51 males, n=52 women). Initially, all women were screened for the presence of Ab to FV2 FCR3 to ensure women were exposed to CSA-binding *P. falciparum* (since gravidity data was not available) and FV2 FCR3 seropositive women were used in the analysis. Horizontal bar represent median and whiskers represent IQR.

Figure S3.5A **B**

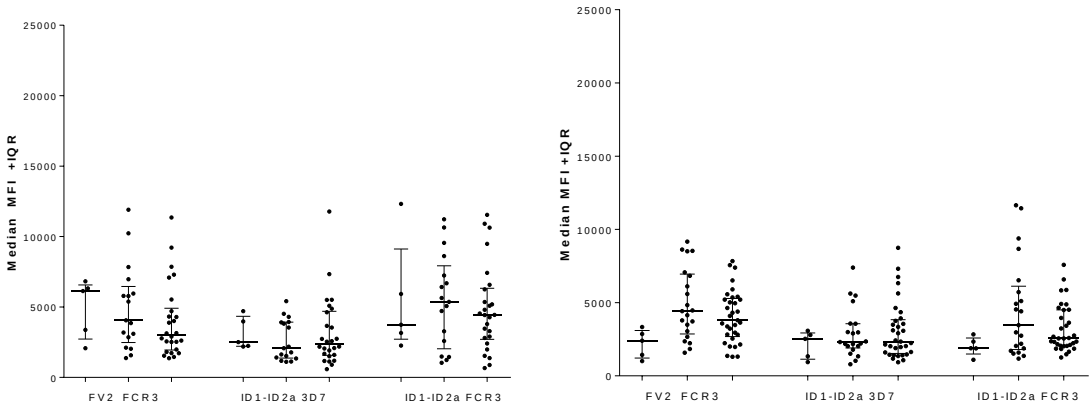

Figure S3.5. IgM levels to FV2 and ID1-ID2a in Cameroonian women. IgM levels to FV2 FCR3 and ID1-ID2a (3D7 and FCR3) were measured in plasma collected at first smear positive visit and the following visit from women living in **A)** city (PG n=17, MG=26) and **B)** Ngali II village (PG=21, MG=33). Second visit IgM data are presented. No significant differences were found between PG and MG IgM levels to ID1-ID2a 3D7 and FCR3 strains, as wells as FV2 FCR3 (both for city and Ngali II village). Small ♀- PG, big ♀- MG.

Figure S3.6

NYIKGDPYFAEYATKLSFILNPSDANNPSGETANHNDEACNCNESGISSVGQAQT
SGPSSNKTCITHSSIKTNKKKECKDVKLGVRENDKDLKICVIEDTSLSGVDNCCC
QDLLGILQENCSDNKRGSSSNDSCDNKNQDECQKKLEKVFASLTNGYKCDKCKS
GTSRSKKKWIWKKSSGNEEGLQEEYANTIGLPPRTQSLYLGNLPKLENVCEDVK
DINFDTKEKFLAGCLIVSFHEGKNLKKRYPQNKNSGNKENLCKALEYSFADYGD
LIKGTSIWDNEYTKDLELNLQNNFGKLFGKYIKKNNTAEQDTSYSSLDELRESW
WNTNKKYIWTAMKHGAEMNITTCNADGSVTGSGSSCDDIPTIDLIPQYLRFLQE
WVENFCEQRQAKVKDVITNCKSCKESGNKCKTECKTKCKDECEKYKKFIEACG
TAGGGIGTAGSPWSKRWDQIYKRYSKHIEDAKRNRKAGTKNCGTSSTTNAAAST
DENKCVQSDIDSFFKHLIDIGLTTPSSYLSNVLDDNICGADKAPWTTYTTYTTTEK
CNKERDKSKSQSSDTLVVVNVPSPLGNTPYRYKYACQCKIPTNEETCDDRKEYM
NQWSCGSARTMKRGYKNDNYELCKYNGVDVKPTTVRSNSSKLD.

Figure S3.6. The sequence of the recombinant ID1-ID2a construct.

CHAPTER 4

DEFINING CORRELATES OF PROTECTION FOR PLACENTAL MALARIA USING A MULTI-ASSAY APPROACH

Running title: VAR2CSA: Correlates of Protection

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Anna Babakhanyan[1], Miao Wang[2], John J. Chen[1,2], Grace Sama[3], Canisia Efundem[3], Robert J.I. Leke[2], Rose G.F. Leke[2], Diane Wallace Taylor[1*]

[1] Department of Tropical Medicine, Medical Microbiology and Pharmacology, University of Hawaii at Manoa, John A. Burns School of Medicine, Honolulu, Hawaii, 96813, U.S.A. Email: A. Babakhayan: annab8@hawaii.edu; J. J.Chen: jjchen.hawaii.edu; D.W. Taylor: dwtaylor@hawaii.edu

[2] Biostatistics and Data Management Core, JABSOM, University of Hawai'i. M. Wang: miaowang@hawaii.edu

[3]The Biotechnology Center, Faculty of Medicine and Biomedical Research, University of Yaounde 1, Yaounde, Cameroon.
Email: G. Sama: menyensg@yahoo.com; C. Efundem: caniciaearthlink@yahoo.com; R.J.I. Leke: robertjleker@yahoo.fr; R.G.F. Leke: roseleke@yahoo.com

*Corresponding author: Mailing address: 651 Ilalo Street, BSB 320, Department of Tropical Medicine, Medical Microbiology and Pharmacology, University of Hawaii at Manoa, Honolulu, HI 96813, USA. Phone: (808) 692-1608. E-mail: dwtaylor@hawaii.edu

4.1 Abstract

Antibodies to VAR2CSA prevent *Plasmodium falciparum* infected erythrocytes from sequestrating in the placenta, i.e., prevent placental malaria. Accordingly, researchers are trying to develop VAR2CSA-based vaccines; however, field testing almost impossible without serological assays that serve as correlates of protection. Thus, the current study screened plasma collected from Cameroonian multigravidae with (n=116) and without (n=348) placental malaria in 24 assays that measured antibody levels to full length VAR2CSA, individual DBL domains, strain variants, and avidity to VAR2CSA, plus antibodies to other malarial antigens. The sensitivity and specificity for each assay was determined. High antibody levels and avidity to VAR2CSA correlated with absence of placental malaria; whereas, high antibodies to AMA-1, MSP-1 and MSP-2 correlated with presence of placental malaria. Women who had <5 pregnancies were more likely to have placental malaria if they had low IgG levels (p=0.038) and avidity to the full-length VAR2CSA (p=0.0003), recognized fewer domains (p=0.01), had low IgG levels to DBL2 (0.04) and DBL3 (0.02). Assays with the best predictive potential were incorporated into multivariate regression models. Parsimonious models containing a) gravidity, VAR2CSA seropositivity, AMA-1 and b) gravidity, VAR2CSA, DBL1+2 and AMA-1had the best discriminatory power (A: Youden index: 0.39, sensitivity: 0.67, specificity: 0.71; B: Youden index: 0.39, sensitivity: 0.74, specificity: 0.65). Further analysis using the CART approach with gravidity, high avidity antibodies to VAR2CSA, VAR2CSA seropositivity, number of domains recognized, MSP-2 and DBL 1+2 resulted in Youden index of 0.49 (sensitivity: 0.59, specificity: 0.89). Another CART model including gravidity, average avidity, number of domains recognized, MSP-2, DBL1+2 and full-length VAR2CSA resulted in Youden index of 0.45 (sensitivity: 0.59, specificity: 0.86). These data show that a multi-assay approach has better discriminatory power than univariate models and redundancy of the immune system allows for multiple mechanisms of protection from natural infection.

4.2 Introduction

In pregnant women, *Plasmodium falciparum*-infected erythrocytes (IE) express the VAR2CSA malarial adhesin that mediates binding of IE to placental chondroitin sulfate A (CSA) [30, 77]. Pathology results from the accumulation of IE causing a condition called placental malaria (PM). PM endangers the health of pregnant women and the developing fetus [30]. Antibodies (Ab) to VAR2CSA play an important role in protection from adverse outcomes of PM in women living in endemic areas. Over successive pregnancies, women produce Ab to VAR2CSA that inhibit the binding of IE to CSA *in vitro* [89, 104], reduce maternal anemia [105], lower placental parasitemia at delivery [106, 107], increase the length of gestation [108], and improve infant birth weight [108]. VAR2CSA is a primary vaccine candidate for reducing the severe effects of PM. Researchers are exploring various sequences of VAR2CSA to develop a vaccine, including VAR2CSA C-terminal domains 4 (DBL4ε) and 5 (DBL5ε) [137] and the N-terminal ID1-ID2a sequence that contains the CSA-minimal binding site [84].

Although VAR2CSA-based vaccines for placental malaria are feasible, field-testing of vaccines is going to be challenging in the absence of defined correlates of protection. Determining correlates of protection is one of the first steps in vaccine development and provides an objective criterion for efficacy testing. For example, it is known that a quantitative correlate of protection from measles is 120mIU/mL of neutralizing Ab [138] and from tetanus it is 0.1IU/mL toxin-neutralizing Ab [139]. Yet, there are no definitive correlates of protection for PM. We have previously shown that high Ab levels to multiple VAR2CSA domains [109] and high avidity Ab to the full-length VAR2CSA early in pregnancy are associated with the absence of PM at delivery [110]. However, all previous studies have used only a single assay to measure protection from PM. We hypothesize that using a multiple assays simultaneously in one model will lead to improved predictive power. This study screened 464 plasma samples from multigravid women with and without placental malaria in 24 serological assays that measured immunoglobulin G (IgG) to full-length VAR2CSA (FV2); the six DBL domains, including variants from different malarial strains Ab avidity to FV2; total number of DBL domains recognized, as well as Ab to non-pregnancy specific antigens

(MSP-1, MSP-2, AMA-1, RESA, CSP). Univariate, multivariate regression models and recursive partitioning approaches were used to analyze the results. Since the plasma samples were collected before implementation of Intermittent Preventive Treatment with Sulfadoxine and Pyrimethamine (IPT) and Insecticide Treated Bednets, the immune responses measured in this study were not confounded by heterogenous exposure to *P. falciparum*. To our knowledge this is a first study to use such a large panel of VAR2CSA domains and moieties as well as non-pregnancy associated malarial antigens to define correlate of protection from placental malaria. Identifying correlates of protection from PM would expedite VAR2CSA-based vaccine development, a vaccine that could protect an estimated 85 million women and their fetuses worldwide from the severe effects of malaria [4].

4.3Methods

IRB approvals. The archival coded samples used in the current study were exempt from human subject research by the Committee on Human Studies, University of Hawaii, Manoa (CHS#19912). The original studies were conducted according to the Helsinki Declaration principles and approved by the National Ethics Committee, Cameroon and the Institutional Review Board at Georgetown University. All participants gave written informed consent to use their blood samples to measure Ab to malaria.

Study design and plasma samples. Archival plasma samples from a previous large cross-sectional study 1996-2001 [65, 131] were used; all the samples were collected at delivery from Cameroonian women living in Yaounde. Yaounde, the capitol of Cameroon, it is a malaria endemic area, where entomological inoculation rates are estimated to be 0.1-1.1 infectious bites/person/night [129]. Since the samples were collected before implementation of IPT and long-lasting insecticide treated bednets, all of the women were likely to have become infected several times during pregnancy. To rule out the possibility that women might not have become infected, plasma samples were pre-screened for the presence of Ab to FV2. Thus, only plasma samples from women with evidence of infection during pregnancy were included in the study. To determine

the fine-specificity of protective Ab, only multigravidae were included in the study. Samples from all multigravidae (≥3 pregnancies; n=116) who had placental malaria and were ≥20 years of age and delivered live babies >28 weeks of gestation were included. These women should have developed protective immunity during previous pregnancies, but the presence of PM demonstrated they had failed to obtained adequate protective immunity. For comparison, three times the number (n=348) of placental malaria-negative women were randomly selected, who met the inclusion criteria. Archival plasma samples from US adults (n=25), US pregnant women (n=42), and 20 Cameroonian males were used as negative controls.

Diagnosis of placental malaria. Thick and thin blood smears were prepared using maternal peripheral and placental intervillous space blood, and impression smears were made from biopsies of placental tissues. Slides were stained with Diff-Quick (Polysciences, Warrington, PA, Catalog Number: 24606-250) and read by two microscopists to determine parasitemia. Placental biopsies were also fixed in buffered formalin, embedded, stained with hemotoxylin-eosin, and examined for parasites. A woman was considered to have PM if IE were detected in blood smears of intervillous space blood, impression smears of villous tissue, or histological sections of the placenta [109].

Recombinant proteins. The panel of recombinant proteins of different regions of VAR2CSA have been described previously [109], and included: full length VAR2CSA, DBL1+2, ID1-ID2a (contains the minimal CSA binding site), DBL 2, DBL 3, DBL 4, DBL 5, DBL 6 of the FCR3 strains expressed in Baculovirus from A. Salanti (University of Copenhagen, Denmark); DBL-1, DBL3, DBL4, DBL5, and DBL6 for the 7G8 and 3D7 strains expressed in *Pichia pastoris* from J.D. Smith and colleagues (Seattle Biomedical Research Institute), and DBL 3 from A4 parasites expressed in *Escherichia coli* provided by K. Singh (NIAID, NIH, Bethesda, MD). In addition, non-pregnancy-associated malarial antigens were used, including recombinant AMA-1 3D7 (expressed in yeast), MSP-1_{42} 3D7 (*E. coli*), and MSP-2 3D7 were provided by C. Long, Malaria Vaccine Development Branch (MVDB), National Institute of Allergy and Infectious Disease, National Institutes of Health, Rockville, MD. Synthetic peptides containing B-

cell epitopes from CSP and RESA were synthesized by AnaSpec, Inc. (San Jose, CA) and reported previously in detail [132].

Coupling of recombinant proteins to SeroMAP microspheres. The method used for coupling was reported previously [132]. Proteins were coupled at optimal concentrations to 1 million microspheres: DBL1 – DBL6 at 1 µg; FV2 at 3µg; AMA-1 3D7 and MSP-1 3D7 at 1µg; RESA at 40µg; CSP at 15µg and MSP-2 3D7 at 0.2µg.

Measuring IgG using a Multi-analyte Platform (MAP) assay. The MAP assay was performed as previously described [109, 110, 132]. Briefly, 50µl of antigen-coupled microspheres (2,000 microspheres/test) were incubated with 50µl of a 1:100 dilution of plasma in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) in pre-wetted filter plates (96 well Multiscreen BV; Millipore, Billerica, MA), for 1hr at 25°C on a rotating shaker at 500 rpm (Microplate Shaker, Lab-line, Melrose Park, IL). Microspheres were washed twice with PBS-0.05% Tween20 and once with PBS-1% BSA. Then, 100µl of secondary Ab (R-phycoerythrin-conjugated, Affini Pure F(ab')$_2$ fragment, Goat anti-human IgG Fc fragment specific, Jackson Immunoresearch, West Grove, PA, Cat # 109-116-170) diluted to 2µg/ml in PBS-1% BSA was added to each well and incubated as above in the dark for 1 hr. Wells were washed as described above, microspheres were re-suspended in 100µl PBS-1% BSA and 85µl of the microsphere suspension was analyzed using a Liquichip M100 reader (Qiagen, Valencia, CA). The reader was programmed to read a minimum 100 beads per spectral address, DD Gate 7500-15000 and 35sec timeout. The results were expressed as mean fluorescence intensity (MFI). Positive and negative controls were included on each plate consisting of 3 different pools of plasma from 8 Cameroonian multigravidae with high Ab levels to VAR2CSA and pools of plasma from 40 Americans who had never travelled to malaria endemic areas.

Avidity to the FV2. The avidity assay was performed as previously described [110]. Briefly, plasma was diluted 1:300, 1:1,000 and 1:3,000 in PBS-1% BSA and 50µl of diluted plasma was added to six wells (each dilution in duplicate) containing 50µl of FV2-coupled microspheres (2,000 microspheres/test) and incubated for 1 hr on a shaker. After incubation, 100µL of 3M NH_4SCN in PBS – % BSA was added to half of the wells

and 100µL of PBS-1% BSA was added to the other half (matching control wells for each dilution). After 30 min of incubation, the wells were washed and incubated with secondary Ab, wash, and analyzed by LiquiChip 100 as described in the MAP assay. Avidity was determined for each dilution by the following formula: (MFI obtained from wells incubated with salt)/(MFI obtained from corresponding control wells)*100%, and the average was taken across 3 dilutions. Positive control consisting of pool of plasma from multigravida Cameroonian women and negative control consisting of pool of US plasma were included on each plate. Ab that had avidity of 35% were considered high avidity Ab.

Determining the breadth of the VAR2CSA antibody response. The panel included 15 recombinant variants from 3 different strains (i.e., 3D7, FCR3, 7G8) that represent the 6 DBL domains. A woman was considered to be Ab-positive for each of the 15 antigens if her plasma had a mean MFI plus 2 standard deviations greater than 20 Cameroonian males. In determining the breadth of the Ab response (repertoire) , the total number of variants a women recognized was determine, giving a score of 0-15 variants (note: ID1-ID2a and DBL1+2 were excluded from this analysis). A woman was considered Ab positive for a particular domain, if she had Ab to one or more variant of that domain, i.e., giving a breadth of DBL domains of 0-6. Mean Ab levels plus two standard deviations of US control was used as a cut-off for seropositivity to non-pregnancy specific malarial antigens.

Statistical Analysis. Demographic, clinical and assay variables were first summarized using descriptive statistics: mean and standard deviation for continuous variables, such as age and assay results; frequency and percentage for categorical variables, e.g., gravidity. We first evaluated the associations between outcome variables, placental malaria status and each of the 24 assay results through univariate logistic regression. Based on the significance levels of the assays and biological plausibility, we assessed models for possible interactive effects among the assays. Receiver Operating Curve (ROC) curves were generated for the univariate models and models with interaction effects. The area (AUC) under the ROC was calculated for each model to assess the discrimination power of the model. The cut-off point for each model was determined based on Youden Index

(YI). The sensitivity, specificity, odds ratios, and their 95% confidence intervals were also derived. Several candidate models with the highest YI values were further evaluated. In each case, based on p-values, non-significant terms were sequentially removed, one at a time, following the hierarchical principle [140]. As an alternative, we also utilized the classification and regression trees (CART) approach to classify these subjects. CART is a non-parametric recursive and iterative procedure for classifying multivariate data points into categories based on qualitative and or quantitative variables [141]. The recursive partitioning algorithm creates a classification tree by a series of binary splits of the data based on selected value of selected covariate. The covariate and the splitting value used are optimal in the sense that they minimize the misclassifications into the categories of the output variable. CART requires the specifications of only a few elements, such as, a rule for selecting the best split at any node and the criterion for choosing the right-sized tree. It makes powerful use of conditional information in handling nonhomogeneous relationships.

Tree structured classifiers, e.g., assay measurements, were constructed by repeated splits of subsets of the parent mode into two descendant subsets, based on Gini Index, until reach the terminal mode, i.e., at least 40 observations in parent node, at least 15 observations in any terminal mode, and any split that does not decrease the overall lack of fit by 0.01 is not attempted in our study. Each terminal node was designated by a class label. The partition corresponding to the classifier was obtained by putting together all terminal subsets corresponding to the same class.

When there were missing values, we used the best surrogate splits to decide whether those missing values went to left node or right node. If it was missing the variable containing the best surrogate split, use the second best, and so on. CART was implemented using the RPART algorithm in the R statistical package [142]. All statistical analyses were performed using R package and a two-tailed p-value of less than 0.05 was regarded as statistically significant.

4.4 Results

Characteristics of study population. Plasma samples from 464 multigravidae with live singleton deliveries were included. Of these women, 116 were PM-positive and 348 were PM-negative. The study population is described in Table 4.1. PM-positive women were ~1 year younger (p=0.0036) and had fewer pregnancies (p=0.0001) compared to the PM-negative women. No significant differences were observed between the two study populations in terms of prevalence of pre-term deliveries (p=0.32), intrauterine growth restriction (p=1.0), and low birth weight babies (p=0.7). PM-positive women had higher prevalence of anemia at delivery (p=0.004) and delivered babies with lower birth weights (p=0.04). Furthermore, PM-positive women with term deliveries had slight shorter lengths of pregnancy (p=0.028) and lower birth weight babies (p=0.002) compared to PM-negative women.

Antibody responses to malarial antigens in pregnant women with and without PM. Because the women resided in a relatively low transmission area, it was possible that the multigravid women had different levels of protective immunity, i.e., immunity increased with gravidity. Thus, pregnant women in each malaria group were stratified into two groups based on gravidity: 1) those who had 3 to 4 pregnancies and 2) those who had 5 or more pregnancies. Data on the Ab levels to FV2, its various domains, as well as non-pregnancy associated malarial antigens were compared between PM-positive and PM-negative women for each of the groups (Tables 4.2 and 4.3).

When immune responses to malarial antigens in women with "<5 pregnancies" were analyzed, significant differences were found between pregnant women with and without PM. PM-negative women had higher average avidity to FV2 (p=0.0003), IgG levels to DBL2 FCR3 (p=0.04), DBL3 7G8 (p=0.02), DBL3 FCR3 (p=0.025), FV2 (p=0.038) and recognized more DBL domains (p=0.016) (Table 4.2). Prevalence of pregnant women who were seropositive to FV2 (p=0.0018) and had high avidity IgG to FV2 (p=0.016) was also higher in PM-negative women (Table 4.2). Further, PM-negative women with "<5 pregnancies" had lower IgG levels to AMA-1 3D7 p=0.0005 and MSP-1 3D7 p=0.0088 (Table 4.2). No significant differences in Ab levels to DBL1+2, DBL 4, DBL5 and DBL6 were observed in these women (Table 4.2).

Women who had ≥5 pregnancies were more likely to have PM if they had high IgG levels to MSP-1 3D7 (p=0.026), MSP-2 3D7 (0.026) and low avidity IgG to FV2 (p=0.039) (Table 4.3). In addition, the prevalence of women with ≥5 pregnancies who were seropositive for FV2 was significantly lower in the PM-positive group (p=0.032) compared to PM-negative group (Table 4.3). Interestingly, women with and without PM with ≥5 pregnancies did not differ in the number of DBL domains recognized (p=0.9), level of IgG levels to FV2 (p=0.62), or Ab levels to the different domains. Thus, in women with ≥5 pregnancies immunity is higher in women with ≥5 pregnancies. This finding is in line with the prevalence of PM in the two groups. Prevalence of PM drops from 34% in women with 3 pregnancies to 20% in women with 5 pregnancies to 6% in women with 6 or more pregnancies.

Note that pregnant women with and without PM in both gravidity categories did not have significant differences in IgG levels to CSP (p=0.18 for ≥5 pregnancies and p=0.63 for women with <5 pregnancies) (Table 4.2 and 4.3). Since high IgG levels to CSP signify recent infection, data suggest that women with and without PM has similar exposure to *P. falciparum*.

Assessing single assays as correlates of protection from PM in univariate models. In order to evaluate fine differences in Ab responses to VAR2CSA and other malarial antigens that correlate with protection from PM, we first evaluated the association between the outcome variable, i.e., placental malaria status, with each of the 24 assay results using the univariate logistic regression model. We compared the Ab response in placenta malaria-negative women with placental malaria-positive women to FV2, the 6 DBL domains, 15 DBL strain variants, ID1-ID2a (CSA minimal binding site), AMA-1, MSP-1, MSP-2, CSP, RESA, as well as breadth of Ab response to VAR2CSA [109] and Ab avidity to FV2 [110]. We assessed predictive accuracy of each assay by determining the AUC using the ROC method. Table 4.4 summarizes the AUC, odds ratios (OR), sensitivity, specificity and YI for each assay. The discriminative power of each one of the individual assays was not impressive. Of interest, 1 standard deviation (1SD of non-transformed data) increase in average avidity to FV2 assay value decreased the risk of PM by 3% (YI=0.19, p<0.00010), having high avidity Ab to FV2 decreased the risk of

PM by 40% (YI=0.12, p=0.026), being seropositive for FV2 decreased the risk of PM by 73% (YI=0.14, p=0.0001) and 1SD increase in Ab levels to AMA-1, MSP-1 or MSP-2 increased that risk of PM by 56%, 40% and 33%, respectively (all $p<0.05$) (Table 4.4). Overall, single assay did not have both high sensitivity and specificity for predicting risk of PM.

A combination of assays measuring Ab to VAR2CSA and non-pregnancy antigens in a multivariate model improved prediction of PM. We hypothesized that a combination of assays would improve discriminatory power, as each assay would contribute unique information to a predictive model. Therefore, we generated several candidate multivariate regression models using single assays with the highest YI. Non-significant terms were sequentially removed from the models and ROC curves were generated for univariate models and multivariate models. The three parsimonious models with the highest predictive power were selected based on AUC and YI. The first model included assays from Table 4.4 with the best predictive scores: gravidity, high avidity Ab to FV2, FV2 seropositivity, number of domains recognized, AMA-1, DBL1+2 FCR3 and interactions between the variables. After non-significant terms were removed, the simplified model included gravidity, FV2 seropositivity and AMA-1 (Figure 4.1); the model had AUC of 0.73 and YI of 0.39 (sensitivity: 0.67, specificity: 0.71). In the second model, we explored biologically important covariates, regardless of their predictive score in univariate models and included gravidity, average avidity to FV2, AMA-1, FV2 and interaction between AMA-1 3D7 and FV2 FCR3 (Figure 4.2); the model had AUC of 0.74 (95% CI) and YI of 0.36 (sensitivity: 0.66, specificity: 0.71). In the third model, we incorporated DBL1+2 FCR3, which improved predictive power of the model: AUC of 0.74 and YI of 0.39 (sensitivity: 0.74, specificity: 0.65) (Figure 4.3).

Partition of placental malaria classification using the CART approach. Next, we sought to utilize an alternative approach and classified PM-positive and PM-negative women using CART analysis. Three best resulting classification trees are presented in Figures 4.4-4.7. The first CART model was composed of co-variates from the best multivariate model, including: gravidity, high avidity Ab to FV2, FV2 seropositivity, number of domains recognized, AMA-1 3D7, DBL1+2 FCR3 and interactions between

the variables (Figure 4.4). The 464 pregnant women were first classified by number of domains recognized, followed by IgG levels to MSP-2, gravidity, IgG levels to DBL1+2 and so on (Figure 4.4). Interestingly, FV2 seropositivity and high avidity Ab to FV2 were not used by the algorithm in the CART approach, possibly because they were not important in classifying the data into PM-positive and PM-negative groups. The specificity of 0.59 and sensitivity of 0.89 was achieved using this approach, resulting in YI of 0.46 (Table S4.1).

We explored other combinations of assays presented in Figures 4.5-4.7. Based on recursive partitioning in Figure 4.5, 464 pregnant women in our data set were initially classified based on Ab levels to DBL5. Nineteen women were classified as PM-positive correctly and 2 were misclassified in PM-positive group. Women were further classified according to their Ab levels to AMA-1, gravidity, and percent of DBL domains they recognized (Figure 4.5), yielding to overall sensitivity of 0.43, specificity of 0.95 and YI of 0.38 (Table S4.1). Women were also classified according to gravidity, average avidity, percent of DBL domains, FV2, DBL1+2 and MSP1 or MSP2 and results are presented in Figure 4.6 and Figure 4.7, respectively. We have integrated DBL1+2 FCR3 in the model, since this recombinant protein contains ID1-ID2a, the CSA-minimal binding site. In the latter two classification trees, women were first categorized by Ab levels to FV2. In the model in Figure 4.7, we were able to achieve YI of 0.45 (sensitivity 0.59, specificity 0.86). Thus, decision on what co-variates should be included in CART models should be guided, but not restricted by predictive scores of univariate models.

4.5 Discussion

This study evaluated the potential of using a multi-assay approach to define correlates of protection from PM. We used different combinations of immunological assays measuring immunity to VAR2CSA and non-pregnancy malarial antigens in PM-positive and PM-negative Cameroonian multigravidae to derive the best predictive model. The principle findings are that multiple assays lead to improved predictive power compared to the univariate approach. Specifically, combination of gravidity, AMA-1,

DBL1+2 and FV2 yielded to YI of 0.39 (sensitivity 0.74, specificity 0.65) (Figure 4.3), while each of the assays alone resulted in YI ranging from 0.06-0.26 (Figure 4.3B). Alternative approach using CART classification trees, presented in Figure 4.4, including gravidity, high avidity Ab to FV2, FV2 seropositivity, number of DBL domains recognized, MSP-2 and DBL1+2 resulted in YI of 0.49 (sensitivity 0.59, specificity 0.89) (Table S4.1).

It is well established that multigravidae should be protected from PM as they acquired Ab to VAR2CSA over successive pregnancies. However, the archival plasma samples included some multigravidae (≥3 pregnancies) who had PM at delivery and hence lacked protective immunity to PM. Therefore, this study sought to identify fine differences in immune responses to VAR2CSA between PM-positive and PM-negative multigravidae living in malaria endemic area. Interestingly, women who had 3 or 4 pregnancies were still acquiring Ab to more DBL domains (p=0.016), FV2 (p=0.038), and avidity to FV2 (0.0003) (Table 4.2); while women who had 5 or more pregnancies reached plateau for IgG levels to FV2 and number of domains recognized and were only fine-tuning their immune response as determined by average avidity (p=0.039) (Table 4.3).

On the other hand, univarite regression analysis suggested that Ab levels to AMA-1 (Table 4.3 p=0.0005), MSP-1 (Table 4.2 p=0.026; Table 4.3 p=0.0088) and MSP-2 (Table 4.2 p=0.026) were associated with infection (Table 4.2 and Table 4.3) in women of all gravidities. IgG levels to DBL 1, 4, 5 and 6 domains were not significantly different between PM-positive and PM-negative pregnant women of all gravidities (Table 4.2 and 4.3).

To construct multivariate models, we included assays that had high AUC and YI in univariate models. In addition, we hypothesized that inclusion of Ab to non-pregnancy-specific malarial antigens, such as AMA-1 and MSP2 would improve predictive power, since it is likely that Ab levels to the latter antigens will be elevated in PM-positive women. Further, gravidity was included as a co-variant, since it is possible that women continue fine-tuning immunity to VAR2CSA over more and more pregnancies.

Parsimonious multivariate models containing a) gravidity, VAR2CSA seropositivity, AMA-1 (Figure 4.1) and b) gravidity, VAR2CSA, DBL1+2 and AMA-1 (Figure 4.3) had the best discriminatory power (A: YI: 0.39, sensitivity: 0.67, specificity: 0.71; B: YI: 0.39, sensitivity: 0.74, specificity: 0.65). Univariate models for AMA-1 and avidity were promising and were associated with significantly increased (AMA-1 p=0.0002) or decreased (avidity p=0.0000) risk of PM (Table 4.4). Although FV2 did not perform as well in the univariate model when women were not stratified by gravidity (p=0.059 Table 4.4), the variable was included into the model since confidence interval for the odds ratio ranged from 0.66 to 1 (Table 4.4). DBL1+2 was included in the model since we hypothesized that it is possible that larger recombinant protein DBL1+2, that contains the ID1-ID2a minimal binding site, would allow for proper folding of the protein. Overall, multi-assay approach resulted in improved YI=0.39 and therefore prediction of PM at delivery. Note that caution should be taken not to exclude co-variates with poor predictive score in univariate models. Our results demonstrate that even co-variates with poor predictive scores when used in multivariate model can lead to strong predictive power that is equal to the one observed for best-scoring assays incorporated together (Figure 4.3).

The fact that more than one combination of assays yielded to comparatively similar predictive powers (YI=0.39 in Figure 4.1 and Figure 4.3), it is likely that more than one correlate of protection exists. Some PM-negative multigravidae in our study had low Ab levels to FV2, but were seropositive for multiple VAR2CSA individual domains. It is possible each woman can have a unique immunological profile to VAR2CSA and correlates of protection might be influenced by age, gravidity, exposure dynamics to VAR2CSA and *P. falciparum*, as well as other intrinsic factors (e.g., MHC) outside the pregnancy. Hence, redundancy of the immune system allows for multiple mechanisms of protection from natural infection.

The CART models proved to be extremely useful as well. Recursive partitioning was advantageous, since it is adaptable to missing data and allows for many covariates. The use of CART analysis further refined the models by adding additional co-variates and resulted in improved predictive power of PM status. The best models presented in

Figure 4.4 and 4.7 (YI of 0.49 and 0.45, respectively) included gravidity, average avidity to FV2, high avidity to FV2, seropositivity to FV2, number of domains recognized, MSP-2, DBL1+2 and FV2. Of interest, using MSP-2 instead of MSP-1 (Figure 4.7) improved YI from 0.37 to 0.45, although MSP-2 performed inferior to MSP-1 in the univariate analysis (Table 4.4). The fact that a higher level of prediction was achieved using more assays in the model speaks of relative nature of correlates of protection defined in this study, i.e., with more assays predictive power will increase. This fact was shown for correlate of protection for influenza vaccine. With 1/40 titer as a correlate of protection 70% subjects were protected; however, protection increased gradually to 90% with higher titers [143].

Some of the limitations of this study include absence of data on clinical and malaria exposure of the pregnant women throughout pregnancy, which would allow us to categorize women into better defined groups based on their clinical scores. In addition, the models did not include functional assays, such as inhibition of binding or phagocytosis. It is well established that not all Ab are the same and vaccines available today are protective on the assumption that they elicit functional Ab [144]. For instance, correct immunoglobulin G subclass is important for *Meningococcal* polysaccharide vaccine [145], lower avidity maturation was implicated to decreased efficacy of Hib *Haemophiluys influenzae* type B vaccine in combination with pertussis vaccine [146], *Pneumococcal* polysaccharide vaccine is highly effective in young adults but has poor efficacy in elderly, due to differences in opsono-phagocytic Ab [147] and one of the strains of *Rubella* induces higher titers of neutralizing Ab [148, 149].

We believe that this is the first comprehensive step towards identification of correlates of protection from placental malaria using a multi-assay approach and therefore developing a method for validating PM vaccines in efficacy trials. We are currently investigating functional properties of Ab to VAR2CSA and hope to further refine current predictive models using data on VAR2CSA Ab functions.

Acknowledgements

We wish to acknowledge the support of the entire Malaria Research Team at the Biotechnology Center, University of Yaoundé I, Cameroon, for their outstanding work, and the women and their families who participated in the studies. We also acknowledge Dr. J. Smith (Seattle Biomedical Research Institute), A. Salanti (Copenhagen University Hospital) and C. Long and K. Singh (NIAID) for providing recombinant proteins used in this study. Finally, we thank A. Kayatani (WRAIR, US Army Forces) for providing expertise with the avidity assay. The work was supported by grants from NIAID, NIH, UO1AI43888 and RO1AI071160 (DWT, RGFL), NIMHD U54MD007584 and NIH G12MD007601 (JJC and MW). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Table 4.1. Characteristics of pregnant women and their babies in the study

Characteristic	PM positive (n=116)	PM negative (n=348)	p value
Age (mean years and SEM)*	27.8 ± 0.4	29.5 ± 0.3	**0.0036**
Gravidity (mean and SEM)*	4.3 ± 0.2	5.2 ± 0.1	**0.0001**
Pre-term deliveries (%)**	14.6	19.3	0.33
Length of pregnancy (mean weeks and SEM)*	39.1 ± 0.3	38.9 ± 0.2	0.71
Full term deliveries ***	40.0 ± 0.16	40.5 ± 0.1	**0.028**
Pre-term deliveries ***	35.1 ± 0.4	34.2 ± 0.4	0.15
Intrauterine growth restriction (%)**	11.3	9.9	1.0
Anemia at delivery (%)**	27.7	14.6	**0.0043**
Low birth weight babies (%)**	2.6	2.6	0.723
Full term deliveries ***	4.7	3.1	0.51
Pre-term deliveries	17.4	32.9	0.63
Baby weight (mean grams and SEM)*	3,111 ± 52	3,245 ± 33	**0.041**
Full term deliveries ***	3,252 ± 47	3,423 ± 29	**0.0025**
Pre-term deliveries	2,544 ± 133	2,714 ± 82	0.32

* unpaired t-test
** Fisher's exact test
*** Included only samples with known gestational age (PM positive n=92, PM negative n=251)

Table 4.2. Antibody response to malarial antigens in pregnant women with 3 or 4 pregnancies (mean and standard error tabulated)

	PM positive (n=75)	PM negative (n=160)	p value§
Immune responses associated with protection			
Average avidity to FV2	**23.53 ± 1.9**	**31.4 ± 1**	**0.0003**
Prevalence of high avidity Ab to FV2 Ω	**29.3**	**46.25**	**0.016**
Prevalence of FV2 seropositive women	**80**	**94**	**0.0018**
% positive domains out of 6	**2.76 ± 0.2**	**3.3 ± 0.13**	**0.016**
% positive variants out of 15	7.7 ± 0.54	8.7 ± 0.32	0.068
Antibody levels associated with protection (mean MFI ± SEM)			
CSP	802 ± 108	866 ± 76	0.63
RESA	863.1 ± 197	1017 ± 153	0.56
DBL1 3D7 *(Pichia pastoris)*	3544 ± 383	4505 ± 312	0.07
DBL1 7G8 *(Pichia pastoris)*	5903 ± 437	6446 ± 305	0.31
ID1-ID2a FCR3 (Baculovirus)	4119 ± 331	4876 ± 255	0.084
DBL1+2 FCR3 (Baculovirus)	7061 ± 777	8091 ± 502	0.26
DBL2 FCR3 (Baculovirus)	**2033 ± 299**	**2922 ± 257**	**0.04**
DBL3 7G8 (*Pichia pastoris)*	**8129 ± 754**	**10390 ± 558**	**0.02**
DBL3 FCR3 *(E. coli)*	6217 ± 661	7606 ± 494	0.10
DBL3 FCR3 (Baculovirus)	**5720 ± 607**	**7550 ± 477**	**0.025**
DBL4 7G8 *(Pichia pastoris)*	3916 ± 429	4304 ± 345	0.51
DBL4 FCR3 (Baculovirus)	5263 ± 516	5250 ± 374	0.98
DBL4 FCR3/IT4 (*Pichia pastoris)*	6721 ± 582	7944 ± 445	0.11
DBL5 3D7*(Pichia pastoris)*	11089 ± 803	11640 ± 486	0.54
DBL5 7G8 *(Pichia pastoris)*	10780 ± 789	11961 ± 466	0.18
DBL5 FCR3 (Baculovirus)	13135 ± 969	14833 ± 521	0.094
DBL6 7G8 *(Pichia pastoris)*	6847 ± 590	7434 ± 376	0.39
DBL6 FCR3 (Baculovirus)	6792 ± 684	7544 ± 507	0.39
DBL6 FCR3/IT4 *(Pichia pastoris)*	3756 ± 376	4433 ± 280	0.16
FV2 FCR3 (Baculovirus)	**9347 ± 700**	**11076 ± 462**	**0.038**
Antibody levels associated with infection (mean MFI ± SEM)			
AMA-1 3D7	**14312 ± 542**	**11856 ± 404**	**0.0005**
MSP-1 3D7	**9000 ± 800**	**6691 ± 466**	**0.0088**
MSP-2 3D7	14917 ± 1008	13179 ± 677	0.15

§ Unpaired 2-sided t-test was used for continuous variables and Fisher's exact test was used for prevalence data.

ℵ Recombinant proteins produced in *Pichia pastoris* are from J. Smith, in *E. coli* from K. Singh and in Baculovirus from A. Salanti (see materials and methods for more details).

Ω Women in which ≥35% of the Ab to FV2 are high avidity.

Table 4.3. Antibody response to malarial antigens in pregnant women with 5 or more pregnancies (mean and standard error of mean)

	PM positive (n=41)	PM negative (n=188)	p value§
Immune responses associated with protection			
Average avidity to FV2	**26.3 ± 2.3**	**31.5 ± 1**	**0.039**
Prevalence of high avidity Ab to FV2 Ω	36.6	41.5	0.60
Prevalence of FV2 seropositive women	**81**	**93**	**0.032**
% positive domains out of 6	3.2 ± 0.26	3.17 ± 0.1	0.90
% positive variants out of 15	8.6 ± 0.69	8.6 ± 0.28	0.98
Antibody levels associated with protection (mean MFI ± SEM)			
CSP	1297 ± 247	1019 ± 81	0.18
RESA	1333 ± 403	1081 ± 130	0.45
DBL1 3D7 (*Pichia pastoris*)	5684 ± 869	4262 ± 292	0.06
DBL1 7G8 (*Pichia pastoris*)	6776 ± 709	6345 ± 325	0.58
ID1-ID2a FCR3 (Baculovirus)	5119 ± 562	4747 ± 250	0.53
DBL1+2 FCR3 (Baculovirus)	8240 ± 1069	6918 ± 416	0.20
DBL2 FCR3 (Baculovirus)	2527 ± 376	2158 ± 195	0.42
DBL3 7G8 (*Pichia pastoris*)	9701 ± 1034	10528 ± 477	0.47
DBL3 FCR3 (*E. coli*)	7128 ± 820	7865 ± 430	0.46
DBL3 FCR3 (Baculovirus)	6440 ± 743	6736 ± 397	0.75
DBL4 7G8 (*Pichia pastoris*)	4622 ± 728	4480 ± 332	0.86
DBL4 FCR3 (Baculovirus)	7579 ± 697	7598 ± 397	0.98
DBL4 FCR3/IT4 *(Pichia pastoris)*	6026 ± 629	4930 ± 294	0.12
DBL5 3D7(*Pichia pastoris*)	12062 ± 1034	11468 ± 427	0.57
DBL5 7G8 (*Pichia pastoris*)	11811 ± 1015	11712 ± 419	0.92
DBL5 FCR3 (Baculovirus)	13876 ± 1268	14298 ± 487	0.73
DBL6 7G8 (*Pichia pastoris*)	6211 ± 695	6964 ± 310	0.31
DBL6 FCR3 (Baculovirus)	6384 ± 836	7695 ± 452	0.21
DBL6 FCR3/IT4 (*Pichia pastoris*)	4290 ± 562	4533 ± 257	0.69
FV2 FCR3 (Baculovirus)	10138 ± 996	10665 ± 445	0.62
Antibody levels associated with infection (mean MFI ± SEM)			
AMA-1 3D7	12931 ± 911	11572 ± 379	0.14
MSP-1 3D7	**10151 ± 1116**	**7643 ± 462**	**0.026**
MSP-2 3D7	**15961 ± 1374**	**12537 ± 646**	**0.026**

§ Unpaired 2-sided t-test was used for continuous variables and Fisher's exact test was used for prevalence data.

ﻯ Recombinant proteins produced in *Pichia pastoris* are from J. Smith, in *E. coli* from K. Singh and in Baculovirus from A. Salanti (see materials and methods for more details).

Ω Women in which ≥35% of the Ab to FV2 are high avidity.

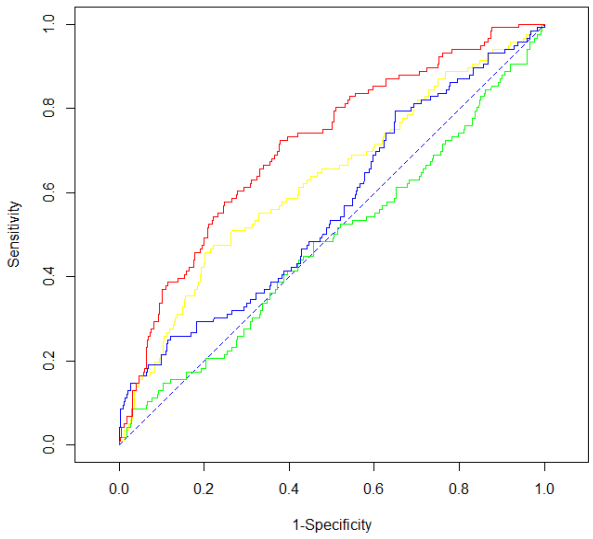
Table 4.4. Single variables as correlates of protection from placental malaria

Variable/Assay	OR	Confidence Intervals	AUC	Sensitivity	Specificity	Youden Index	p value
Average avidity to FV2	**0.97**	**0.96-0.98**	**0.62**	**0.53**	**0.66**	**0.19**	**<0.0001**
High avidity Ab to FV2 Ω	**0.60**	**0.38-0.94**	**0.56**	**0.68**	**0.44**	**0.12**	**0.026**
Seropositive for FV2	**0.27**	**0.14-0.51**	**0.57**	**0.20**	**0.94**	**0.14**	**0.0001**
% positive domains out of 6	0.89	0.78-1.01	0.55	0.68	0.47	0.15	0.066
% positive variants out of 15	0.96	0.91-1.01	0.53	0.16	0.96	0.11	0.13
Antibody levels (mean MFI ± SEM)							
AMA-1 3D7	**1.56**	**1.24-1.98**	**0.63**	**0.46**	**0.80**	**0.26**	**0.0002**
MSP-1 3D7	**1.40**	**1.14-1.73**	**0.60**	**0.28**	**0.86**	**0.15**	**0.0016**
MSP-2 3D7	**1.33**	**1.07-1.65**	**0.59**	**0.62**	**0.53**	**0.15**	**0.0095**
ID1-ID2a FCR3 (Baculovirus)	0.90	0.72-1.11	0.52	0.80	0.30	0.10	0.34
DBL1+2 FCR3 (Baculovirus)	1.00	0.81-1.23	0.48	0.09	0.97	0.06	0.98
DBL2 FCR3 (Baculovirus)	0.89	0.7-1.11	0.52	0.47	0.61	0.08	0.33
DBL3 FCR3 (Baculovirus)	0.81	0.64-1.0	0.56	0.26	0.86	0.11	0.059
DBL4 FCR3 (Baculovirus)	0.87	0.69-1.08	0.53	0.85	0.24	0.10	0.20
DBL5 FCR3 (Baculovirus)	0.85	0.69-1.05	0.53	0.21	0.95	0.16	0.13
DBL6 FCR3 (Baculovirus)	0.85	0.68-1.05	0.55	0.21	0.89	0.09	0.14
FV2 FCR3 (Baculovirus)	0.81	0.66-1.01	0.56	0.79	0.35	0.14	0.059

ﬡ Data on antibody levels were normalized by one standard deviation.
§ VAR2CSA domains expressed in other strains (3D7 and 7G8) had similar patterns.
Ω Women in which ≥35% of the Ab to FV2 are high avidity.

Figure 4.1

A



B. Results from a Multivariate Model: Gravidity, FV2 seropositive and AMA-1.

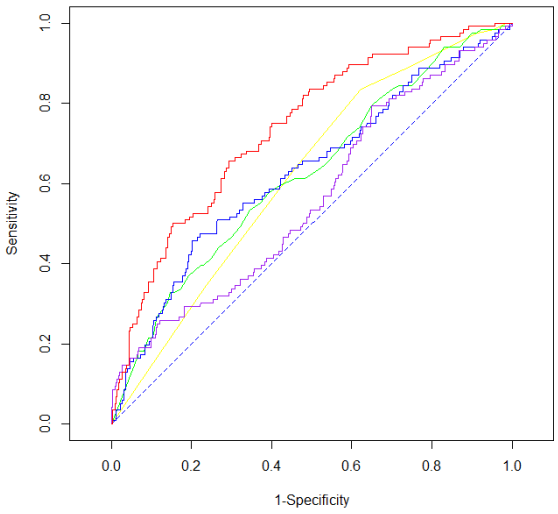
	AUC	Sensitivity	Specificity	Youden Index
Gravidity	0.62	0.84	0.38	0.21
FV2 seropositive	0.57	0.2	0.94	0.14
AMA-1 3D7	0.63	0.46	0.8	0.26
Multi-assay model	0.73	0.67	0.71	0.39

* p values of individual co-variates in the multi-assay model: gravidity p=0.00028, FV2 seropositivity p<0.0001, AMA-1 p=0.00001

Figure 4.1. A multivariate model improved prediction of PM. A. ROC curves of gravidity (yellow), FV2 seropositivity (blue) and AMA-1 (brown) considered individually in univariate models and together in the multivariate model (red). **B.** AUC, sensitivity, specificity and YI are presented in the table for univariate models and assays used in a multivariate model. AUC and YI increased when assays were considered together in the multivariate model (YI was 0.39).

Figure 4.2

A.



B. Multivariate model based on Gravidity, Average Avidity, FV2 and AMA-1.

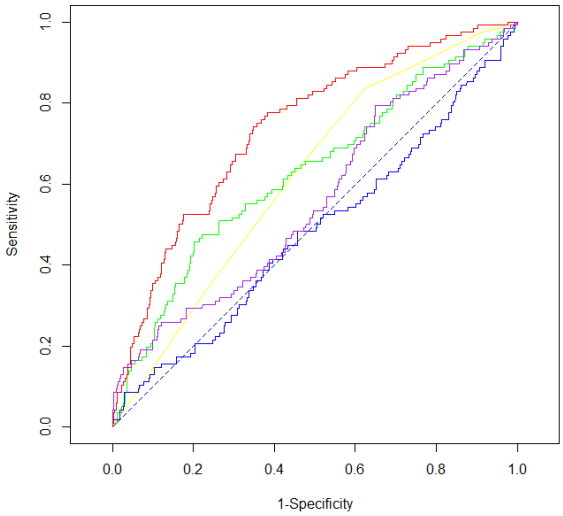
	AUC	Sensitivity	Specificity	Youden Index
Gravidity	0.62	0.84	0.38	0.21
Average avidity to FV2	0.62	0.53	0.66	0.19
AMA-1 3D7	0.63	0.46	0.8	0.26
FV2 FCR3	0.56	0.79	0.35	0.14
Multi-assay model	0.74	0.66	0.71	0.36

*p values of individual co-variates in the multi-assay model: gravidity p=0.0004, average avidity p=0.003, AMA-1 p=0.78, FV2 FCR3 p=0.0027, AMA-1 and FV2 interaction p=0.002

Figure 4.2. A combination of assays measuring pregnancy-associated and non-pregnancy associated immune responses in a multivariate model improved prediction of PM. A. ROC curves of gravidity (yellow), average avidity (green), AMA-1 (blue) and FV2 FCR3 (purple) considered individually in univariate models and together in the multivariate model (red). **B.** AUC, sensitivity, specificity and YI are presented in the table for univariate models and assays together in the multivariate model. AUC and YI increased when assays were used together in the multivariate model (YI was 0.36).

Figure 4.3

A



B. Multivariate model including Gravidity, AMA-1, DBL1+2, FV2.

	AUC	Sensitivity	Specificity	Youden Index
Gravidity	0.62	0.84	0.38	0.21
AMA-1 3D7	0.63	0.46	0.8	0.26
DBL 1+2 FCR3	0.48	0.09	0.97	0.06
FV2 FCR3	0.56	0.79	0.35	0.14
Multi-assay model	0.74	0.74	0.65	0.39

*p values of individual co-variates in the multi-assay model: gravidity p=0.001, AMA-1 p=0.14, DBL1+2 p=0.28, FV2 p=0.001, AMA-1 and DBL 1+2 interaction p=0.16, AMA-1 and FV2 interaction p=0.009, DBL1+2 and FV2 interaction p=0.05, AMA-1 and DBL1+2 and FV2 interaction p=0.05.

Figure 4.3. Combination of assays including assay measuring immune responses to DBL1+2 containing minimal CSA-binding site used in a multivariate model improve prediction of PM. A. ROC curves of gravidity (yellow), AMA-1 (green), DBL1+2 FCR3 (blue) and FV2 FCR3 (purple) considered individually in univariate models and together in the multivariate model (red). **B.** AUC, sensitivity, specificity and YI are presented in the table for univariate model and assays in the multivariate model. AUC and YI increased when assays were considered together in the multivariate model (YI was 0.39).

Figure 4.4

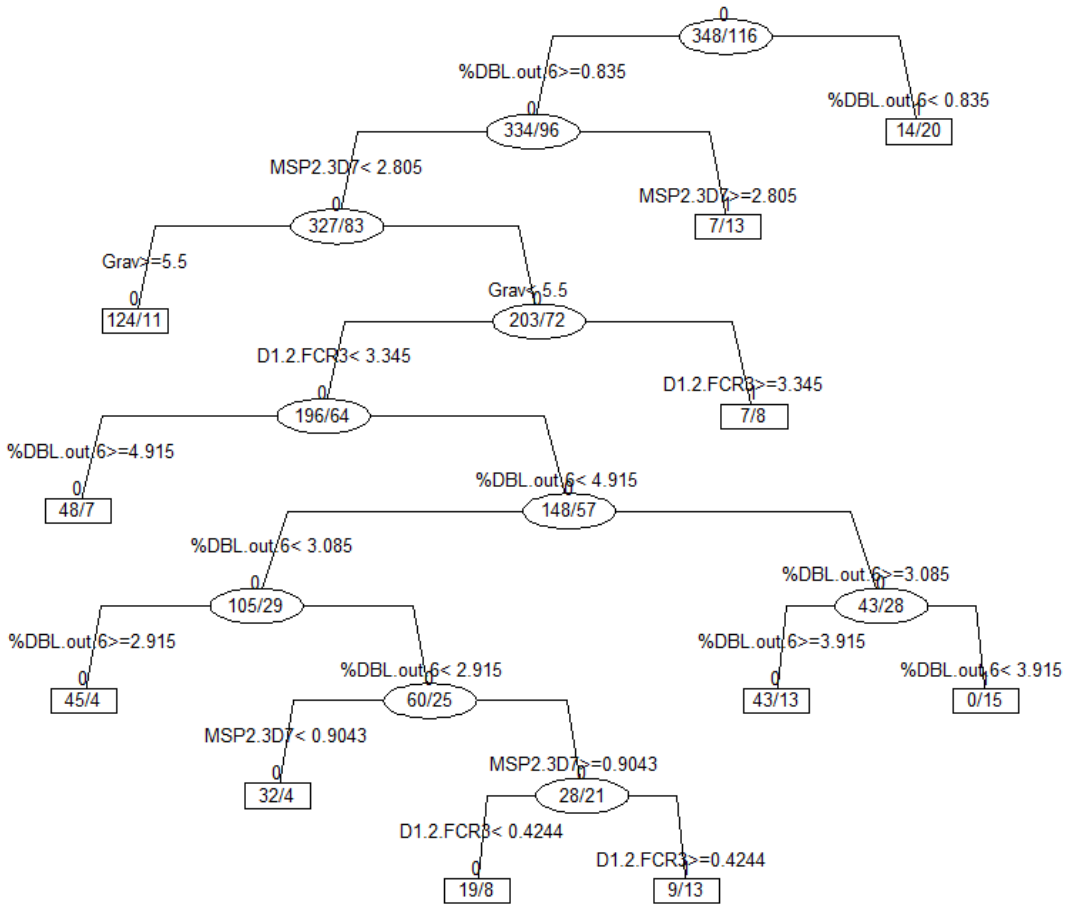


Figure 4.4. Partition of placental malaria classification using gravidity, high avidity antibodies to FV2, FV2 seropositivity, % positive domains, MSP-2 and DBL1+2 FCR3 classifiers in the CART approach. X/Y X represents controls (PM negative women) and Y represents cases (PM positive women). "1"represents predictive PM positive women and "0" represents predictive PM negative women. Each parent node is labeled with corresponding classifier or assay, i.e., MSP-2, DBL1+2 FCR3, etc. and Gini index used for classification. Sensitivity: 0.59, specificity: 0.89, YI: 0.49 (CI and OR presented in Supplementary Table S1). "Grav" is gravidity, "X DBL positive out of 6" represent % of VAR2CSA domains women recognized out of 6 possible, "D1.2" is DBL 1+2 FCR3.

Figure 4.5

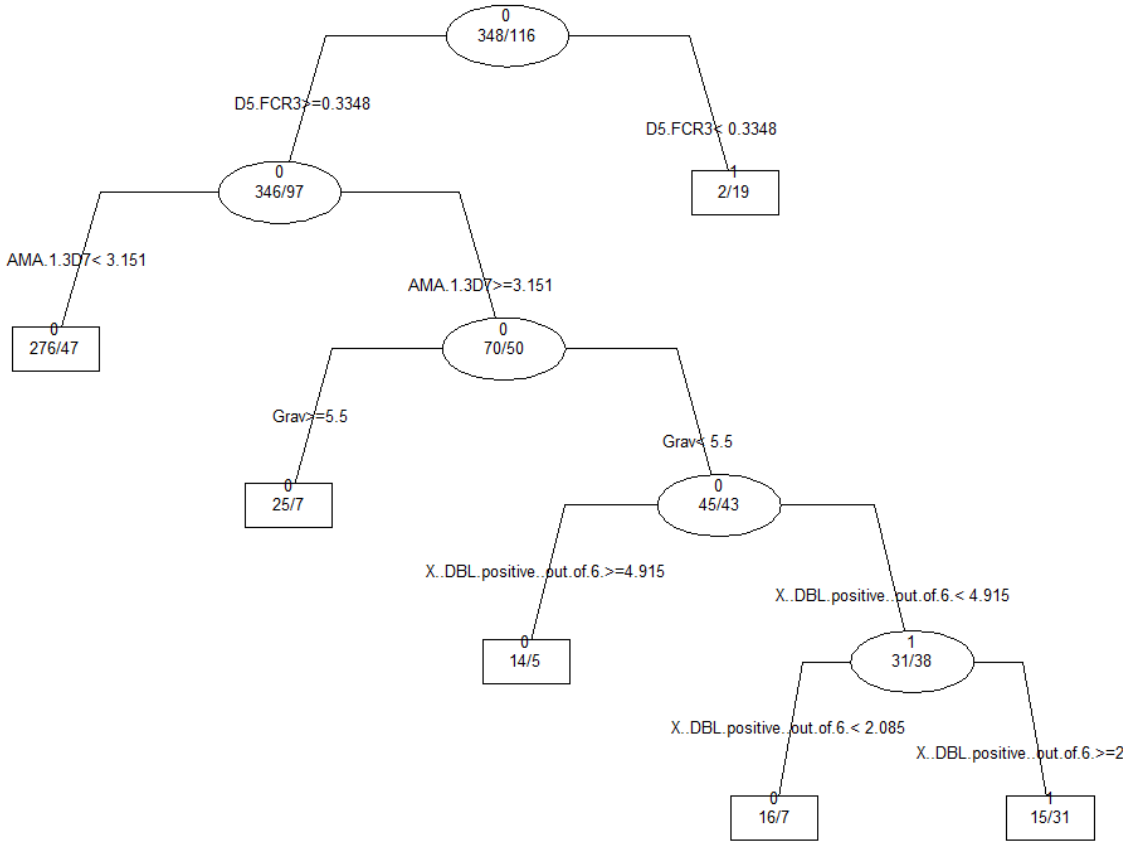


Figure 4.5. Partition of placental malaria classification using gravidity, average avidity, % positive domains, AMA-1, DBL1+2 FCR3 and DBL5 FCR3 classifiers in CART approach. X/Y X represents controls (PM negative women) and Y represents cases (PM positive women). "1"represents predictive PM positive women and "0" represents predictive PM negative women. Each parent node is labeled with corresponding classifier or assay, i.e. DBL5 FCR3, AMA-1, etc. and Gini index used for classification. Sensitivity: 0.43, specificity: 0.95, YI: 0.38 (CI and OR presented in Supplementary Table S1). "Grav" is gravidity, "X DBL positive out of 6" represent % of VAR2CSA domains women recognized out of 6 possible, "D5" is DBL5.

Figure 4.6

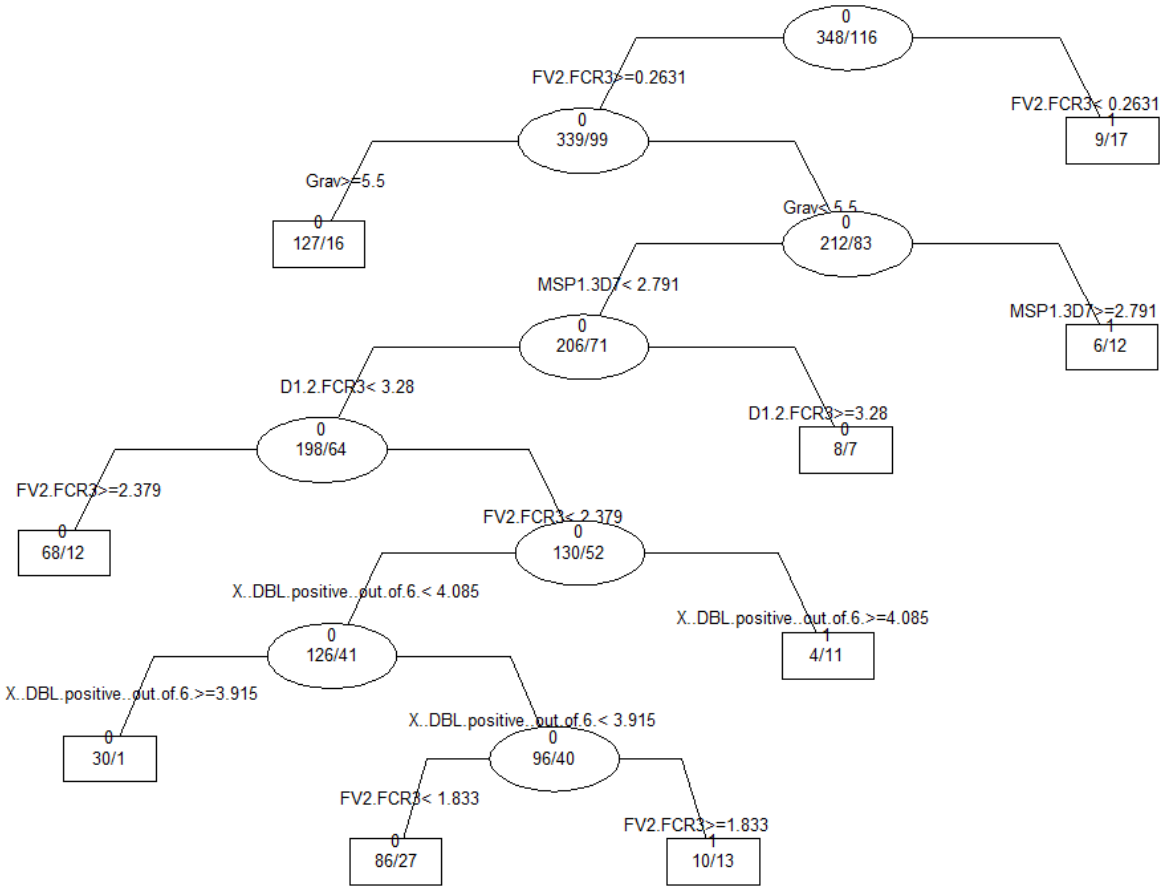


Figure 4.6. Partition of placental malaria classification using gravidity, average avidity, % positive domains, MSP-1, DBL1+2 FCR3 and FV2 FCR3 classifiers in the CART approach. X/Y X represents controls (PM negative women) and Y represents cases (PM positive women). "1"represents predictive PM positive women and "0" represents predictive PM negative women. Each parent node is labeled with corresponding classifier or assay, i.e. MSP-1, etc. and Gini index used for classification. Sensitivity: 0.46, specificity: 0.92, YI: 0.37 (CI and OR presented in Supplementary Table S1). "Grav" is gravidity, "X DBL positive out of 6" represent % of VAR2CSA domains women recognized out of 6 possible, "D1.2 FCR3" is DBL1+2 FCR3.

Figure 4.7

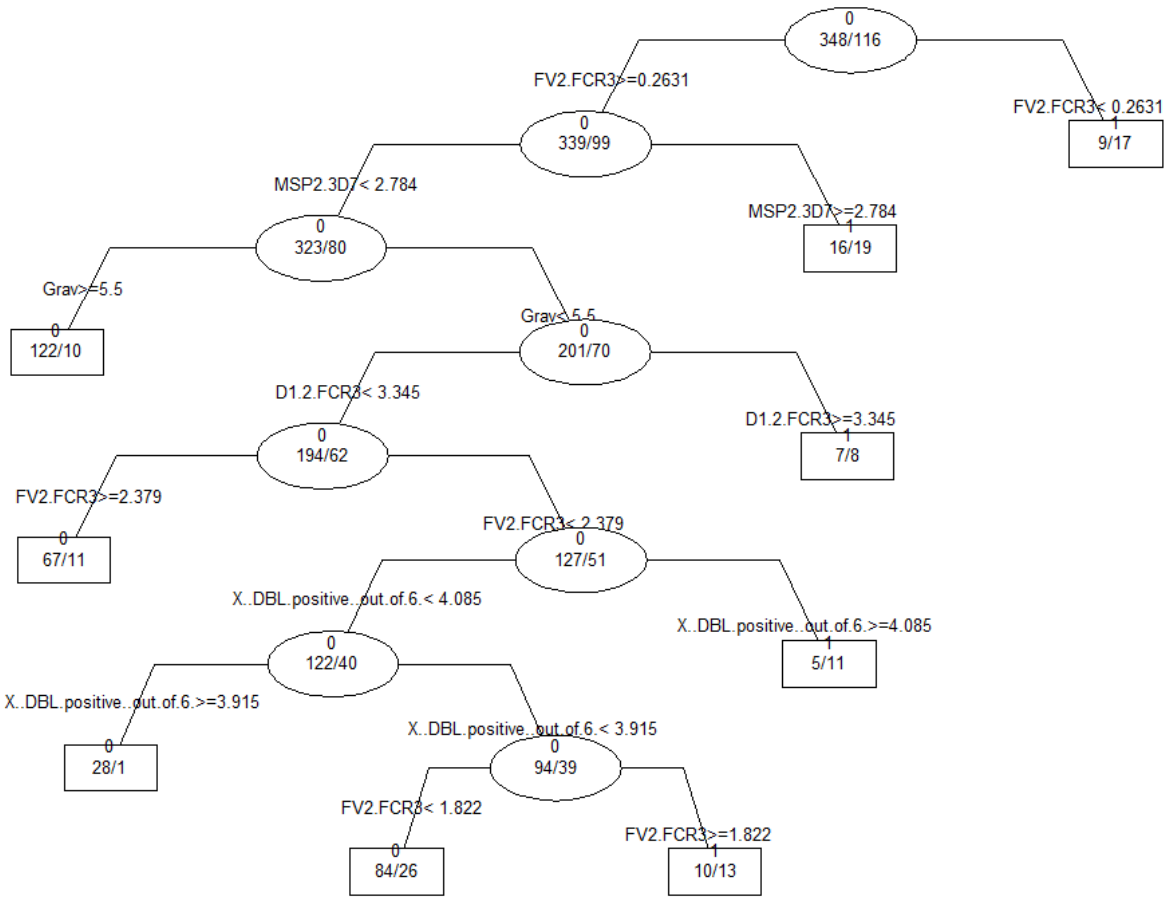


Figure 4.7. Partition of placental malaria classification using gravidity, average avidity, % positive domains, MSP-2, DBL1+2 FCR3 and FV2 FCR3 classifiers in CART approach. X/Y X represents controls (PM negative women) and Y represents cases (PM positive women). "1"represents predictive PM positive women and "0" represents predictive PM negative women. Each parent node is labeled with corresponding classifier or assay, i.e. FV2 FCR3, MSP-2, etc. and Gini index used for classification. Sensitivity: 0.59, specificity: 0.86, YI: 0.45 (CI and OR presented in Supplementary Table S1). "Grav" is gravidity, "X DBL positive out of 6" represent % of VAR2CSA domains women recognized out of 6 possible, "D1.2 FCR3" is DBL1+2 FCR3.

Supplementary

Table S4.1. Sensitivity, specificity and Youden index for CART models.

Classifiers	OR	95% CI	Sensitivity	95% CI	Specificity	95% CI	Youden Index
gravidity, high avidity Ab to FV2, FV2 seropositive, % positive domains, MSP-2 3D7, DBL1+2 FCR3 (Figure 4.4)	12.34	7.46 to 20.42	0.59	0.49 to 0.69	0.89	0.86 to 0.92	0.49
gravidity, average avidity, % positive domains, AMA-1 3D7, DBL§1+2 FCR3 and DBL5 FCR3 (Figure 4.5)	14.75	8.01 to 27.16	0.43	0.34 to 0.53	0.95	0.92 to 0.97	0.38
gravidity, average avidity, % positive domains, MSP-1 3D7, DBL1+2 FCR3 and FV2 FCR3 (Figure 4.6)	9.25	5.46 to 15.68	0.46	0.36 to 0.55	0.92	0.88 to 0.94	0.37
gravidity, average avidity, % positive domains, MSP-2 3D7, DBL1+2 FCR3 and FV2 FCR3 (Figure 4.7)	9.07	5.61 to 14.67	0.59	0.49 to 0.68	0.86	0.82 to 0.89	0.45

§ Duffy binding like domain (DBL)

CHAPTER 5

DISCUSSION

By the 1950s, control measures, based on chemotherapy with chloroquine and use of the insecticide dichlorodiphenyltrichloroethane (DDT) to kill mosquitoes, reduced the burden of malaria substantially, so policy makers optimistically believed in eradication of malaria. However, hopes for complete malaria control diminished in the middle of 20th century, with the emergence of widespread resistance of *P. falciparum* to antimalarial drugs and resistance of *Anopheles gambiae,* the primary vector in sub-Saharan Africa to insecticides. The resurgence of malaria morbidity and mortality is especially devastating in pregnant women, since VAR2CSA adhesin allows parasite to evade the immune system by sequestering in the placenta.

Currently, widely employed prevention of malaria during pregnancy using Intermittent Preventive Treatment with sulfadoxine pyrimethamine (IPT-SP) and long-lasting insecticide treated bednets (ITN) allows temporary relief from the adverse effects of malaria during pregnancy. However, spreading resistance to IPT/ITN in East Africa is alarming and clearly a vaccine for placental malaria is urgently needed. Today, VAR2CSA is a leading vaccine candidate for placental malaria, since Ab to VAR2CSA improve pregnancy outcome, the antigen is relatively conserved across different geographic regions, it is immunogenic and high Ab levels are detected in MG women, the antigen is present on the surface of the IE for prolonged periods of time (compared to merozoite antigens), Ab to VAR2CSA are strain-transdecent, and recombinant constructs of the full-length protein and its domains are available.

The PM field today is experiencing expansion in the number of VAR2CSA-based vaccines that are under consideration for clinical evaluation. Two big consortia are developing VAR2CSA subunit vaccines using recombinant protein-based platforms. Recombinant protein-based platforms were chosen, since Ab-mediated immunity is thought to be the most important mechanism for controlling PM. One group, lead by researchers at the Seattle Biomedical Research Institute and NIAID are focusing on

VAR2CSA C-terminal DBL 4 and DBL5 (REF). because Ab generated in rats bind to placental *P. falciparum* parasite isolates and inhibit the binding of IE to CSA on placental tissue [134, 137]. The recombinant proteins under consideration are based on the DNA sequence found in the 3D7 laboratory strain of *P. falciparum* and are being produced in *E. coli*. The second major consortia, lead by researchers participating in the PlaMalVac project coordinated by the University of Copenhagen, recently received an FP7 European grant for first clinical trial of a VAR2CSA vaccine based on the ID1-ID2a construct, containing the minimal sequence in VAR2CSA required for binding to CSA [84]. This group produces recombinant proteins based on the FCR3 laboratory strain of *P. falciparum* and the proteins are expressed in proprietary Drosophila Schneider-2 (S2) cells in partnership with ExpreSion Biotechnologies, Denmark. Thus, the precise region of VAR2CSA that is important for inducing protection remains unclear, since one group believes the DBL 4 and DBL5 in the c-terminus is critical and the other group is focused on the minimal binding site in the N-terminus. However, both groups hypothesize that naturally acquired Ab which prevent binding of IE to the placental CSA are associated with reduction of clinical disease. Therefore, the tertiary structure of recombinant VAR2CSA subunit protein antigen is likely to be critical for vaccine induction of Ab-based protection.

Some of the challenges with this approach include conformationally sensitive protein production, difficulty producing recombinant proteins on a large scale, and absence of information on which domains of VAR2CSA are important in protection. However, the biggest challenge is the absence of correlates of protection from PM, which is necessary for preclinical evaluation and evaluation of efficacy of vaccine candidates. Currently employed ortholog and experimental animal model systems and *in vitro* assays that assess function of the generated immune response are important as they provide information that antigen was correctly chosen and presented to the immune system. Yet, the predictive value of these tools cannot be determined until the results are correlated with clinical efficacy of vaccine candidates. Since the use of IPT-SP and ITN as the standard of care throughout Africa and other regions significantly reduced the prevalence of women with PM, field-studies testing potential vaccines are not possible. Finally, it is impossible to use parsitological endpoints (presence of IE in the placenta) and clinical

endpoints (pregnancy outcome) to evaluate efficacy of vaccine, as these data are not available until the end of pregnancy, correlates of protection from PM take an even greater importance when developing a vaccine for pregnant women.

The study reported herein was aimed at identification of surrogate correlates of protection from PM disease. The unique study design included only pregnant women with at least 3 pregnancies, which allowed us to delineate fine immunologic differences between PM-positive and PM- negative women. Moreover, with the widespread use of IPT/ITN and reduced exposure to malaria during pregnancy, it is difficult to study naturally acquired immune responses to PM. The use of archival plasma samples collected prior to implementation of IPT/ITN policy allowed us to circumvent challenges associated with confounding effects of IPT/ITN on immunity to PM. Finally, availability of a full panel of VAR2CSA domains and variants enriched our toolbox and allowed us to study immunity to malaria in pregnancy in a comprehensive manner.

In the first part of the study, we evaluated naturally acquired immunity to recombinant ID1-ID2a, CSA- minimal binding site. Unfortunately, our data showed that Ab responses to ID1-ID2a in naturally infected women were not pregnancy-associated. Ab levels to ID1-ID2a were the same in Cameroonian males and pregnant women. Thus, the native conformation of VAR2CSA on the IE does not expose the minimal CSA-binding site epitopes and the Ab measured to the recombinant ID1-ID2a in the Cameroonian women were due to cross-reactive Ab that were induced either by some other malarial antigen or other pathogen. Our data does not reject the possibility that a ID1-ID2a -based vaccine could be efficacious. We hypothesized that naturally women do not acquire protection from PM by producing high levels of Ab to the CSA-binding site.

In order to define correlates of protection from PM, we undertook an innovative multi-assay approach. Naturally acquired immunity to malaria is thought to be conferred primarily by the gradual acquisition of Ab to variant antigens expressed on the surface of IE [150]. Thus, we have measured Ab levels to various domains of VAR2CSA expressed in different parasite strains. In addition, we hypothesized that since women live in malaria endemic area and are exposed to malarial antigen throughout life; it is likely that Ab responses to other malarial antigens might play role in protection. Thus, we included

CSP, RESA, AMA-1, MSP-1 and MSP-2 in the panel of antigens, as all of these antigens have been associated with protection in non-pregnant children and adults. Finally, since all Ab are not the same, we wanted to determine the functional properties of Ab VAR2CSA Ab by measuring Ab to the surface of IE, Ab avidity to FV2, FcγR-mediated opsonic phagocytosis, and inhibition of binding to the CSA. Unfortunately, due to major technical difficulties, some of the functional assays were not completed as originally proposed. However, the reported data herein provides substantial insight into correlates of protection from PM.

One of the key findings is that women living in the city of Yaounde who are in their 3rd and 4th pregnancy are still acquiring immunity to PM, since they have lower Ab levels to FV2, recognize fewer domains, and have lower avidity to FV2, and thus, are more likely to have PM. This is not surprising, since compared to the rural villages, where entomological inoculation rate is much higher; women in the city are bitten by infectious mosquito about once a month. Moreover, our unpublished data suggests that with increasing gravidity prevalence of MG who have PM decreases. In this study, prevalence of PM drops from 34% in women with 3 pregnancies to 20% in women with 5 pregnancies to 6% in women with 6 or more pregnancies. On the other hand, women with 5 or more pregnancies have reached maximal breadth of response, i.e., number of DBL domains and strains they recognize and maximal Ab levels to various VAR2CSA domains. No significant differences were detected in Ab levels to any of the domains in these women. Women with 5 or more pregnancies were more likely to have malaria only if they had low avidity Ab to FV2. This indicates that after 5 pregnancies women are only fine-tuning their immune responses. Since some of the women with 5 or more pregnancies had placental malaria, these women are missing the functional aspects of protective Ab, i.e. opsono-phagocytic and inhibitory properties and high levels of Ab to DBL and FV2 do not correlate with protection.

Another important finding was that no significant differences were observed for domains DBL4, DBL5 and DBL6 between women with and without PM in all gravidities. DBL 5 is the most immunogenic domain and pregnant women quickly obtain high levels of Ab to epitopes of DBL5. DBL4 is the least immunogenic and most

conserved, with 88% sequence homology recorded for protein isolates from different parts of the world. DBL6 is the least conserved domain with only 61% sequence homology between strains from Asia, Africa and S. America. This is especially important in light of the fact that DBL4 and DBL5 are major candidates for a subunit vaccine. Thus, it is important that researchers establish functional properties of Ab to DBL4 and DBL5 for efficacious PM vaccine, such as their capacity to inhibit binding to placenta and/or mediate opsonic phagocytosis.

Interestingly, but not surprisingly, Ab to AMA-1, MSP-1 and MSP-2 antigens were correlated with infection. Despite the fact that adults already have high levels of Ab to these antigens, exposure to malaria during pregnancy appears to boost the immune response to non-pregnancy associated malarial antigens. However, this finding is in contrast to the recent findings by Mayor et al. (2013) [151] who reported that high levels of IgG to AMA-1, DBL3 and DBL6 were associated with improved pregnancy outcome in Mozambican women at delivery. The data are controversial, since the women in this study were on IPT/ITN prophylaxis, and therefore the acquisition of natural immunity to VAR2CSA and AMA-1 was likely affected by the limited antigen exposure. Also, half of the women in the reported study were PG and secundigravidae, which further diluted detection of the fine specificity of the protective response.

Next, we used multivariate and CART methods to identify combination of assays that correlated with protection from PM. For both approaches we used not only the best scoring assays in univariate models, but also biologically relevant combinations. Of importance, both methods of assay selection lead to models with the same high predictive power (Youden index 0.39). Some of the parameters that were important for prediction of PM included gravidity, AMA-1, MSP-2, high avidity IgG to FV2, DBL1+2, FV2, number of domains recognized and FV2 seropositivity. AMA-1 and MSP-2 discriminated between women who were infected with malaria and were positively associated with *infection*. The rest of the parameters were associated with *protection* from PM. As predicted, high levels of IgG to FV2, high avidity Ab to FV2 and recognition of more domains predicted protection from PM. Interestingly, DBL1+2 was important for prediction. We hypothesize that although Ab to ID1-ID2a are not pregnancy-specific in

women exposed to VAR2CSA naturally, it is likely that since DBL1+2 encompasses ID1-ID2a, the larger recombinant protein allows for better folding and preservation of native tertiary structure. Yet, Ab levels to DBL1+2 alone in univariate model do not correlate with protection.

Finally, multiple assays were screened in CART using RPART algorithm. This recursive partitioning method was unique in that it allowed us to add even more assays to the model and as a result improved prediction power. We were able to reach Youden index of 0.45 and 0.49 in two independent CART models. Again, assay selection was based on univariate model results, but biological relevance proved to be equally valuable. We believe that addition of functional assays would greatly improve predictive score of the multivariate and CART models. Thus, to our knowledge this is first comprehensive attempt to identify correlates of protection from PM using multi-assay approaches. Using multi-assay approaches to assess protection from PM could provide a way to evaluate vaccine efficacy in field trials.

Knowing what correlates with protective response from PM is important. However, another imperative factor is how long protective immunity lasts. Long term protection after vaccination is a key for ensuring women remain protected throughout pregnancy and possibly during their next pregnancy. Protection after vaccination differs depending on a vaccine and is found to be anywhere from 1 year (meningococcal) to ten years (tetanus, measles, mumps, Hep B). However, longevity of Ab to VAR2CSA is poorly understood. There is only one report on longevity of immunity to DBL5, which states that Ab half-life in infected women is about 142 years [125]. However, the study was conducted in the area of malaria low transmission and does not provide information on Ab longevity to other DBL domains and FV2.

Thus, as a part of the current study, Ab half-life of FV2, its domains and non-pregnancy associated malarial antigens, as a reference, were estimated based on women who received IPT/ITN and live in malaria high transmission area. We established that Ab to non-pregnancy malarial antigens such as MSP-1 and MSP-2 are short-lived in Cameroonian women, requiring exposure to antigens, in order to sustain serum IgG. These results are in line with findings in Thai pregnant women by Fowkes et al. [125].

Ab to VAR2CSA and its domains in Cameroonian MG persists post-partum, as was suggested before [95, 125], and half-life estimates for MG population ranges from 4 years to lifetime. We hypothesize that long half-life for VAR2CSA could is due to the relatively conserved nature of VAR2CSA. Thus, MG have long-lived B cells that were generated during the previous pregnancies. Ab to FV2 are short-lived in Cameroonian PG (<1 year), possibly indicating that plasmablasts did not mature into functional long-lived plasma cells or memory B cells during a single pregnancy. The discrepancies in VAR2CSA Ab decay rate between PG and MG is due to the fact that PG experience primary immune response, while MG experience a secondary immune response. Knowing how long antibodies to VAR2CSA persist is important for establishing continuous protective immunity against malaria in pregnant women.

As with any effort to develop a new health intervention, managing high level of expectations with unknown timeline to delivery, while ensuring advocacy for the development poses a challenge. Any current PM vaccine candidates could lead to a highly effective vaccine. The more likely scenario is that the first generation vaccine for PM is going to yield partial efficacy. It is possible that cycles of iterative product development will be needed before a vaccine is sufficiently efficacious to be widely deployed. Meanwhile, even partially efficacious vaccine would have a tremendous public health impact.

REFERENCES

1. Laveran, A., *De la nature parasitaire de l'impaludisme.* Bulletins et Memoires de la Societe Medicale des Hopitaux de Paris, 1882. **18**: p. 168-176.
2. Clark, H.C., *The diagnostic value of the placental blood film in aestivo-autumnal malaria.* J. of Experimental Medicine, 1915. **22**: p. 427-444.
3. Wickramasuriya, G.A., *Some observations on malaria occuring in association with pregnancy. .* J. of Obstetrics and Gynaecology of the British Empire, 1935. **42**: p. 816-834.
4. Dellicour, S., et al., *Quantifying the Number of Pregnancies at Risk of Malaria in 2007: A Demographic Study.* Plos Medicine, 2010. **7**(1).
5. Shulman, C.E., et al., *Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae.* Trop Med Int Health, 2001. **6**(10): p. 770-8.
6. McGregor, I.A., *Epidemiology, malaria and pregnancy.* Am J Trop Med Hyg, 1984. **33**(4): p. 517-25.
7. ter Kuile, F.O., et al., *The burden of co-infection with human immunodeficiency virus type 1 and malaria in pregnant women in sub-saharan Africa.* Am J Trop Med Hyg, 2004. **71**(2 Suppl): p. 41-54.
8. Singh, N., R.K. Mehra, and N. Srivastava, *Malaria during pregnancy and infancy, in an area of intense malaria transmission in central India.* Ann Trop Med Parasitol, 2001. **95**(1): p. 19-29.
9. Desai, M., et al., *Epidemiology and burden of malaria in pregnancy.* Lancet Infect Dis, 2007. **7**(2): p. 93-104.
10. Menendez, C., et al., *Malaria prevention with IPTp during pregnancy reduces neonatal mortality.* PLoS One, 2010. **5**(2): p. e9438.
11. Steketee, R.W., et al., *The effect of malaria and malaria prevention in pregnancy on offspring birthweight, prematurity, and intrauterine growth retardation in rural Malawi.* Am J Trop Med Hyg, 1996. **55**(1 Suppl): p. 33-41.
12. Oestergaard, M.Z., et al., *Neonatal mortality levels for 193 countries in 2009 with trends since 1990: a systematic analysis of progress, projections, and priorities.* Plos Medicine, 2011. **8**(8): p. e1001080.
13. Eisele, T.P., et al., *Malaria prevention in pregnancy, birthweight, and neonatal mortality: a meta-analysis of 32 national cross-sectional datasets in Africa.* Lancet Infect Dis, 2012. **12**(12): p. 942-9.
14. Blacklock, D.B.a.G., R. M., *Malaria infection as it occurs in late pregnancy; its relationship to labor and early infancy.* Annals of Tropical Medicine and Parasitology, 1925b. **19**: p. 327-363.
15. Chiarleoni, G., *Malaria e atti funzionali della generazione nella donna.* Annali Universali di Medicina e Chirurgia, 1886. **275**(249-305).
16. Goth, E., *Ueber den Einfluss der Malariainfection auf Schwagerschaft, Geburt und Wochenbett.* Zeitschrift fur Geburtshilfe und Gynakologies, 1881. **6**: p. 17-34.
17. Wickramasuriya, G.A., *Malaria and Ankylostomiasis in the Pregnant Woman*. 1st ed1936, London: Oxford University Press.
18. Edmonds, F.H., *Malaria and pregnancy.* British Medical Journal, 1899. **i**: p. 1023.
19. Laffont, A., *Le plaudisme dans ses rapports avec la grossesse, l'accouchmenet et les suites de couches.* L'Obstetrique, 1911. **4**: p. 777-819.

20. Menon, R., *Pregnancy and malaria.* Medical Journal of Malaysia, 1972. **27**: p. 115-119.
21. Granja, A.C., *Malaria-related maternal mortality in urban Mozambique.* Annals of Tropical Medicine and Parasitology, 1998. **92**: p. 257-263.
22. Duffy P., M.F., *Malaria in pregnancy: deadly parasite, susceptible host.*2001, New York: Taylor & Francis.
23. Brabin, B.J., *The risks and severity of malaria in pregnant women.*, in *Applied field research report No 1.*1991, UNDP/Wrold Bank/WHO Special Programme for Research and Training in Tropical Diseases: Geneva. p. 1-52.
24. White, N.J., et al., *Severe hypoglycemia and hyperinsulinemia in falciparum malaria.* N Engl J Med, 1983. **309**(2): p. 61-6.
25. Organization, W.H., *Severe and Comlicated Malaria.* , 2000, Transactions of the Royal Society of Tropical Medicine and Hygiene. p. s1-s90.
26. Verhoeff, F.H., et al., *An analysis of the determinants of anaemia in pregnant women in rural Malawi--a basis for action.* Ann Trop Med Parasitol, 1999. **93**(2): p. 119-33.
27. Fleming, A.F., *Tropical obstetrics and gynaecology. 1. Anaemia in pregnancy in tropical Africa.* Trans R Soc Trop Med Hyg, 1989. **83**(4): p. 441-8.
28. van den Broek, N.R., S.A. White, and J.P. Neilson, *The relationship between asymptomatic human immunodeficiency virus infection and the prevalence and severity of anemia in pregnant Malawian women.* Am J Trop Med Hyg, 1998. **59**(6): p. 1004-7.
29. van den Broek, N.R. and E.A. Letsky, *Etiology of anemia in pregnancy in south Malawi.* Am J Clin Nutr, 2000. **72**(1 Suppl): p. 247S-256S.
30. Brabin, B.J., Z. Premji, and F. Verhoeff, *An analysis of anemia and child mortality.* J Nutr, 2001. **131**(2S-2): p. 636S-645S; discussion 646S-648S.
31. Jarcho, S., *Quinine's Predecessor: Francesco Torti and the Early History of Cinchona*. 1st ed1989, Baltimore: The Johns Hopkins University Press.
32. Le Dantec, A., *Precis de Pathologie Exotique*. 5th ed1929, Paris: Octave Doin.
33. Steketee, R.W., et al., *The burden of malaria in pregnancy in malaria-endemic areas.* Am J Trop Med Hyg, 2001. **64**(1-2 Suppl): p. 28-35.
34. Martignetti, A., *L'influenza della malaria materna sullo sviluppo somatico del bambino nel primo anno di vita.* Rivista di Clinica Pediatrica, 1932. **30**: p. 11-24.
35. Bruce-Chwatt, L.J., *Malaria in African infants and children in southern Nigeria.* Annals of Tropical Medicine and Parasitology, 1952. **46**: p. 173-200.
36. Archibald, H.M., *Influence of maternal malaria on newborn infants.* British Medical Jounral, 19958. **ii**: p. 1512-1514.
37. Cannon, D.S.H., *Malaria and prematurity in the western region of Nigeria.* British Medical Jounral, 1958. **ii**: p. 877-878.
38. Jelliffe, E.F.P., *Low birth-weight and malarial infection of the placenta.* Bulletin of the World Health Organization, 1968. **38**: p. 69-78.
39. Bignami, A., *Sulla questione della malaria congenita.* Al Policlinico Cupplemento, 1898. **4**: p. 763-767.
40. Bastianelli, R., *Sulla trasmissione dei parassiti della malaria dalla madre al feto.* Bulletino della Societa Lancisiana degli Ospedali di Roma, 1892. **12**: p. 48-49.
41. Caccini, Cited in La Medecine Moderne, 1896. **6**: p. 154.
42. Sereni, S., *Sulla tranmissibilita dei parassiti della malaria dalla madre al feto.* Bulletino della Reale Accademia Medica di Roma, 1902. **29**: p. 55-88.
43. Economos, M., *La permeabilite du placenta aux hematozoaires, le paludisme congenital.* Bulletin de la Societe d'Obstetrique de Paris, 1907. **10**: p. 70-73.
44. Covell, G., *Congenital malaria.* Tropical Disease Bulletin, 1950. **47**: p. 1147-1167.

45. Cardamatis, J.P., *La malaria infantile.* Archives de Medecine des Enfants, 1910. **13**: p. 641-665.
46. Butler, G.G., *Malaria in parturient women*, in *In report on the Medical and Sanitary Department for the Year 1929-1930, Gold Coast Colony*1930, Accra: Goverment Printing Office. p. 102-104.
47. Blacklock, D.B.a.G., R. M, *Malaria parasites in the placental blood.* Annals of Tropical Medicine and Parasitology, 1925a. **19**: p. 37-45.
48. Ferreira, M.U., M. da Silva Nunes, and G. Wunderlich, *Antigenic diversity and immune evasion by malaria parasites.* Clin Diagn Lab Immunol, 2004. **11**(6): p. 987-95.
49. Rogerson, S.J., et al., *Malaria in pregnancy: pathogenesis and immunity.* Lancet Infect Dis, 2007. **7**(2): p. 105-17.
50. Ismail, M.R., et al., *Placental pathology in malaria: a histological, immunohistochemical, and quantitative study.* Hum Pathol, 2000. **31**(1): p. 85-93.
51. Brabin, B.J., *An analysis of malaria in pregnancy in Africa.* Bull World Health Organ, 1983. **61**(6): p. 1005-16.
52. Wislocki, G.B., *Observations on the placenta from a case of malaria.* Bulletin of the Johns Hopkins Hospital, 1930. **47**: p. 157-163.
53. Garnham, P.C.C., *The placenta in malaria with special reference to reticulo-endothelial immunity.* Transactions of the Royal Society of Tropical Medicine and Hygene, 1938. **32**: p. 13-48.
54. Lucchi, N.W., et al., *Natural hemozoin stimulates syncytiotrophoblast to secrete chemokines and recruit peripheral blood mononuclear cells.* Placenta, 2011. **32**(8): p. 579-85.
55. Parroche, P., et al., *Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9.* Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1919-24.
56. Fried, M., et al., *Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes.* J Immunol, 1998. **160**(5): p. 2523-30.
57. Muehlenbachs, A., et al., *Hypertension and maternal-fetal conflict during placental malaria.* Plos Medicine, 2006. **3**(11): p. e446.
58. Lucchi, N.W., et al., *Plasmodium falciparum-infected red blood cells selected for binding to cultured syncytiotrophoblast bind to chondroitin sulfate A and induce tyrosine phosphorylation in the syncytiotrophoblast.* Placenta, 2006. **27**(4-5): p. 384-94.
59. Lucchi, N.W., D.S. Peterson, and J.M. Moore, *Immunologic activation of human syncytiotrophoblast by Plasmodium falciparum.* Malar J, 2008. **7**: p. 42.
60. Chaisavaneeyakorn, S., et al., *Immunohistological characterization of macrophage migration inhibitory factor expression in Plasmodium falciparum-infected placentas.* Infect Immun, 2005. **73**(6): p. 3287-93.
61. Guyatt, H.L. and R.W. Snow, *Malaria in pregnancy as an indirect cause of infant mortality in sub-Saharan Africa.* Trans R Soc Trop Med Hyg, 2001. **95**(6): p. 569-76.
62. Conroy, A.L., et al., *Performance characteristics of combinations of host biomarkers to identify women with occult placental malaria: a case-control study from Malawi.* PLoS One, 2011. **6**(12): p. e28540.
63. Umbers, A.J., et al., *Placental malaria-associated inflammation disturbs the insulin-like growth factor axis of fetal growth regulation.* J Infect Dis, 2011. **203**(4): p. 561-9.
64. Silver, K.L., et al., *Circulating soluble endoglin levels in pregnant women in Cameroon and Malawi--associations with placental malaria and fetal growth restriction.* PLoS One, 2011. **6**(9): p. e24985.

65. Suguitan, A.L., Jr., et al., *Malaria-associated cytokine changes in the placenta of women with pre-term deliveries in Yaounde, Cameroon.* Am J Trop Med Hyg, 2003. **69**(6): p. 574-81.
66. Kalanda, B.F., et al., *Adverse birth outcomes in a malarious area.* Epidemiol Infect, 2006. **134**(3): p. 659-66.
67. Brabin, B., *An analysis of malaria parasite rates in infants- 40 years after Macdonald.* Tropical DIseases Bulletin, 1990. **87**: p. 1-21.
68. Metenou, S., et al., *Fetal immune responses to Plasmodium falciparum antigens in a malaria-endemic region of Cameroon.* J Immunol, 2007. **178**(5): p. 2770-7.
69. Mackroth, M.S., et al., *Human cord blood CD4+CD25hi regulatory T cells suppress prenatally acquired T cell responses to Plasmodium falciparum antigens.* J Immunol, 2011. **186**(5): p. 2780-91.
70. Gardner, M.J., et al., *Genome sequence of the human malaria parasite Plasmodium falciparum.* Nature, 2002. **419**(6906): p. 498-511.
71. Newbold, C., et al., *Cytoadherence, pathogenesis and the infected red cell surface in Plasmodium falciparum.* Int J Parasitol, 1999. **29**(6): p. 927-37.
72. Pasternak, N.D. and R. Dzikowski, *PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum.* Int J Biochem Cell Biol, 2009. **41**(7): p. 1463-6.
73. Kyes, S., et al., *Plasmodium falciparum var gene expression is developmentally controlled at the level of RNA polymerase II-mediated transcription initiation.* Mol Microbiol, 2007. **63**(4): p. 1237-47.
74. Scherf, A., et al., *Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum.* EMBO J, 1998. **17**(18): p. 5418-26.
75. Chen, Q., et al., *Developmental selection of var gene expression in Plasmodium falciparum.* Nature, 1998. **394**(6691): p. 392-5.
76. Mok, B.W., et al., *Default Pathway of var2csa switching and translational repression in Plasmodium falciparum.* PLoS One, 2008. **3**(4): p. e1982.
77. Bockhorst, J., et al., *Structural polymorphism and diversifying selection on the pregnancy malaria vaccine candidate VAR2CSA.* Mol Biochem Parasitol, 2007. **155**(2): p. 103-12.
78. Andersen, P., et al., *Structural insight into epitopes in the pregnancy-associated malaria protein VAR2CSA.* PLoS Pathog, 2008. **4**(2): p. e42.
79. Salanti, A., et al., *Several domains from VAR2CSA can induce Plasmodium falciparum adhesion-blocking antibodies.* Malar J, 2010. **9**: p. 11.
80. Srivastava, A., et al., *Full-length extracellular region of the var2CSA variant of PfEMP1 is required for specific, high-affinity binding to CSA.* Proc Natl Acad Sci U S A, 2010. **107**(11): p. 4884-9.
81. Smith, J.D., et al., *Classification of adhesive domains in the Plasmodium falciparum erythrocyte membrane protein 1 family.* Mol Biochem Parasitol, 2000. **110**(2): p. 293-310.
82. Srivastava, A., et al., *Var2CSA minimal CSA binding region is located within the N-terminal region.* PLoS One, 2011. **6**(5): p. e20270.
83. Dahlback, M., et al., *The chondroitin sulfate A-binding site of the VAR2CSA protein involves multiple N-terminal domains.* J Biol Chem, 2011. **286**(18): p. 15908-17.
84. Clausen, T.M., et al., *Structural and functional insight into how the Plasmodium falciparum VAR2CSA protein mediates binding to chondroitin sulfate A in placental malaria.* J Biol Chem, 2012. **287**(28): p. 23332-45.

85. Buffet, P.A., et al., *Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection.* Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12743-8.
86. Reeder, J.C., et al., *The adhesion of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1.* Proc Natl Acad Sci U S A, 1999. **96**(9): p. 5198-202.
87. Degen, R., N. Weiss, and H.P. Beck, *Plasmodium falciparum: cloned and expressed CIDR domains of PfEMP1 bind to chondroitin sulfate A.* Exp Parasitol, 2000. **95**(2): p. 113-21.
88. Khattab, A., et al., *Variants of Plasmodium falciparum erythrocyte membrane protein 1 expressed by different placental parasites are closely related and adhere to chondroitin sulfate A.* J Infect Dis, 2001. **183**(7): p. 1165-9.
89. Khunrae, P., et al., *Full-length recombinant Plasmodium falciparum VAR2CSA binds specifically to CSPG and induces potent parasite adhesion-blocking antibodies.* J Mol Biol, 2010. **397**(3): p. 826-34.
90. Viebig, N.K., et al., *A single member of the Plasmodium falciparum var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A.* EMBO Rep, 2005. **6**(8): p. 775-81.
91. Viebig, N.K., et al., *Disruption of var2csa gene impairs placental malaria associated adhesion phenotype.* PLoS One, 2007. **2**(9): p. e910.
92. Duffy, M.F., et al., *VAR2CSA is the principal ligand for chondroitin sulfate A in two allogeneic isolates of Plasmodium falciparum.* Mol Biochem Parasitol, 2006. **148**(2): p. 117-24.
93. Fox, H., *Pathology of the placenta*, ed. L. V.1997, London: Saunders Company Ltd
94. Smith, J.D. and K.W. Deitsch, *Pregnancy-associated malaria and the prospects for syndrome-specific antimalaria vaccines.* J Exp Med, 2004. **200**(9): p. 1093-7.
95. Aitken, E.H., et al., *Antibodies to chondroitin sulfate A-binding infected erythrocytes: dynamics and protection during pregnancy in women receiving intermittent preventive treatment.* J Infect Dis, 2010. **201**(9): p. 1316-25.
96. Hviid, L., *Naturally acquired immunity to Plasmodium falciparum malaria in Africa.* Acta Trop, 2005. **95**(3): p. 270-5.
97. Beeson, J.G., et al., *Antigenic differences and conservation among placental Plasmodium falciparum-infected erythrocytes and acquisition of variant-specific and cross-reactive antibodies.* J Infect Dis, 2006. **193**(5): p. 721-30.
98. Ricke, C.H., et al., *Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on Plasmodium falciparum-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A.* J Immunol, 2000. **165**(6): p. 3309-16.
99. Avril, M., et al., *Immunization with VAR2CSA-DBL5 recombinant protein elicits broadly cross-reactive antibodies to placental Plasmodium falciparum-infected erythrocytes.* Infect Immun, 2010. **78**(5): p. 2248-56.
100. Fernandez, P., et al., *Strain-transcendent immune response to recombinant Var2CSA DBL5-epsilon domain block P. falciparum adhesion to placenta-derived BeWo cells under flow conditions.* PLoS One, 2010. **5**(9): p. e12558.
101. Avril, M., et al., *Induction of strain-transcendent antibodies to placental-type isolates with VAR2CSA DBL3 or DBL5 recombinant proteins.* Malar J, 2011. **10**(1): p. 36.

102. Avril, M., et al., *Antibodies to a full-length VAR2CSA immunogen are broadly strain-transcendent but do not cross-inhibit different placental-type parasite isolates.* PLoS One, 2011. **6**(2): p. e16622.
103. Hommel, M., et al., *Evaluation of the antigenic diversity of placenta-binding Plasmodium falciparum variants and the antibody repertoire among pregnant women.* Infect Immun, 2010. **78**(5): p. 1963-78.
104. Barfod, L., et al., *Chondroitin sulfate A-adhering Plasmodium falciparum-infected erythrocytes express functionally important antibody epitopes shared by multiple variants.* J Immunol, 2010. **185**(12): p. 7553-61.
105. Feng, G., et al., *Antibodies to variant surface antigens of Plasmodium falciparum-infected erythrocytes are associated with protection from treatment failure and the development of anemia in pregnancy.* J Infect Dis, 2009. **200**(2): p. 299-306.
106. O'Neil-Dunne, I., et al., *Gravidity-dependent production of antibodies that inhibit binding of Plasmodium falciparum-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy.* Infect Immun, 2001. **69**(12): p. 7487-92.
107. Staalsoe, T., et al., *Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of Plasmodium falciparum-infected erythrocytes that protect against placental parasitemia.* J Infect Dis, 2001. **184**(5): p. 618-26.
108. Duffy, P.E. and M. Fried, *Antibodies that inhibit Plasmodium falciparum adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns.* Infect Immun, 2003. **71**(11): p. 6620-3.
109. Tutterrow, Y.L., et al., *High levels of antibodies to multiple domains and strains of VAR2CSA correlate with the absence of placental malaria in Cameroonian women living in an area of high Plasmodium falciparum transmission.* Infect Immun, 2012. **80**(4): p. 1479-90.
110. Tutterrow, Y.L., et al., *High avidity antibodies to full-length VAR2CSA correlate with absence of placental malaria.* PLoS One, 2012. **7**(6): p. e40049.
111. Fried, M., et al., *Maternal antibodies block malaria.* Nature, 1998. **395**(6705): p. 851-2.
112. Joergensen, L.M., et al., *The kinetics of antibody binding to Plasmodium falciparum VAR2CSA PfEMP1 antigen and modelling of PfEMP1 antigen packing on the membrane knobs.* Malar J, 2010. **9**: p. 100.
113. Ghumra, A., et al., *Immunisation with recombinant PfEMP1 domains elicits functional rosette-inhibiting and phagocytosis-inducing antibodies to Plasmodium falciparum.* PLoS One, 2011. **6**(1): p. e16414.
114. Abrams, E.T., et al., *Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated beta chemokine expression.* J Immunol, 2003. **170**(5): p. 2759-64.
115. Keen, J., et al., *HIV impairs opsonic phagocytic clearance of pregnancy-associated malaria parasites.* Plos Medicine, 2007. **4**(5): p. e181.
116. WHO *A strategic framework for malaria prevention and control during pregnancy in the African region.* . Geneva: World Health Organization AFR/MAL, 2004.
117. Leke, R.G. and D.W. Taylor, *The use of intermittent preventive treatment with sulfadoxine-pyrimethamine for preventing malaria in pregnant women.* Clin Infect Dis, 2011. **53**(3): p. 231-3.
118. van Eijk, A.M., et al., *Coverage of malaria protection in pregnant women in sub-Saharan Africa: a synthesis and analysis of national survey data.* Lancet Infect Dis, 2011. **11**(3): p. 190-207.

119. White, N.J., *Intermittent presumptive treatment for malaria.* Plos Medicine, 2005. **2**(1): p. e3.
120. WHO, *Updated WHO Policy Recommendation (October 2012). Intermittent Preventive Treatment of malaria in pregnancy using Sulfadoxine-*

Pyrimethamine (IPTp-SP). 2012.
121. Greenwood, A.M., et al., *Can malaria chemoprophylaxis be restricted to first pregnancies?* Trans R Soc Trop Med Hyg, 1994. **88**(6): p. 681-2.
122. Staalsoe, T., et al., *Intermittent preventive sulfadoxine-pyrimethamine treatment of primigravidae reduces levels of plasma immunoglobulin G, which protects against pregnancy-associated Plasmodium falciparum malaria.* Infect Immun, 2004. **72**(9): p. 5027-30.
123. Serra-Casas, E., et al., *The effect of intermittent preventive treatment during pregnancy on malarial antibodies depends on HIV status and is not associated with poor delivery outcomes.* J Infect Dis, 2010. **201**(1): p. 123-31.
124. Diouf, I., et al., *[Effect of intermittent presumptive treatment with sulfadoxine-pyrimethamine on the acquisition of anti-VAR2CSA antibodies in pregnant women living in a hypoendemic area in Senegal].* Bull Soc Pathol Exot, 2011. **104**(4): p. 277-83.
125. Fowkes, F.J., et al., *New insights into acquisition, boosting, and longevity of immunity to malaria in pregnant women.* J Infect Dis, 2012. **206**(10): p. 1612-21.
126. Salanti, A., et al., *Evidence for the involvement of VAR2CSA in pregnancy-associated malaria.* J Exp Med, 2004. **200**(9): p. 1197-203.
127. Salanti, A., et al., *Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria.* Mol Microbiol, 2003. **49**(1): p. 179-91.
128. Leke, R.F., et al., *Longitudinal studies of Plasmodium falciparum malaria in pregnant women living in a rural Cameroonian village with high perennial transmission.* Am J Trop Med Hyg, 2010. **83**(5): p. 996-1004.
129. Louis, J.P., et al., *[Malaria in Yaounde (Cameroon). Cost and antivectorial control at the family level].* Bull Soc Pathol Exot, 1992. **85**(1): p. 26-30.
130. Quakyi, I.A., et al., *The epidemiology of Plasmodium falciparum malaria in two Cameroonian villages: Simbok and Etoa.* Am J Trop Med Hyg, 2000. **63**(5-6): p. 222-30.
131. Tako, E.A., et al., *Risk factors for placental malaria and its effect on pregnancy outcome in Yaounde, Cameroon.* Am J Trop Med Hyg, 2005. **72**(3): p. 236-42.
132. Fouda, G.G., et al., *Multiplex assay for simultaneous measurement of antibodies to multiple Plasmodium falciparum antigens.* Clin Vaccine Immunol, 2006. **13**(12): p. 1307-13.
133. Barfod, L., et al., *Evasion of immunity to Plasmodium falciparum malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1.* Proc Natl Acad Sci U S A, 2011. **108**(30): p. 12485-90.
134. Fried, M., et al., *Multilaboratory approach to preclinical evaluation of vaccine immunogens for placental malaria.* Infect Immun, 2013. **81**(2): p. 487-95.
135. Fusco, P.C., et al., *Protective meningococcal capsular polysaccharide epitopes and the role of O acetylation.* Clin Vaccine Immunol, 2007. **14**(5): p. 577-84.
136. Bordbar, B., et al., *Identification of Id1-DBL2X of VAR2CSA as a key domain inducing highly inhibitory and cross-reactive antibodies.* Vaccine, 2012. **30**(7): p. 1343-8.
137. Saveria, T., et al., *Antibodies to Escherichia coli-expressed C-terminal domains of Plasmodium falciparum variant surface antigen 2-chondroitin sulfate A (VAR2CSA)*

inhibit binding of CSA-adherent parasites to placental tissue. Infect Immun, 2013. **81**(4): p. 1031-9.
138. Chen, R.T., et al., *Measles antibody: reevaluation of protective titers.* J Infect Dis, 1990. **162**(5): p. 1036-42.
139. McComb, J.A., *The Prophylactic Dose of Homologous Tetanus Antitoxin.* N Engl J Med, 1964. **270**: p. 175-8.
140. Burnham, K.P., and Anderson, D.R. and *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approac*. 2nd ed2002: Springer-Verlag.
141. Breiman L, F.J., Olshen RA, Stone CJ., *Classification and Regression Trees.*1984, Monterey, CA: Wadsworth.
142. Therneau TM, A.B. *RPART: recursive partitioning function of R-package ("The R project for Statistical Computing")*.
143. Hobson, D., et al., *The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses.* J Hyg (Lond), 1972. **70**(4): p. 767-77.
144. Plotkin, S.A., *Vaccination against the major infectious diseases.* C R Acad Sci III, 1999. **322**(11): p. 943-51.
145. Maslanka, S.E., et al., *Age-dependent Neisseria meningitidis serogroup C class-specific antibody concentrations and bactericidal titers in sera from young children from Montana immunized with a licensed polysaccharide vaccine.* Infect Immun, 1998. **66**(6): p. 2453-9.
146. Denoel, P.A., et al., *Quality of the Haemophilus influenzae type b (Hib) antibody response induced by diphtheria-tetanus-acellular pertussis/Hib combination vaccines.* Clin Vaccine Immunol, 2007. **14**(10): p. 1362-9.
147. Romero-Steiner, S., et al., *Reduction in functional antibody activity against Streptococcus pneumoniae in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity.* Clin Infect Dis, 1999. **29**(2): p. 281-8.
148. Horstmann, D.M., *Maxwell Finland lecture: viral vaccines and their ways.* Rev Infect Dis, 1979. **1**(3): p. 502-16.
149. Plotkin, S.A., J.D. Farquhar, and P.L. Ogra, *Immunologic properties of RA27-3 rubella virus vaccine. A comparison with strains presently licensed in the United States.* JAMA, 1973. **225**(6): p. 585-90.
150. Marsh, K. and R.J. Howard, *Antigens induced on erythrocytes by P. falciparum: expression of diverse and conserved determinants.* Science, 1986. **231**(4734): p. 150-3.
151. Mayor, A., et al., *Improved Pregnancy Outcomes in Women Exposed to Malaria With High Antibody Levels Against Plasmodium falciparum.* J Infect Dis, 2013. **207**(11): p. 1664-74.