

HIGH AVIDITY ANTIBODIES TO VAR2CSA: ARE THEY ASSOCIATED WITH  
PROTECTION FROM PLACENTAL MALARIA IN A LOW TRANSMISSION SETTING?

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE  
UNIVERSITY OF HAWAII AT MĀNOA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

TROPICAL MEDICINE

DECEMBER 2015

By Vanda Koko

Thesis Committee:

Dr. Diane Wallace Taylor, Chairperson

Dr. Saguna Verma

Dr. John Chen

## **Acknowledgments**

My greatest appreciation goes to my PI and mentor, Dr. Diane Taylor for having me in her laboratory and letting me participate in this project. Many techniques I used were new to me, but her encouragement, support and patience helped me to get this project in shape.

I would also like to thank my committee members Dr. Saguna Verma and Dr. John Chen. Thank you for your important insights, encouragement and your precious time that you shared for me.

Without your support I could not have achieved what I have with my project. I also greatly appreciate the statistical input of the Biostatistics core, Dr. John chen, Rui Fang and Dr. Andrew Wey. Their analysis helped me a great deal to understand the results of the study.

I would like to thank the faculty, staff and students of the Department of Tropical Medicine. I would especially like to send my appreciations to my lab members, most especially Naveen for his constant encouragement and training and his contribution in this study.

This work received support through Dr. Diane Taylor from NIAID, NIH R21AI105286 and Fogarty International Center 5D43TW009074.

## Abstract

Placental malaria is characterized by the sequestration of *Plasmodium falciparum* infected erythrocytes in the intervillous space of the placenta. Sequestration is mediated by the binding of a parasite membrane protein called VAR2CSA to Chondroitin Sulfate A on the surface of placental syncytiotrophoblasts. The presence of  $\geq 35\%$  high avidity antibodies (Ab) to VAR2CSA early in pregnancy correlates with the absence of placental malaria at delivery in a high transmission setting. Little is known about high avidity Ab to VAR2CSA in low transmission areas, and if one or multiple pregnancies are required to produce such Ab. Therefore, blood samples obtained at delivery between 1995 and 2001 from women living in Yaoundé, Cameroon (low transmission setting) were used in this study. The plasma samples collected prior to use of chemoprophylaxis (IPT) and bed nets, so natural immunity determined presence/absence of placental malaria. These samples were screened for IgG Ab to Full Length VAR2CSA (FV2) using a bead-based immunoassay and 494 samples were identified as being Ab-positive. The percentage of strong binding (high avidity) IgG equals the amount of Ab that remain bound to FV2 after 30 minutes of incubation with 3M NH<sub>4</sub>SCN. Results showed that 178 of the 494 women had  $\geq 35\%$  high avidity Ab at delivery. Ab avidity increased significantly between the 1<sup>st</sup> and 2<sup>nd</sup> pregnancies however, it remained constant thereafter. After adjusting for age and malaria status, a significant increase in avidity with gravidity was found (P=0.0008). Avidity increased gradually, with only a 0.9% increase per pregnancy. A higher mean avidity was found in PM-negative compared to PM-positive secundigravidae (P=0.022), but no difference was seen between PM+ and PM- in the other gravidity groups. A modest correlation between high avidity Ab to FV2 and Ab levels. Affinity maturation peaked at G2 and tended to be almost stable with subsequent pregnancies. The Ab specificity of the DBL domains suggest the N-

terminal may have a role in the production of high avidity Ab. Ab avidity may serve as an important correlate of protection, hence can be considered an important marker for the assessment of the efficacy of VAR2CSA vaccine candidate.

Keywords: *Malaria, pregnant women, antibody avidity*

## Table of Contents

Abstract.....	3
List of abbreviations .....	8
<b>Introduction</b> .....	9
Disease pathogenesis .....	10
Placental pathology.....	11
<i>P. falciparum</i> infection in pregnancy is the major cause of placental pathology.....	11
Parasites that cause malaria in pregnancy are unique.....	12
Malaria-associated placental pathology.....	12
Primigravid and multigravid women.....	13
Antibody response gravidity.....	14
VAR2CSA: Structure and function .....	15
Natural acquisition of antibodies to VAR2CSA.....	16
Immunity to placental malaria: antibody response with gravidity .....	17
Antibodies to different domains .....	18
Affinity maturation .....	20
High avidity antibodies to VAR2CSA and other malarial antigens.....	22
Persistence of immunity .....	24
Summary.....	27
Specific Aims .....	28
Hypothesis .....	29
<b>Materials and methods</b> .....	29
Study design and plasma samples .....	29
Diagnosis of placental malaria and anemia .....	30
Screening of plasma samples for ab to recombinant VAR2CSA using a multi-analyte assay.....	31
Antibody avidity to FV2.....	32
Measuring Ab avidity to FV2 and its domains.....	33
Antibody avidity to the 6 DBL domains .....	34
Statistical analysis.....	34
<b>Results</b> .....	35

Prevalence of placental malaria .....	35
Prevalence of Ab to FV2 .....	35
Comparison of ab levels and avidity to FV2 .....	36
Pregnancy outcomes of women with $\geq 35\%$ high avidity Ab to FV2 .....	38
High avidity antibodies to FV2 Increase minimally with gravidity .....	39
.....	
Affinity maturation is acquired by secundigravidae.....	41
Prevalence of women with $\geq 35$ high avidity in Yaoundé .....	42
Proportion of high avidity ab to individual DBL domains.....	42
Immunogenic differences between FCR3-FV2 and its domains.....	44
Is a specific DBL domain of the FV2 molecule necessary to achieve protection from PM?.....	45
Percent high avidity across different gravidity groups .....	47
Multivariable prediction model to identify women who are protected from placental malaria. ....	49
<b>Discussion</b> .....	51
Conclusion.....	56
<b>References</b> .....	59

### List of Figures

Fig. 1: Structure of VAR2CSA gene and protein.....	15
Fig. 2: Normal course of antibody response to antigen.....	16
Fig. 3: Change in antibody level and affinity during a primary and secondary challenge with antigen.....	20
Figure 4: High avidity antibodies to var2csa were associated with absence of placental malaria in Ngali II (high transmission setting).....	27
Figure 5: The prevalence of placental malaria in Yaoundé.....	35
Figure 6a: Relationship between Ab Avidity and Ab Level for FV2.....	37
Fig. 6b Relationship between Ab avidity and levels for FV2 in PM+ and PM- women.....	38
Figure 7: Relationship between Ab avidity and gravidity in PM+ and PM- women for FV2.....	40
Figure 8: Avidity stratified by gravidity between PM+ and PM- groups for FV2.....	41

Figure 9: Average avidity of 3 strains of FV2 and their corresponding DBL domains.....43

Figure 10: Percent high avidity IgG against FV2 and its domains.....45

Figure 11: Percent high avidity Ab to FV2 is higher across different gravidity groups in a high transmission setting compared to a low transmission setting.....48

**List of tables**

Table1: Summary of literature on Ab avidity and protection from malaria.....24

Table 2: Summary of the VAR2CSA DBL domains used.....33

Table 3: Characteristics of study population for FV2 Ab positive samples stratified by PM status.....36

Table 4: Characteristics of study population for FV2 Ab positive women stratified by low and high avidity.....39

Table 5a: Significant difference proportion of high avidity antibodies to FV2 by gravidity.....40

Table 5b: Prevalence of PM in women in Yaoundé.....42

Table 6: Percentage of women who had Ab to FV2 and its domains (n=103).....44

Table 7: Avidity variation for various DBL domains between PM+ and PM- women.....46

Table 8: Multivariable logistic regression model predicted presence of PM.....50

## List of Abbreviations

Ab Abbreviation	Antibodies Meaning
AMA1	Apical Membrane Protein 1
Ang	angiopoietin
CSA	Chondroitin Sulfate A
CSPG	Chondroitin Sulfate Proteoglycans
DBL	Duffy-Binding-Like
FV2	Full length VAR2CSA
IE	Infected erythrocytes
IP10	Interferon-inducible protein 10
IPT	Intermittent preventive treatment
ITNs	Insecticide-treated bed nets
IVS	Intervillous space
MCP1	Monocyte chemoattractant protein 1
MG	Multigravidae
MSP 2	Merozoite Surface Protein 2
MSP1	Merozoite Surface Protein 1
<i>pfemp1</i>	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
Pf-IEs	<i>Plasmodium falciparum</i> - infected erythrocytes
PG	Primigravidae
PM	Placental malaria
PM	Placental malaria
VAR2CSA	Variant Surface Antigen 2 CSA

## Introduction

Malaria is an entirely preventable and treatable mosquito-borne illness. It is transmitted between humans by female Anopheles mosquitoes that are mostly found in the tropics. In 2014, 97 countries (40% of the world's population) and territories had ongoing malaria transmission (WHO 2014). An estimated 3.3 billion people are at risk of malaria, of which 1.2 billion are at high risk, i.e., live in areas that have more than one malaria case occurs per 1000 population. Today, there are five known species of *Plasmodium* that can cause malaria in humans: *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi*. Amongst these, *P. falciparum* is the most virulent species and is common in sub-Saharan Africa.

Malaria in pregnant women is unique, because pregnant women are more susceptible than the general population. An estimated 85.3 million pregnant women are at risk of becoming infected with *P. falciparum* annually (Dellicour S1 *et al.*, 2010). In areas where *P. falciparum* is infrequent or sporadic, pregnant women have limited malarial immunity and suffer from spontaneous abortions and premature deliveries, hence increasing maternal and fetal mortality. On the other hand, in areas where malarial transmission is stable and high (i.e., areas where pregnant women have considerable immunity), malaria leads to increased risk of maternal anemia and low birth weight babies (Babrin *et al.*, 2001).

The increased burden of *P. falciparum* infection in pregnant women can lead to detrimental effects, that have been attributed to both elevated parasite densities and the placental sequestration of *P. falciparum*-infected erythrocytes (IE) (Hviid and Salanti, 2007; Rogerson, 2010; Umbers *et al.*, 2011). *P. falciparum* infection during pregnancy is typically associated with

a selective accumulation of mature forms of blood-stage parasites in the placenta with placental parasitemia many fold higher than that observed in the peripheral blood (Walter *et al.*, 1982, Beeson *et al.*, 2002). The accumulation of large numbers of IEs in the placenta results in changes to placental histology which include inflammation, deposition of pigment in fibrin or inflammatory cells, syncytial knotting, and thickening of the trophoblastic basement membrane (Ismail *et al.*, 2000; Rogerson *et al.*, 2003). Thus, *P. falciparum* should be prevented during pregnancy.

### **Disease pathogenesis**

When pregnant women become infected, mosquitoes inject sporozoites that grow in the liver, enter erythrocytes, and divide asexually. Illness is caused by asexual stage parasites, i.e., trophozoites, schizonts, and rings. Trophozoites express knobs and a unique protein called VAR2CSA that causes sequestration of IE in the placental intervillous space (IVS). This leads to anemia, parasite growth and may induce inflammation. Placental malaria (PM) is caused by the sequestration of parasites in the placenta, that is mediated by VAR2CSA interacting with chondroitin sulfate A (CSA) expressed on syncytiotrophoblasts (Viebig *et al.*, 2007; Duffy and Fried, 1997). Infected erythrocytes expressing VAR2CSA only reach immunogenic levels during pregnancy and antibodies (Ab) to VAR2CSA are produced in a sex-specific and parity-dependent manner (Salanti *et al.*, 2004). It should be noted that in males and children become infected with strains of *P. falciparum* that have the ability to express VAR2CA , but the IE are rapidly eliminated in the spleen without inducing an Ab response (Beeson *et al.*, 2007; Gnedehou *et al.*, 2014).

## **Placental pathology**

Increased susceptibility of *P. falciparum* has been largely attributed to the lack of immunity to VAR2CSA-expressing IE that sequester in the placenta (Duffy, 2007; Hviid and Salanti, 2007; Rogerson, 2010; Umbers *et al.* 2011). In areas where *falciparum* malaria is highly endemic, adults usually have only asymptomatic disease. Many adults will become infected, but their parasite densities usually remain at a low level. However, during pregnancy, there is a dramatic increase in both malaria prevalence and parasite densities. This often leads to anemia which is one of the major hazards to pregnant women in these areas. One of the most remarkable features is the intense accumulation of *P. falciparum* in the placenta. In these heavy placental infections, all the asexual developmental stages of *P. falciparum*, including trophozoites and schizonts, are seen. The very high parasitemias seen in placental blood are not reflected in peripheral blood smears made from the mother.

### ***P. falciparum* infection in pregnancy is the major cause of placental pathology**

Trophozoite and schizont stages, which are absent from peripheral blood, sequester in the placenta (Beeson *et al.*, 2002). Other findings related with PM include increased numbers of maternal phagocytic cells, especially monocytes in the IVS, and deposition of haemozoin or malaria pigment in phagocytic leucocytes, and within fibrin deposits in the IVS. Accurate detection of placental parasitization, and of these other findings, requires examination of histological sections of fixed placental tissue. A careful classification of placental histological changes based on histological appearances of a normal placenta and of a malaria-infected placenta shows parasites and monocyte macrophage infiltration as reviewed by Rogerson *et al.*, 2007. Babrin and collaborators found that chronic infection has been most closely associated

with decreased birthweight due to fetal growth restriction, whereas acute infection (especially with high parasitemia) has been more closely associated with preterm delivery.

### **Parasites that cause malaria in pregnancy are unique**

Erythrocytes infected with *P. falciparum* that are obtained from the placenta differ in important ways from infected erythrocytes isolated from non-pregnant individuals. Placental IE adhere to glycosaminoglycan receptors not exploited by other IE, and do not bind to receptors commonly used for sequestration by non-placental IE (Fried and Duffy, 1996, Beeson *et al.*, 1999). Placental sequestration occurs throughout the IVS, by contrast with sequestration in other tissues, where infected erythrocytes are usually found in close apposition to the vascular wall.

### **Malaria-associated placental pathology**

The sequestration of infected erythrocytes in the placenta stimulates maternal mononuclear cells to secrete  $\beta$ -chemokines that are chemotactic for monocytes and macrophages, including macrophage inflammatory protein-1 $\alpha$  and  $\beta$  (MIP1 $\alpha$  and  $\beta$ ), interferon-inducible protein 10 (IP10) and monocyte chemoattractant protein 1 (MCP1) (Chaisavaneeyakorn *et al.*, 2002). Macrophage migration inhibitory factor (MIF), a cytokine that aids in retention and activation of macrophages, is also found in raised concentrations in women with placental malaria (Chaisavaneeyakorn *et al.*, 2002, Chaisavaneeyakorn *et al.*, 2005). Thus, induction of these chemokines provides a physiological explanation as to why monocytes and macrophages, and no other types of leucocytes, predominate in the IVS in response to parasite sequestration. Macrophages in the IVS can be activated (Suguitan *et al.*, 2003) and have the ability to process and present antigens to T cells (Chaisavaneeyakorn *et al.*, 2005).

Significant changes in nutrient transport, vasculogenesis, angiogenesis, and the presence or absence of inflammatory cytokines leads to LBW in addition to other complications. The angiogenic factors angiopoietin (Ang)-1, Ang-2 and Tie-2 (which serves as a receptor for both Ang-1 and Ang-2) have been found to have important roles not only in vasculogenesis and angiogenesis, but also important in mediating inflammation in infectious diseases (Page *et al.*, 2013, Conroy *et al.*, 2013). In 2010, Silver and colleagues, based on data from their mice model as well as malaria exposed pregnant women, hypothesized that dysregulation of angiopoietins is associated with PM and LBW outcomes, and suggest that ANG-1 and ANG-2 levels may be clinically informative biomarkers to identify *P. falciparum* infected mothers at risk of LBW deliveries. Ataide *et al.*, (2015) conducted a cross-sectional study in Brazil in an area of low transmission where both *P. vivax* and *P. falciparum* circulate, and found no differences in pregnancy outcome between PM- and PM+ women. However, they found that, lower levels of Angiopoietin-1 in women who experienced malaria during pregnancy were associated with specific modifications occurring in the placenta. Griffin *et al.*, (2012) found that primigravidae who became infected with malaria early pregnancy had 3.6 times the risk of subsequently delivering LBW babies due to intrauterine growth restriction compared to multigravidae with no early parasitemia during pregnancy. Thus parasitemia early in pregnancy affects uterine and umbilical artery blood flow, possibly due to alterations in placentation and angiogenesis, respectively.

### **Primigravid and multigravid women**

Women who become pregnant for the first time i.e., primigravidae (PG) have a primary infection which makes it interesting to study because a primary response is followed by a secondary response in multigravidae (MG). In PG, Ab against placental-type parasites

demonstrate exposure to the parasite (Staalsoe *et al.*, 2001) and do not confer protection against adverse outcomes at delivery, such as PM, maternal anemia, or low birth weight (Fievet *et al.*, 2006). Specific immune responses are usually initiated during the first pregnancy and result in decrease infection in subsequent pregnancies, thus PG, often have the most severe consequences of pregnancy-associated malaria because they lack specific protective immunity to VAR2CSA.

### **Antibody response gravidity**

Women with PM have higher Ab levels to VAR2CSA than uninfected women, and infected PG have lower levels of IgG to VAR2CSA than do infected MG (Mayor *et al.*, 2011). Nevertheless, Ab levels can fluctuate during pregnancy (Aitken *et al.*, 2010; reviewed by Fowkes *et al.*, 2012) i.e., PG have a primary Ab response upon first infection, but they may have secondary responses late in pregnancy. O’Neil-Dunne and colleagues (2001) reported a difference in antibody production between PG and MG that correlated with the prevalence of malaria in these groups, suggesting that Ab are produced during pregnancy in response to PM infection. In addition, the early onset of efficient antibody response in MG and the delayed production to Ab in PG appears to account for the gravidity-dependent differential susceptibilities of pregnant women to placental malaria (O’Neil-Dunne *et al.*, 2001).

In areas of stable *P. falciparum* transmission, susceptibility to PM rapidly declines with increasing parity consistent with acquisition of PM-specific protective immunity over several pregnancies. *P. falciparum* infection rates during the course of pregnancy have been shown to be similar between PG and MG women. However, PM at delivery is more frequently observed in PG than in MG (Dechavanne *et al.*, 2015). Recently, it was found that the ability of Ab in plasma to inhibit IE from binding to Chondroitin Sulfate Proteoglycans (CSPG) was higher for MG than PG at enrollment early in pregnancy and at delivery. Whereas, early in pregnancy Ab levels to

VAR2CSA were similar in PG and men who lack Ab to VAR2CSA (Dechavanne *et al.*, 2015). This clearly shows that multigravidae have higher Ab levels compared to women who are only pregnant for the first time.

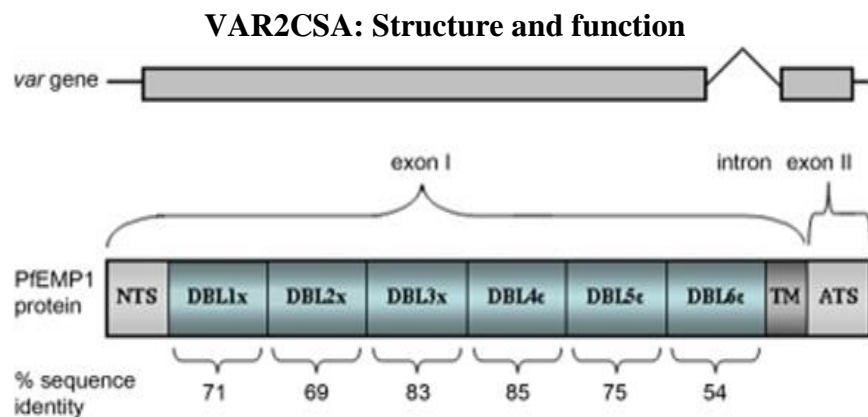


Fig. 1: Diagram shows VARCSA gene and protein (Salanti *et al.*, 2003)

VAR2CSA is an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite *P. falciparum*. The molecule is a 350-kDa transmembrane protein composed of six Duffy-Binding-Like (DBL) domains (Salanti *et al.*, 2003).

Placental isolates are functionally distinct from those in the peripheral blood, because they do not bind to CD36, but instead bind to CSA (Fried and Duffy, 1996, Salanti *et al.*, 2004) and hyaluronic acid (Beeson *et al.*, 2000). Today this multi-domain protein is considered the leading vaccine candidate against PM. VAR2CSA is encoded for by the *Pfemp1* gene family and is expressed on IE. There are over 60 different genes in the PfEMP1 family, but only one gene is expressed at a time by each IE. The *var2csa* gene encodes a *P. falciparum* adhesion receptor which binds to CSA. This *var* gene is more conserved than other PfEMP1/*var* genes and is found

in all *P. falciparum* isolates. Specific differences exist between isolates with respect to structural motifs for adhesion to CSA, and this creates polymorphisms in VAR2CSA encoded by *var2csa*-type genes probably has an influence on parasite virulence (Beeson *et al.*, 2007).

### Natural acquisition of antibodies to VAR2CSA

How the immune system responds to exposure to malaria in pregnant women is not completely understood. Normally, immunity to many pathogens is long-lived after a single infection (Fig. 2). However, this is not true with immunity to *P. falciparum*. Pathogens vary in their antigenic complexity. For

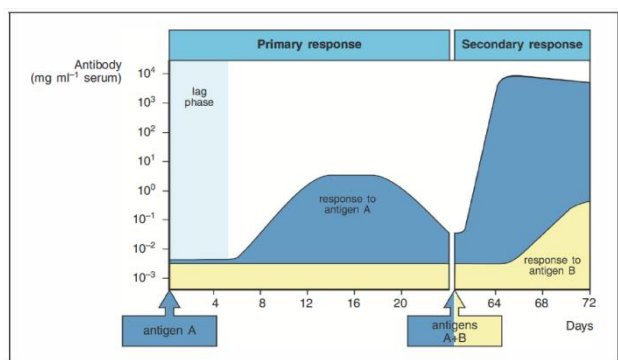


Fig. 2: Normal course of antibody response to antigen. Janeway, 8<sup>th</sup> edition

example, some pathogens, such as measles virus, present only a few relatively invariant targets (antigens or epitopes) to the immune system; whereas other pathogens, such as malaria, display considerable antigenic diversity. How the immune response copes with the presence of multiple antigens, and whether a trade-off exists between the breadth and efficacy of the antibody-mediated immune response, is difficult to evaluate. With regards to malaria, complete protection is probably never obtained, even in adults who have lived in high-transmission areas all their lives. In pregnant women, Ab to VAR2CSA are highly variable over time, and maintenance of high levels of these Ab involves highly dynamic responses resulting from intermittent exposure to infection (Fowkes *et al.*, 2012). It is thought that there is limited, infrequent, or no exposure to VAR2CSA prior to the first pregnancy, in contrast to most regular malarial antigens, which are generally encountered throughout life. Some studies have shown an association between Ab levels to FV2 and its domains, i.e., DBL1, 2, 3 etc. increasing with age in areas where women

are exposed multiple times to mosquito bites during multiple pregnancies. But it is unclear if Ab levels (quantity) positively correlate with protection or if protection is achieved by high avidity Ab (quality) as a result of affinity maturation. High avidity Ab to full-length VAR2CSA (FV2) and multiple domains have been shown to be associated with reduced risk of PM in a high transmission setting (Tutterrow *et al.*, 2012), but it is unclear if Ab quantity or quality is important in a region where women are exposed to just a few mosquito bites per pregnancy.

Antibodies to VAR2CSA improve pregnancy outcome, but what domains should be included in the vaccine is not certain. Salanti's group showed that Ab raised against FV2 in laboratory animals completely inhibit recombinant VAR2CSA binding to CSA, as well as IE binding to CSPG (Khunrae *et al.*, 2010). Ndam *et al.*, (2015) recently reported that Ab responses to VAR2CSA DBL domains increased in women in Benin with gravidity, except to DBL4 and DBL6 and that high responses to DBL3X at enrollment were associated with reduced prevalence of placental infection at delivery (Ndam *et al.*, 2015). Investigating the relationships between Ab responses at enrollment and LBW, this group found that strong IgG responses against DBL1–DBL2 were associated with reduced prevalence of LBW babies, a trend that they also observed for responses to DBL3X (Ndam *et al.*, 2015). Thus, it remains unclear which DBL domain or domains should be used in a vaccine.

### **Immunity to placental malaria: antibody response with gravidity**

Numerous studies have demonstrated the important role of anti-VAR2CSA humoral immunity in *P. falciparum* infections during pregnancy (Duffy, 2007; Hviid and Salanti, 2007; Rogerson, 2010; Ataide *et al.*, 2013). Antibody reactivity increase with gravidity against recombinant VAR2CSA, the surface of erythrocytes infected with *P. falciparum* placental isolates and *P. falciparum* lines selected by their adhesion to CSA (Ataide *et al.*, 2013), indicating

that immunity to VAR2CSA is acquired or boosted progressively with successive pregnancies, and is associated with parasite clearance of placental infection (Feng *et al.*, 2009; Tutterrow *et al.*, 2012). In addition, other mechanisms have been shown to influence susceptibility to both species (*P. falciparum* and *P. vivax*) during pregnancies, including increased cortisol concentrations (Bouyou-Akotet *et al.*, 2005) and reduced NK cell activity (Bouyou-Akotet *et al.*, 2004) particularly in primigravid pregnancies.

### **Antibodies to different DBL domains**

It is not clear if protection is associated with the Ab to the full length molecule or the individual domains. Bentley & Benoit (2008) propose that protection is associated with Ab to DBL3. Whereas Dahlback *et al.*, (2011) showed that full-length recombinant VAR2CSA binds specifically to CSA with nanomolar affinity and that the CSA-binding site lies in the N-terminal part of the protein. However, contrary to this idea, other groups have reported that single domains possess the structural requirements for specific binding to CSA, i.e., that the core-CSA binding site lies within the DBL2X domain and parts of the flanking inter-domain regions (Mayor *et al.* 2005; Clausen *et al.*, 2012). Which domain is really associated with protection is not clear.

Although it is well established that the VAR2CSA molecule is able to elicit immune IgG Ab that are associated with improved pregnancy outcomes, it is not clear if Ab levels or the quality of the Ab that is associated with protection. In addition, a lot of controversies exist with respect to which domain is the most immunogenic. Studies attempting to identify which of the six constitutive extracellular VAR2CSA domains are critical to the protective Ab responses have produced differing results (Oleinikov *et al.*, 2007, Brodin *et al.*, 2010). Ab to DBL5 was the higher in multigravidae, compared to primi- and secundi-gravidae, but there was no significant

difference between Ab affinities to DBL5e for primi- and secundi gravidae. In the same study, the authors found that pregnant women from a malaria-endemic area had increasing levels of anti-DBL5e IgG by parity, indicating this domain of VAR2CSA may be a promising vaccine candidate against PM (Brolin *et al.*, 2010). Recently, it was shown that among women infected during pregnancy, an increase in inhibition of IE binding to CSA was associated with reduced risks for placental infection, preterm birth, and low birthweight (Ndam *et al.*, 2015). Another group also revealed a parity-dependent recognition of the full-length VAR2CSA and of the CSA-binding region, DBL1X-3X (Dechavanne *et al.*, 2015). Indeed, they saw that multigravid women possess significantly higher levels of Ab directed against these constructs than primigravidae, suggesting the importance of Ab targeting the CSA-binding region in the development of immunity against PM. These data provide new insights on how natural protection might be acquired as well as further information for the design of VAR2CSA-based vaccines (Dechavanne *et al.*, 2015).

## Affinity maturation

Affinity maturation is the process that produces Ab with a stronger ability to bind to an antigen over time. Affinity maturation is produced by changes in the genes that encode for immunoglobulin G (IgG) and by increased survival of those B lymphocytes that produce Ab with the greatest ability to bind a particular epitope. Increased affinity occurs only when B-cell activation is stimulated by helper T cells. Normally with repeated exposure to the same antigen, a host will produce Ab of successively greater affinities and in a much faster rate compared to the primary challenge.

B cells play central roles in the establishment and maintenance of protective immunity, including the generation of protective Ab, antigen presentation, and regulatory functions (Frasca *et al.*, 2008). B cells are first activated at the follicular border by a combination of antigens and helper T cells. They migrate to germinal centers where the remaining events occur. Somatic hyper-mutation may result in amino acid replacements in the immunoglobulin V regions that affect the fate of the B cell. Mutations that result in a B cell receptor of lower or no affinity for the antigen will prevent the B cell from being activated efficiently, because both B cell receptor (BCR) cross-linking and the ability of the B cell to present peptide antigen to T cells are reduced.

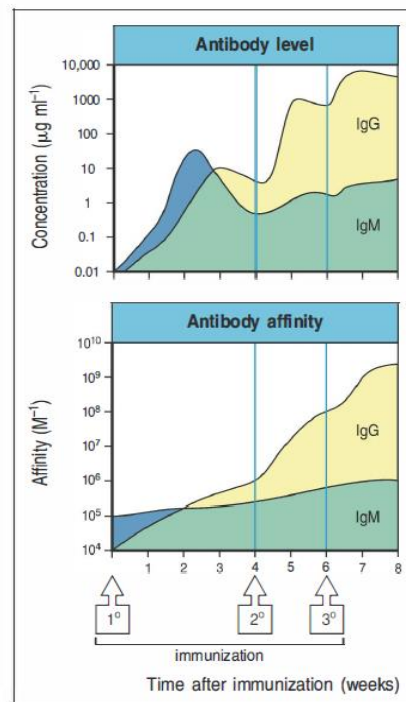


Fig. 3: Change in antibody level and affinity during a primary and secondary challenge with antigen

This results in B cells dying by apoptosis, which purges low affinity B cells from the germinal center. Some mutations, however will improve the ability of B cell receptor to bind antigen. This increases the B cell's chance of interacting with T cells and thus of proliferating and surviving. B cells that successfully undergo somatic hyper mutation have a BCR that interacts with whole or unprocessed antigen presented by follicular dendritic cells (FDC) in the light zone of the germinal center. These BCR receive survival signals from the FDC, which renders them capable of interacting with T follicular helper cells that have also migrated to the light zone.

Surviving cells undergo repeated cycles of mutation and selection during which some of the progeny B cells undergo differentiation into either memory B cells or plasma cells and leave the germinal center. Overall, this process results in high affinity antibody-secreting plasma cells and high affinity memory B cells.

Unlike in a primary immune response which usually consist of Ab made by plasma cells derived from a diverse population of precursor B cells specific for different epitopes of the antigen and with receptors with a range of affinities for the antigen, the secondary response is derived from a far more limited population of high affinity B cells that have undergone clonal expansion. Their receptors and Ab are of higher affinity for antigen and show extensive somatic mutation.

Although affinity maturation consistently produces high-affinity B-cell clones, there is no general theory for the observed diversity of Ab repertoires. For some antigens, the majority of induced B cells within and across hosts target the same epitope, indicating consistent patterns of immunodominance. For other antigens, evolved B-cell populations show adaptation to different epitopes. Ab are a crucial component of naturally acquired immunity against blood stage malaria with multiple roles (e.g., inhibition of merozoite invasion into new red blood cells, blocking

cytoadherence of infected RBCs to endothelial cells, and enhancing phagocytic activity of monocytes and macrophages). However, it is widely believed that periodic re-infection is required to maintain immunity to malaria and that malaria-specific Ab are short-lived in the absence of re-infection; implying that B cell memory to malaria may be defective or suboptimal. In the presence of a high antigen concentration and a diverse BCR repertoire population, the affinity of the B cells for the antigen does not increase because competition for the antigen doesn't occur. On the other hand, when the antigen concentration is limiting, B cell selection occurs due to increased competition of BCRs for the antigen; i.e. those of the highest affinity are selected, resulting to a rise of Ab with increased affinity for the antigen. The number of B cells with high affinity BCRs to a new random antigen is quite small. VAR2CSA being a very complex and diverse malaria antigen, the presence of multiple epitopes promotes the evolution of B cells with different specificities. Data from yet another study on women in Cameroon, suggest that immunity to PM results from high antibody levels to multiple VAR2CSA domains and allelic variants and that Ab breadth is influenced by malaria transmission intensity (Tutterrow *et al.*, 2012).

### **High avidity antibodies to VAR2CSA and other malarial antigens**

Antibody avidity gives a measure of the overall strength of interaction between polyclonal Ab in plasma and an antigen. Being a highly variable protein, VAR2CSA has many epitopes that may reduce the affinity and increase the relative breadth of the Ab repertoire. It has been shown that PG acquire high avidity Ab during the course of pregnancy and malaria transmission force influences the acquisition of Ab (Tutterrow *et al.*, 2012). In 2010, Brolin and collaborators showed that even though multigravid women had higher levels of Ab against DBL5e than primigravidae, the Ab in multigravidae did not show higher affinity against the

DBL5e domain (Brolin *et al.*, 2010). Thus, questions about high avidity Ab to VAR2CSA and its domains remain inconclusive.

Little is known about antibody avidity and the importance of high avidity Ab and immunity to malaria in general (Table 1). Antibody avidity has only been investigated to 5 malarial antigens, including those on the surface of merozoites (MSP1, AMA1, MSP 2), sporozoite (CSP) and VAR2CSA. High avidity Ab have been investigated for prevention of merozoite and sporozoite invasion. The three studies evaluating the influence of Ab avidity to merozoite surface antigens came to different conclusions, finding an association or no association with infection. Two sporozoite vaccine trials have measured Ab avidity to the vaccine and sought to determine if high avidity Ab were associated with protection from infection. Surprisingly, one study reported no association; whereas, the other reported a strong association. Clearly, further studies are needed to understand the role of high avidity Ab in immunity to malaria.

Table1 Summary of literature on Ab avidity and protection from malaria					
Year	Author	Antigen	Type of Immunity/Setting	Association of Avidity with Protection	Age group
2009	Mehrezi et al.	PvMSP1	Natural/Endemic	* YES	Adults
2010	Wapisa et al.	Pf-MSP1 and PfAMA	Natural/ Non-endemic	*YES	Adults
2012	Reddy et al.	AMA-3D7 MSP2-3D7	Natural/Endemic	* YES	1-74 years
2012	Ibosin et al.	AMA1, MSP1, MSP2	Natural/Endemic	NO	≤13years
2012	Tutterrow et al.	VAR2CSA	Natural/Endemic	* YES	16-45 years Pregnant women
2014	Olutu et al.	RTS,S/AS01 <sub>E</sub>	Vaccination	NO	5-17 months
2015	Ajua et al.	CSP	Vaccination/Endemic	** YES	Children
*Protection was defined as association of avidity with age *Reduced risk of placental malaria meant 7.6 fold increase in pregnancy outcome					

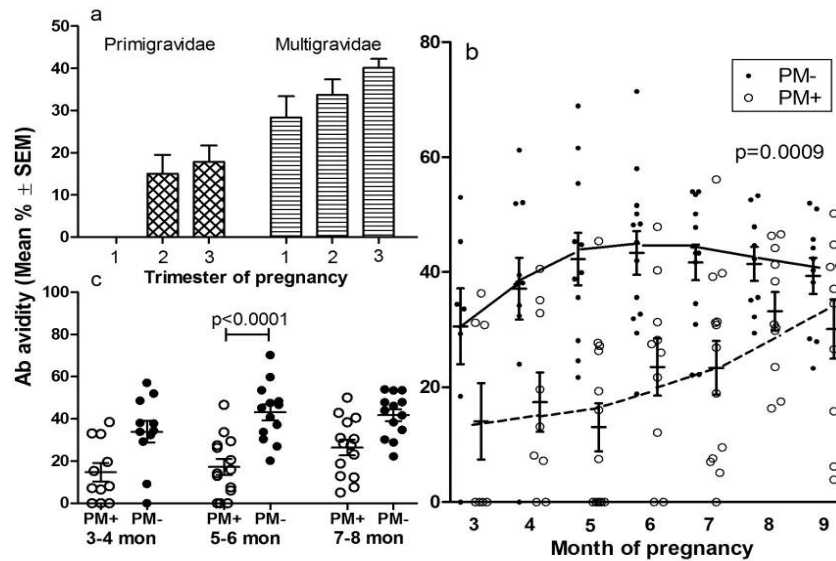
### Persistence of immunity

Antigen-experienced B-cell subsets include recently activated B cells, memory B cells, and Ab-forming plasma cells. Wipasa *et al.*, (2010) analyzed the longevity of both Ab and B cell memory responses to regular malaria antigens among individuals who were living in an area of extremely low malaria transmission in northern Thailand, and who were known either to be malaria naive or to have had a documented clinical attack of *P. falciparum* and/or *P. vivax* in the past 6 years (Wipasa *et al.*, 2010). They found that exposure to malaria resulted in the generation of relatively avid antigen-specific Ab and the establishment of populations of antigen-specific memory B cells in a significant proportion of malaria-exposed individuals. Both Ab and memory B cell responses to malarial antigens were stably maintained over time in the absence of re-

infection. In a number of cases where antigen specific Ab were not detected in plasma, stable frequencies of antigen-specific memory B cells were nonetheless observed, suggesting that circulating memory B cells may be maintained independently of long-lived plasma cells. The authors concluded that infrequent malaria infections are capable of inducing long-lived Ab and memory B cell responses (Wipasa *et al.*, 2010). On the other hand, another group observed that PfEMP1-specific B-cell memory was adequately acquired even when antigen exposure was infrequent (e.g., VAR2CSA-type PfEMP1) (Ampomah *et al.*, 2014). In addition, they found that, immunological memory specific for VAR2CSA can be maintained for many years without antigen re-exposure and after circulating antigen-specific IgG has disappeared. Their study provides evidence that natural exposure to *P. falciparum* leads to formation of durable B-cell immunity to clinically important PfEMP1 antigens (Ampomah *et al.*, 2014).

Tutterrow, et al. (2012) tried to elucidate if anti-FV2 Ab quality is important in parasite clearance, the proportion of high avidity Ab to FV2 (i.e., Ab remaining bound after treatment with 3M NH<sub>4</sub>SCN) was determined in plasma from women in Ngali II and women in Yaoundé (low transmission setting) who were seropositive to FV2. Their data set showed that very few women in Yaoundé developed high avidity Ab to, preventing further analysis. But by comparison, in Ngali II during the first trimester none of the Ab in primigravidae were of high avidity. However, by delivery, about 17.8% of anti-FV2 Ab were found to be high avidity. Furthermore, during the first trimester, about 28.4% of anti-FV2 Ab in multigravidae were high avidity, and this increased to about 40% during the third trimester. The proportion of high avidity Ab was higher throughout pregnancy in multigravidae compared to primigravidae implying the development of high avidity anti-FV2 Ab is gravidity-related. Importantly, they found that proportion of high avidity Ab was significantly higher in PM<sup>-</sup> compared to PM<sup>+</sup> women

throughout pregnancy ( $p=0.0009$ ) (Fig. 4). That is, the proportion of high avidity Ab in  $PM^-$  women rose from  $34\% \pm 12\%$  at 3 months to  $42 \pm 15\%$  at 5 months, and plateaued at 43% until term as shown in the Fig. 4b. In contrast, in  $PM^+$  women, only  $16 \pm 18\%$  of Ab were high avidity at 3 months,  $23 \pm 16\%$  at 6 months, and  $33 \pm 12\%$  at the end of pregnancy. Although the proportion of high avidity Ab was generally higher in multigravids compared to primigravids, some multigravidae still had placental malaria, suggesting high avidity Ab may be needed early in pregnancy for parasite clearance by delivery. Notably, higher proportion of high avidity anti-FV2 Ab at 5–6 months of pregnancy was significantly associated with absence of PM. Women in Ngali II with  $\geq 35\%$  high avidity Ab to FV2 during the 5–6 months of pregnancy had a 7.6 times lower risk of placental malaria than women with  $<35\%$  high avidity Ab. Thus, at least in the high transmission setting, high avidity Ab to FV2 by the 2<sup>nd</sup> trimester was found to be important in reducing PM by delivery. The sample size representing the low transmission setting (Yaoundé) in this study was limiting ( $n=25$ ), so valuable comparison could not be done with respect to the two sites. It is based on these findings that we thought it was important to study a low transmission setting and see the patterns of Ab produced across different pregnancies.



**Figure 4: High Avidity Antibodies to VAR2CSA were Associated with Absence of Placental Malaria in Ngali II (High transmission setting) Tutterrow et al., 2012.**

(a) The proportion (%) of Ab that remains bound to FV2 after incubation with 3M NH<sub>4</sub>SCN (i.e., % high avidity Ab) in the plasma of primigravidae and multigravidae living in Ngali II is shown. Results represent the mean + SEM for 11 to 51 data points per bar. Throughout the course of pregnancy, the proportion of high avidity Ab to FV2 was higher in multigravid than primigravid women (p < 0.0001; multilevel polynomial regression analysis). (b) The scattergram shows that the proportion of high avidity Ab to FV2 was highly variable. However, the proportion (%) of high avidity Ab in plasma of women who were PM<sup>-</sup> was significantly higher than in plasma of PM<sup>+</sup> women during pregnancy (PM<sup>-</sup>, n=13; PM<sup>+</sup>, n=14) (p=0.0009), based on multilevel polynomial regression analysis.

### Summary

Naturally acquired immunity to *P. falciparum* malaria is non-sterile and slowly acquired, requiring repeated infections over a number of transmission seasons. Recent studies have further refined this view, suggesting that the immune repertoire acquired by individuals living in highly endemic areas is different compared with those where malaria infection is less frequent. These differences in acquisition of naturally acquired immunity in humans, not only makes it more complex to elucidate immune mechanisms of protection, but also makes designing a broadly protective vaccine for *P. falciparum* malaria all the more challenging. VAR2CSA is a promising

vaccine candidate because immunity to placental malaria has been directly associated with Ab to this antigen, as high levels of Ab to VAR2CSA are associated with improved pregnancy outcomes. VAR2CSA is less variable than other members of the var family, although polymorphisms exist. The importance of polymorphism in immunity, i.e., do women need to have Ab to multiple variants of the antigens or not, is unclear. The size and complexity of the VAR2CSA protein pose challenges for vaccine development, but smaller domains may be suitable for subunit vaccine development. Thus, additional information on the importance of Ab avidity, to both the full length molecule and individual DBL domains is important for vaccine development.

### **Rationale**

Women who have 35% of high avidity Ab to FV2 in a high transmission setting have 7.6 times lower risk of PM than women who have lower percentage of high avidity Abs (Tutterrow *et al.*, 2012). We don't know what the case may be in a low transmission setting.

### **Specific Aims**

The specific aims of the study is to answer the questions listed below.

- 1) Do high avidity antibodies to FV2 increase with gravidity in women living in Yaoundé, a low malaria transmission setting?
- 2) Is there an association between Ab avidity and the presence or absence of PM in women living in a low transmission setting?
- 3) Are high avidity Ab to the full-length VAR2CSA directed against a specific region of the molecule, i.e., one DBL domain, or multiple domains?

## **Hypothesis**

In a high transmission area, women who have 35% of high avidity Ab to FV2 by the middle of pregnancy have 7.6 times lower risk of PM than women who have lower percentage of high avidity PM (Tutterrow *et al.*, 2012). In this situation, women are infected multiple times during a single pregnancy. In a low transmission setting, i.e., the city of Yaoundé, women are bitten by infectious mosquitoes only a few times. Thus, my working hypothesis is: The prevalence of high avidity Ab will be higher in PM- women with greater than 35% compared to PM+ women in a low transmission setting.

## **Materials and methods**

### **Study design and plasma samples**

This current study used plasma samples collected between 1996 and 2001, prior to the implementation of intermittent preventive treatment (IPT) and insecticide-treated bed nets (ITNs) for prevention of malaria in pregnant women (Tako *et al.*, 2005). The panel consisted of blood samples, placental biopsies, and clinical information from ~2,500 Cameroonian women collected consecutively at delivery from consenting women at Central Maternity and Biyam Assi Hospitals, Yaoundé. Yaoundé is the capital of Cameroon and is a malaria endemic area with low transmission. Estimated entomological inoculation rates at the time when the samples were collected was estimated to be about 13 infectious mosquito bites/year (Manga, *et al.*, 1997a). The prevalence of malaria in pregnant women at delivery in Yaoundé at this time was about 20% (Tako *et al.*, 2005).

Since plasma samples were obtained from Cameroonian women prior to use of (IPT and ITNs) natural immunity helped determine the presence or absence of PM (as only women who

become ill seek treatment or take antimalarial drugs). Because the women did not use preventive measures, it is likely all of the women became infected with *P. falciparum* during pregnancy.

A 1:3 case-control study design was used. Starting with 2,500 samples collected at delivery. Women with multiple births, abortions, and stillbirths were excluded from the study. All the PM+ samples (n=341) were selected and then 3 times PM- samples (n=1,036) were randomly selected, making a total of 1,377 samples. Women with preterm delivery were included (premature was defined as less than 37 weeks of gestation). Low birth weight was defined as less than 2,500g; primigravidae are women with no previous pregnancies; and multigravid women were those who had been pregnant at least once previously.

### **Diagnosis of placental malaria and anemia**

Thick and thin smears were prepared from maternal peripheral and placental IVS blood, and impression smears were made from biopsies of placental tissues. Slides were stained with Diff-Quick and read by two different microscopists to determine parasitemia. Placental biopsies were used to prepare impression smears that were made by imprinting IVS tissue onto a slide, staining with Diff-Quick, and examining for IE. Placental biopsies were also used to prepare histological sections, in which tissues were fixed in buffered formalin, embedded in parafin, and stained with hematoxylin-eosin, and examined for parasites. A woman was considered to have PM if infected erythrocytes were detected in blood smears of IVS blood, impression smears of villous tissue, or histological sections of placenta. Maternal peripheral blood was used to determine the packed cell volume (PCV), i.e., hematocrit.

## **Screening of plasma samples for Ab to recombinant VAR2CSA using a multi-analyte platform (MAP) assay**

To rule out the possibility that women might not have become infected and produced Ab to VAR2CSA, plasma samples were pre-screened for the presence of Ab to FV2 (FCR3 strain). Only plasma samples from women with evidence of infection (seropositive to FV2) were included in the study. All 1,377 samples were screened for Ab to FV2. Using a Luminex bead-based assay, the optimal amount of FV2 was coupled to SeroMap beads as previously described (Fouda *et al.*, 2006; Tutterrow *et al.*, 2012)-). Plasma samples from 1,377 women were diluted 1:200.

The MAP assay was performed as previously described (Tutterrow *et al.*, 2012). Briefly, 50µl of antigen-coupled microspheres (2000 microspheres/test) were incubated with 50µl of 1:200 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 1 hour at 25<sup>0</sup>C on a rotating shaker at 500rpm (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-phycoerthrin-conjugated, Affini Pure F(ab')<sub>2</sub> fragment, Goat anti-human IgG Fc fragment specific)(Jackson Immunoresearch, West Grove, PA, Cat #109-116-170) diluted to 2ug/ml in PBS-1%BSA was added to each well and incubated as above in the dark for 1 hour. Wells were washed as described above, microspheres were re-suspended in 100µl of PBS-1%BSA and 80µl of suspension was analyzed using a Luminex M100 reader (Quiagen, Valencia, CA). The reader was programmed to read a minimum of 100 beads per spectral address, DD Gate 7500-15000 and 32 seconds time out. The results were expressed as Mean Fluorescence Intensity (MFI). A panel of 6 control plasma samples (4 pools of positive

and 2 pools of negative plasma) was included on each plate. A pool of archival plasma from US pregnant women who have never travelled to malaria endemic area was used, as one of the negative controls. In addition, 30 males residing in Yaoundé, Cameroon were pooled used as a second negative control for VAR2CSA. All samples were screened by two different individuals for a total of 34 plates.

Results from the two data sets were compared and any sample with variation was re-screened. The resulting two data sets were very similar with  $r=0.976$ . Control samples on each of the 34 plates were compared, and results were averaged. Samples with MFI greater than the mean + 2 SD of the male controls were considered to be positive for Ab to FV2.

### **Antibody avidity to FV2**

The avidity assay was performed as previously described (Tutterrow *et al.*, 2012) with slight modifications. Briefly, plasma was diluted 1:300 in PBS-1% BSA and 50 $\mu$ l of diluted plasma was added in duplicate to wells containing FV2-coupled microspheres (2,500 microspheres/test) and incubated for one hour on a shaker. After incubation, coupled FV2 – plasma complex were washed twice with PBS-0.05% Tween20 and once with PBS-1% BSA. After incubation, 100 $\mu$ l of 3M NH<sub>4</sub>SCN in PBS-1% BSA was added to half of the wells, and 100 $\mu$ l of PBS-1% BSA was added to the remaining wells. After 30 minutes of incubation, the wells were washed as described above and then 50 $\mu$ l of PE-goat anti-human IgG was added and incubated for 60 minutes. The wells were washed and analyzed using a Microchip100 as described above. Avidity was determined by the following formula: (MFI obtained from wells incubated with salt)/(MFI obtained from corresponding control wells)X100. Positive control consisting of a pool of plasma from multigravidae Cameroonian women and negative control consisting of pool of US plasma were included on each plate. When  $\geq 35\%$  of Ab remained

bound in the presence of 3M NH<sub>4</sub>SCN, women were considered to have high proportion of high avidity Ab.

### Measuring Ab avidity to FV2 and its domains

To determine if there exist an association between high avidity Ab to specific DBL domains and PM, a pilot study was conducted where 30 plasma samples with high (20,000-25,000 MFI to FV2, intermediate (15,000 - 20,000), and lower (10,000 to 15,000) MFI were screened against a panel of 17 different DBL domains from 3 different strains, i.e., 3D7, FCR3 and 7G8 that represent the 6 DBL domains (Table 1). The 17 antigens were coupled to microspheres as described above. Then, 50µl of plasma diluted 1:300 was added for 60 minutes. After 60 minutes of incubation at RT on the shaker, half the beads were incubated with 3M NH<sub>4</sub>SCN or PBS for 60 minutes, washed, incubated with PE-anti-human IgG, and analyzed as described above. MFIs were determined as well as the proportion of high avidity Ab as described above.

Table 2: Summary of the VAR2CSA DBL domains used

Strain	FV2	DBL 1	DBL 1+2	DBL 2	DBL 3	DBL 4	DBL 5	DBL 6	DBL ID1-ID2a
FG8		X			X	X	X	X	
3D7	X	X				X	X		
FcR3	X	X	X	X	X	X	X	X	X

### **Antibody avidity to the 6 DBL domains**

Based on the above results, 100 plasma samples were randomly selected from various MFI groups as mentioned above and avidity was determined for DBL1, DBL2, DBL3, DBL4, DBL5 and DBL6 for the FCR3 strain, as well as the FV2 (8 antigens in total). Every experiment was done twice by two different investigators.

Data from this study was compared with archival data from a high transmission setting. In addition, the data obtained from the current study will be used to predict a woman's PM status i.e., if a woman is immune or not. This model will help to determine if avidity can be a correlate of protection in PM or not.

### **Statistical analysis**

Statistical analysis were done using the Graph pad prism software, version 5, and the Statistical Analysis Software (SAS) version 9.4. The mean + 2 Standard Deviations was used as the cut-off value (3,709 MFI) based on the 30 male negative controls were used to define FV2 Ab-positive women. The cutoff for proportion of high/low avidity Ab to FV2 was 35%. The characteristics were summarized by descriptive statistics with mean  $X \pm SD$  for continuous variables and n (%) for the categorical variables. Two-sample t-tests were used for continuous variables and Chi-square test or Fisher's exact test were used when necessary for categorical variables to compare women in PM+ and PM- groups. The association between the presence of PM and major variables such as gravidity, Ab avidity and Ab levels were evaluated using logistic regression.

## Results

### Prevalence of placental malaria

Analysis of the data on placental malaria status at delivery in the original database (n~2,500 women) showed that the prevalence of PM declined with gravidity (Fig. 5). Therefore, immunity to PM was slowly acquired over multiple pregnancies.

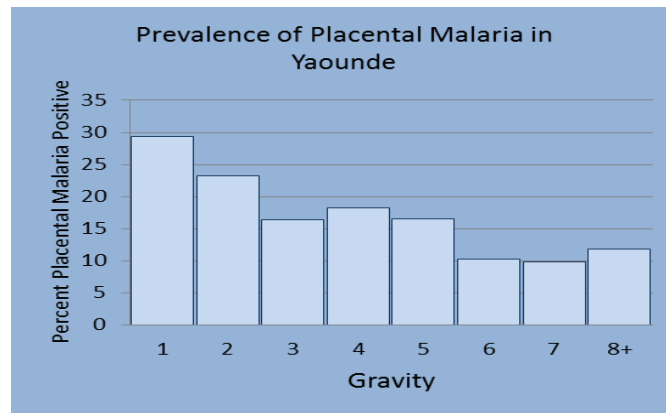


Figure 5. **The Prevalence of Placental Malaria in Yaoundé**

The prevalence (percent placental malaria positive) decreases with gravidity.

### Prevalence of Ab to FV2

Plasma samples from the 1,377 women collected at delivery were screened for Ab to FV2. Using a cut-off based on the mean + 2 SD for males living in Yaoundé (3,709 MFI), only 36% (494/1,377) were Ab positive. Thus, many women in Yaoundé lacked Ab to FV2 at delivery.

Characteristics of the 494 Ab-positive women are shown in Table 3. Of these women, only 178 (36%) were PM+ while 316 were PM- (Table 3). Overall, the PM+ women were 2.4 years younger than the PM- women ( $P<.0001$ ) and had fewer pregnancies ( $P<.0001$ ). These data suggest that gravidity plays a role in the acquisition of immunity to PM. PM+ women had

significantly lower hematocrits ( $P < 0.0001$ ), however, the decrease did not have a significant effect on prevalence of maternal anemia and baby birth weight. There were no significant differences observed between the two groups in terms of pre-term deliveries, length of gestation, and low birth weight due to intrauterine growth restriction. Thus, the only other significant differences between the groups of PM+ and PM- were age and gravidity.

Table 3: Characteristics of study population for FV2 Ab positive samples stratified by PM status

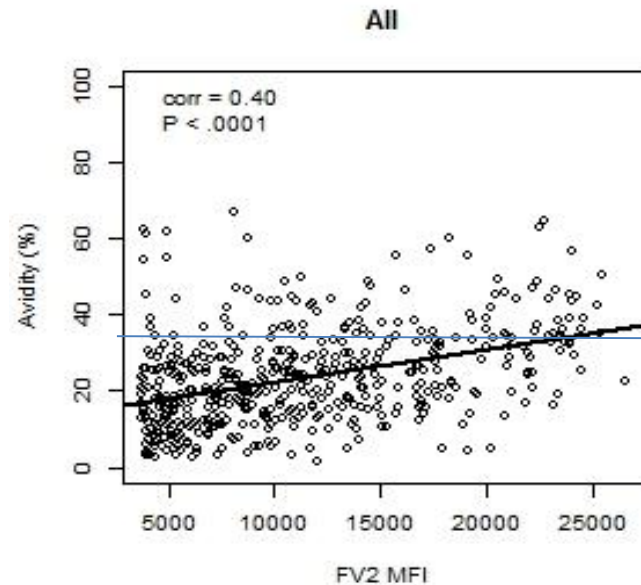
Characteristic	All (n=494)		P-value
	PM+ (n=178)	PM- (n=316)	
Age in years	24.3 (5.5)	26.7 (5.8)	<b>&lt;.0001</b>
Gravidity	3.0 (2.0)	3.9 (2.3)	<b>&lt;.0001</b>
Placental parasitemia by impression smears in %	2.4 (6.4)	0 (0)	<b>na</b>
Pre-term deliveries	36 (20.2%)	71(22.5%)	0.54
Length of pregnancy in weeks	38.6 (6.4)	38.2 (6.0)	0.54
Intrauterine growth restriction	8 (4.5%)	12 (3.8%)	0.72
Maternal anemia at deliveries*	43 (24.2%)	51(16.1%)	0.082
Hematocrit in %	31.2 (5.7)	34.0 (5.4)	<b>&lt;.0001</b>
Low birth weight babies	31 (17.4%)	53(16.8%)	0.89
Baby weight in grams	2997 (633)	3081 (671)	0.17

na – not applicable since the samples were selected  
 \*anemia is defined as having Hb<10g/dL (Hct<30%)

### Comparison of ab levels and avidity to FV2

Ab levels (amount) and avidity (quality) to FV2 were compared (Fig. 6). A modest correlation between high Ab levels and avidity was found ( $P=0.0001$ , Correlation coefficient=0.40); however, some women who had very low Ab levels (less than 5,000MFI), but still had very high quality Ab i.e., up to 60% high avidity Ab. On the other hand, some women made a large amount of Ab (up to 20,000 MFI) with avidity below 10%. These results suggest

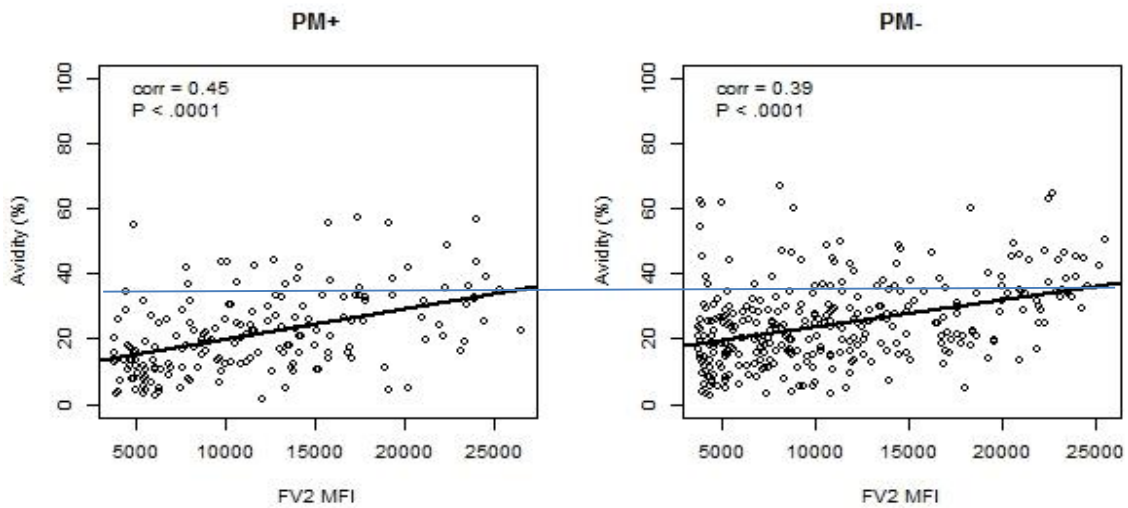
that some of the Ab produced did not undergo affinity maturation, hence resulting in high Ab quantity but low Ab quality (Fig. 6a).



**Figure 6a. Relationship between Ab Avidity and Ab Level for FV2.** All: n=494 women

The blue horizontal line represents 35% high avidity and above. The circles represent individual data points. The X-axis represents the mean fluorescence intensity (MFI) of full length VAR2CSA (FV2).

Figure 6b shows the relationship between Ab levels to FV2 and avidity in women who were PM+ and PM-. In both cases, Ab avidity increased as MFI increased (with correlation coefficients of 0.45 (PM+) and 0.39 (PM-) ( $p < 0.001$ ). Importantly, the prevalence of PM- women with  $\geq 35\%$  high avidity, 18.4% (58/316) exceeds that of PM+ group 13.5% (24/178). Overall, PM- women had a significantly higher proportion of high avidity Ab (mean  $\pm$ SD:  $24.8 \pm 12.6\%$ ) compared to PM+ women ( $21.5 \pm 12.1\%$ ) ( $P = 0.0045$ ). These data show an associated increase in Ab levels and avidity. In addition, the proportion of high avidity Ab was greater in PM- compared to PM+ women.



**Fig. 6b Relationship between Ab Avidity and Levels for FV2 in PM+ and PM- Women.** The blue horizontal line represents 35% high avidity Ab. The circles represent data points for individual women. The Y-axis represents the percentage of Ab avidity, and the X axis shows the mean fluorescence intensity (MFI) of Ab to full length VAR2CSA (FV2).

### **Pregnancy outcomes of women with $\geq 35\%$ high avidity Ab to FV2**

To test our hypothesis that women with  $\geq 35\%$  high avidity Ab would have a lower prevalence of PM and better pregnancy outcomes than women with  $< 35\%$  high avidity Ab, the 494 women were stratified based on Ab avidity to FV2 (Table 3). The percentage of high avidity Ab was significantly different between the PM+ and PM- groups ( $P=0.0045$ ). Most Ab to FV2 were of reasonably low avidity, with only 16.5% of women having  $\geq 35\%$  high avidity Ab to FV2 and 83.5% of the women had  $< 35\%$  avidity Ab to FV2. Women with  $\geq 35\%$  high avidity Ab had had more pregnancies (mean 4.0 vs 3.5) than women with low avidity Ab. There was no significant difference with respect to age anemia/hematocrit, or length of gestation. However, babies in the high avidity group were on average 166 grams bigger than their counterparts ( $P=0.021$ ). Thus, the only impact of high avidity Ab for FV2 was on infant birth weight.

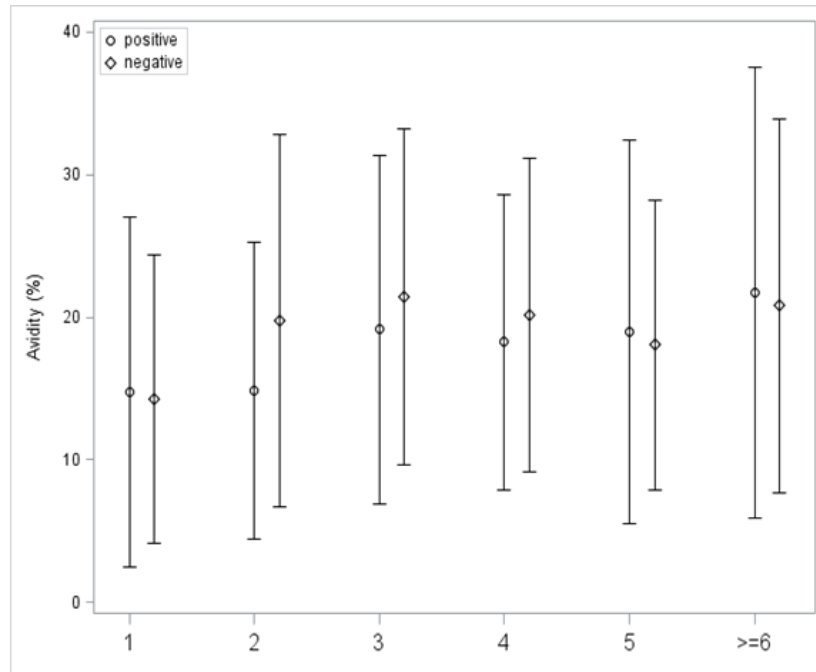
**Table 4. Characteristics of Study Population for FV2 Ab Positive Women Stratified by Low and High Avidity**

Characteristic	All (n=494)		P-value
	High avidity (n=82)	Low avidity (n=412)	
Age in year	26.7 (5.4)	25.7 (5.9)	0.15
Gravidity	4.0 (2.1)	3.5 (2.2)	<b>0.030</b>
Percent Placental parasitemia by impression smears (%)	0.7 (4.5)	0.9 (3.9)	0.72
Pre-term deliveries*	13 (15.9%)	94 (22.8%)	0.19
Length of pregnancy in weeks*	39.0 (3.0)	38.2 (6.5)	0.11
Intrauterine growth restriction*	0 (0%)	20 (4.9%)	0.058†
Maternal anemia at deliveries*	14 (17.1%)	80 (19.4%)	0.86
Hematocrit in %*	33.9 (5.4)	32.7 (5.7)	0.12
Low birth weight babies*	9 (11.0%)	75 (18.2%)	0.12
Baby weight in grams*	3189 (561)	3023 (673)	<b>0.021</b>

\* There were missing data, so data analyses were based on available data points: Values in the table represent means (SD)

#### **High avidity antibodies to FV2 Increase minimally with gravidity**

The proportion of high avidity Ab increased minimally with gravidity (Fig. 7). After adjusting for age and malarial status, an increase of 0.9% (P=0.008) per pregnancy was observed, which may not be biologically relevant. The data suggest that the mean percent of high avidity Ab is similar among PM+ and PM- women (Fig. 7). There was a significant increase in mean Ab avidity from G1 to G2 (Fig. 3), but not among G2, G3 or G4 and above (Table 4).



**Figure 7. Relationship between Ab avidity and gravidity in PM+ and PM- women for FV2.**

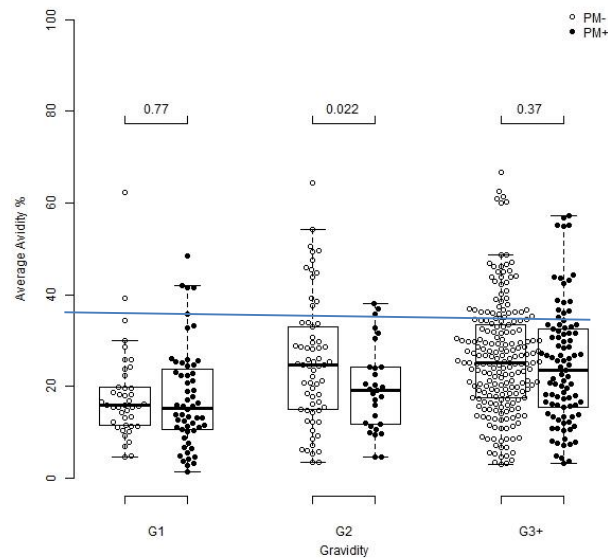
Circles on horizontal bars represent mean % high avidity for PM+ women, while the squares represent mean % high avidity for PM- women. The figures on X-axis represents the different gravidity groups.

Table 5a: Significant difference proportion of high avidity antibodies to FV2 by gravidity

Gravidity	N	Mean % high avidity Ab	StdDev	P-value
1	98	17.7	10.6	<.0001
2	94	23.5	12.9	
3	79	25.9	12.5	
4+	223	25.4	12.5	
Gravidity	N	Mean	StdDev	P-value
1	98	17.7	10.6	<.0001
2+	396	25.0	12.6	P-value

## Affinity maturation is acquired by secundigravidae

Looking at Figure 4, the data suggested that affinity maturation has not yet occurred in most primigravidae during the primary infection, and there were no significant differences in the average avidity between of PM+ and PM- women in each gravidity group. However, a few primigravidae had  $\geq 35\%$  high avidity, suggesting that some primigravid women had been infected early in pregnancy and there was sufficient time for affinity maturation to occur. On the contrary, results show that affinity maturation has occurred in many secundigravidae who were PM-, due to an increase in the average avidity compared to G1. A significant difference was seen PM+ and PM- secundigravid women ( $P=0.022$ ), with a higher average avidity in the PM- group, suggesting the presence of protective Ab (Fig. 8). Thereafter, the Ab avidity remained stationary from G3 throughout subsequent pregnancies.



**Figure 8. Avidity Stratified by Gravidity between PM+ and PM- groups for FV2.** Blue horizontal line represents 35% high avidity. Black and white circles represent individual data points. Vertical bars represent 75th percentile. X-axis represents different gravidity groups from G1 to G3+ and above.

### Prevalence of women with $\geq 35$ high avidity in Yaoundé

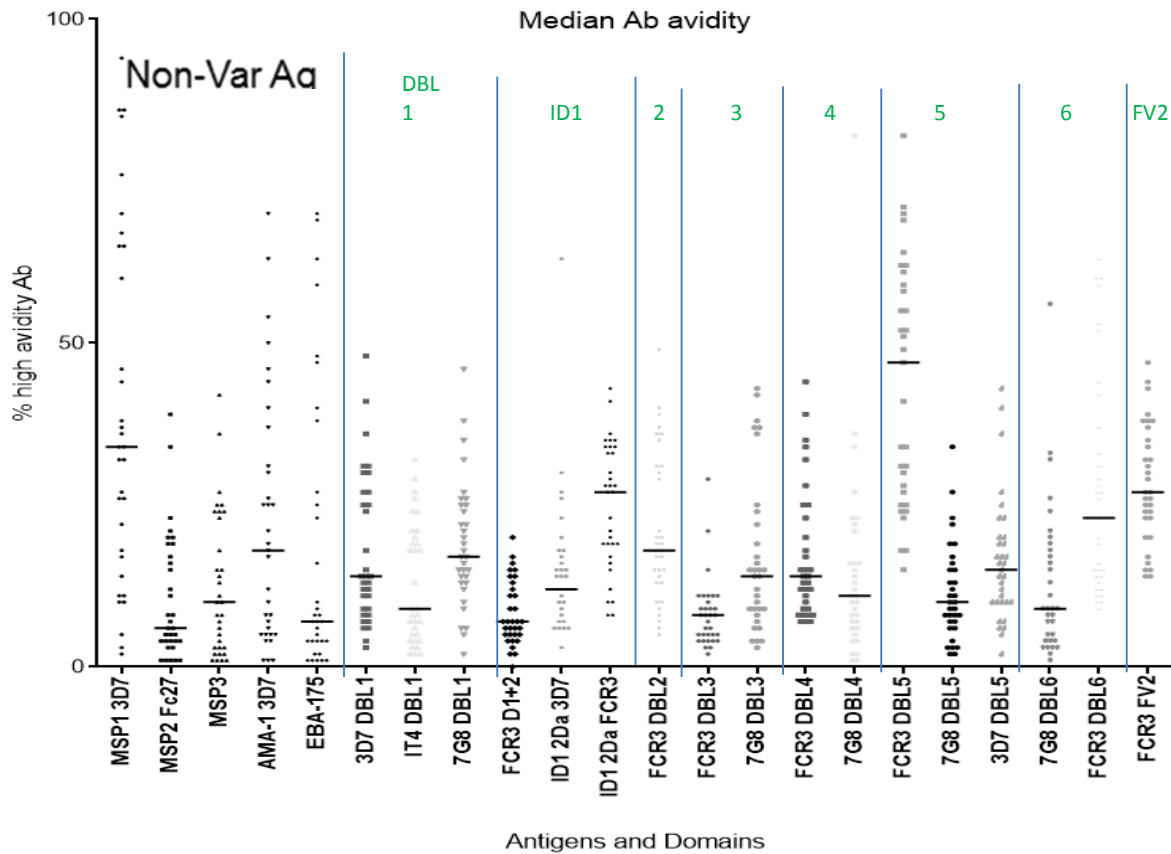
Approximately 36% of the women were PM+ in the study population, while ~16.5% of women with  $\geq 35\%$  avidity Ab had PM. The results actually supports our hypothesis that; the prevalence of PM will be lower in women who have  $\geq 35\%$  high avidity Ab to FV2 in a low transmission setting compared to the prevalence of PM in the study population (Table 4).

Table 5b: Prevalence of PM in Women in Yaoundé			
	Number of women with high avidity Ab $\geq 35\%$	Number of women with low avidity Ab $< 35\%$	All women*
PM+	24	154	<b>13.5%</b>
PM-	58	258	18.4%
	82	412	494
*Number of women; **all women selected 1:3 ratio PM+:PM- P=0.17			

### Proportion of high avidity ab to individual DBL domains

The question remains, why did 29% of women with high avidity Ab still have PM? A possible explanation could be that the Ab produced were against epitopes on a specific DBL domain(s) that are not involved in protection. The VAR2CSA molecule is a very large molecule of about 350kDa, folded in a particular conformation, and some women may not produce Ab against specific domains that are protective. Thus, the specificity of Ab for the individual DBL domains was investigated.

To determine the proportion of high avidity Ab produced to the different DBL domains, a pilot study was conducted where 30 plasma samples with high (20,000-25,000 MFI to FV2), intermediate (15,000 - 20,000), and lower (10,000 to 15,000) MFI were screened against a panel of 17 different DBL domains from 3 different strains of *P. falciparum*. The results suggested that the FCR3 strain was the most immunodominant among the variants for each DBL domain. Overall, FCR3 DBL5 was the most immunogenic domain with the highest median percentage of high avidity Ab values (Fig. 9).



**Figure 9. Average avidity of 3 strains of FV2 and their corresponding DBL domains.** n= 30 plasma samples with high (20,000-25,000 MFI to FV2), intermediate (15,000 - 20,000), and lower (10,000 to 15,000) MFI were screened against a panel of 17 different DBL domains (antigens) from 3 different strains of *P. falciparum* (X-axis). Horizontal bars for individual domains represents median antibody avidity.

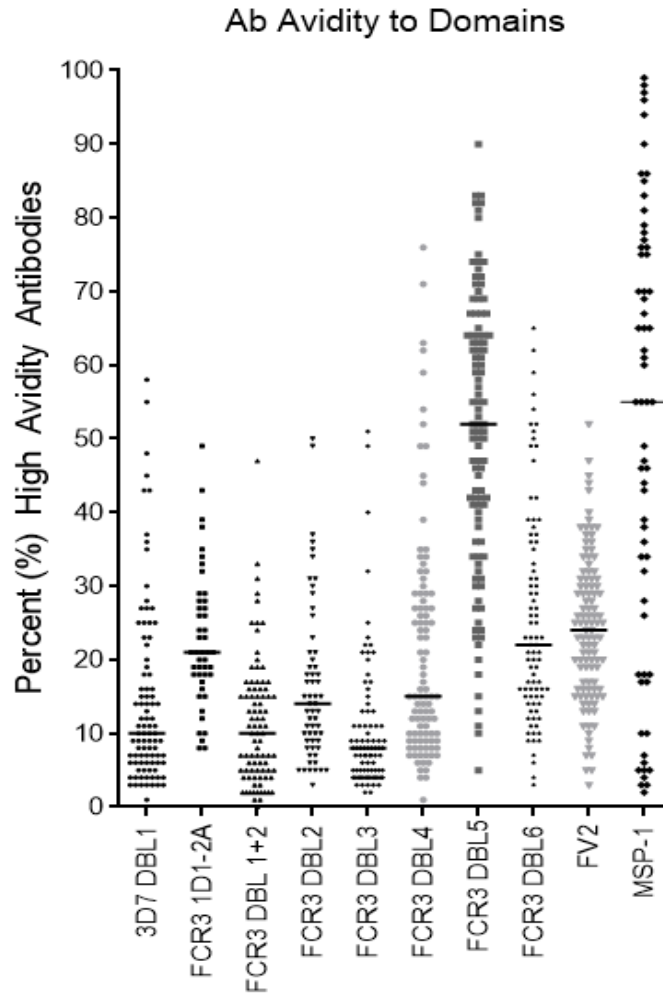
### Immunogenic differences between FCR3-FV2 and its domains

Based on the above results, a larger panel of 103 plasma samples (selected based on MFI value to FV2) was screened for Ab avidity to each of the 6 DBL domains using DBL1 3D7 and the FCR3 strain for DBL 2-6. This was to see the responses of the various domains with respect to avidity and determine which DBL domain(s) might be associated with protection.

The 103 samples were selected based on Ab levels to FV2. Table 6 shows the percent of women who have Ab to the various DBL domains. Overall, most of the women have Ab to most of the domains.

Table 6. Percentage of Women who had Ab to FV2 and its Domains (n=103)									
	FV2	DBL1 3D7	DBL1+2	DBL2	DBL3	DBL4	DBL5	DBL6	ID1- ID2a
3D7- Asia		56%							
FCR3- Africa	100 %		99%	69%	90%	96%	100%	77%	39%

Fig. 10 is a scatter plot showing the percent of high avidity IgG against FV2, DBL1 (3D7), and the individual FCR3 DBL2-6 domains) for 103 Ab-positive women (Fig. 10). Results suggested that DBL5 as the most immunogenic domain with an average of about 50% of the Ab being high avidity.



**Figure 10. Percent High Avidity IgG Against FV2 and its Domains.** A scatter plot of percent high avidity IgG against FV2, the individual FCR3 DBL2-6 domains, and DBL1 (3D7) for 103 Ab-positive women. MSP1=Control and horizontal bars for individual domains represent median antibody avidity.

**Is a specific DBL domain of the FV2 molecule necessary to achieve protection from PM?**

There were no significant differences between PM+ and PM- women in age, gravidity, and all the pregnancy outcomes (such as preterm, anemia and low birth weight), as well as Ab levels. Nevertheless, DBL5 had the highest average percent high avidity Ab levels, but these Ab were probably not associated with protection since there were no big difference between the PM- and PM+ group (Table 7). Generally, for all the antigens, the average percent of high avidity Ab

was higher in PM- group compared to the PM+, except for DBL4 which had a slightly higher average avidity for the PM+ group. However, the data suggest that DBL1 3D7, DBL 1+2, DBL 3 and ID1 2Da may be associated with protection from PM as the differences between PM+ and PM- group were significant compared to all the other domains including FV2 Table 7). The question is; why do 29% of women with high avidity Ab still have PM? A possible explanation for this observation could be that the Ab produced may be against epitopes on a specific DBL domain(s) that may not be protective. The VAR2CSA molecule is a very large molecule of about 350kDa, folded in a particular conformation, probably not in a way that allows for it to mount the production of those Ab that are protective. We therefore went further to look at the specificity of the individual domains.

Table 7: Avidity variation for various DBL domains between PM+ and PM- women

Domain	All (n=103)		
	PM+ (N=40)	PM- (N=63)	P- value
Avidity to FV2 in %, mean (SD)	23.1 (11)	25.4 (14.6)	0.370
Avidity to DBL1 3D7 in %, mean (SD)	12.4 (10.2)	19.7 (15.9)	<b>0.005</b>
Avidity to DBL1+2 FCR3 in %, mean (SD)	11.8 (7.4)	15.2 (12.1)	0.084
Avidity to DBL2 FCR3 in %, mean (SD)	13.9 (7.5)	19.8 (12.6)	<b>0.003</b>
Avidity to DBL3 FCR3 in %, mean (SD)	8.7 (5.7)	12.9 (12.2)	<b>0.024</b>
Avidity to DBL4 FCR3 in %, mean (SD)	23.3 (17.1)	21.5 (15.7)	0.590
Avidity to DBL5 FCR3 in %, mean (SD)	50.2 (20.6)	54.3 (17.7)	0.310
Avidity to DBL6 FCR3 in %, mean (SD)	29.5 (19.1)	35.7 (17.2)	0.100
Avidity to ID1 2Da FCR3 in %, mean (SD)	25.0 (8.0)	29.7 (13.5)	<b>0.029</b>
Avidity to MSP1 in %, mean (SD)	30.7 (22.7)	32.4 (19.6)	0.700

This result suggest that:

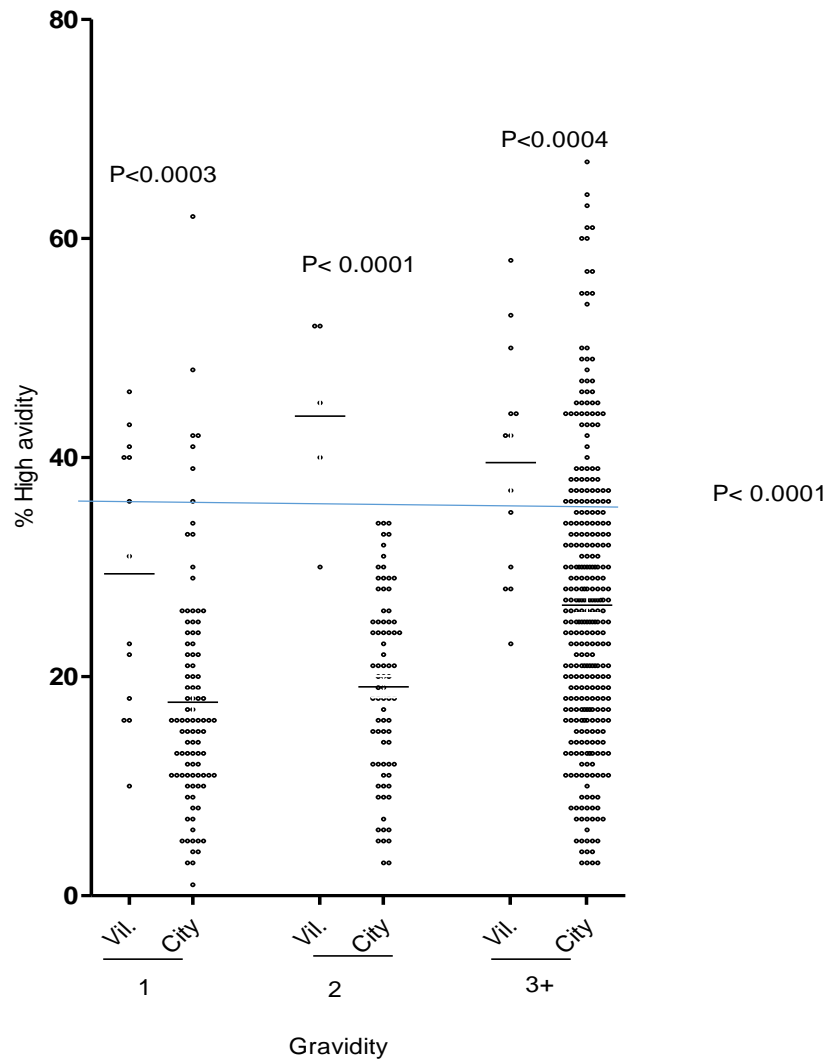
- 1) DBL5 may be highly immunogenic but may not necessarily play a role in protection from PM
- 2) It is possible that a combination of more than one DBL domain may be required for protection

3) The fact that significant differences exist in average avidities between PM+ and PM- women for DBL1 3D7, DBL 2, DBL 3 and ID1-2a suggest that the N-terminus of the FV2 molecule may play an important role in protection.

**The percentage of high avidity was higher across different gravidity groups in a high transmission setting compared to a low transmission setting**

Avidity data to FV2 for the 494 women living in Yaoundé were compared with archival data for women living in Ngali II, a rural village with high transmission (Fig. 11). Generally, the average avidity to FV2 was significantly higher in the village across all gravidity groups compared to the city ( $P < 0.0001$ ). This result was expected as women were exposed to multiple infectious bites (at least 1 infectious bite per night) as opposed to the city where women are exposed to just one infectious bite per month.

Mean Avidity Trend at Delivery Across Different Gravity Groups in Ngali 2, n=38 (High transmission setting) and Yaounde, (Low transmission setting) n=494



**Figure 11. Percent High Avidity Ab to FV2 is Higher Across Different Gravity Groups in a High Transmission Setting Compared to a Low transmission Setting.** Blue horizontal line represents 35% high avidity Ab, horizontal bars represent mean % avidity and dots represent individual samples. X-axis represents different gravity groups for the village and city at delivery.

## **Development of a multivariable prediction model to identify women who are protected from placental malaria**

The following hypothesis was tested: a combination of avidity and/or Ab levels to multiple domains and other subject characteristics will contribute unique information to malaria prediction. In the univariate analysis, avidity to domain DBL1 (3D7), DBL1+2 (FCR3), DBL2 (FCR3), DBL3 (FCR3), D6 (FCR3) and ID1 2Da (FCR3), and gravidity were at least suggestive to be associated with PM status (all P-values were  $P < 0.10$ ). Therefore, a multivariable logistic regression model incorporating gravidity, avidities and antibody levels to domain DBL1 (3D7), DBL1+2 (FCR3), DBL2 (FCR3), DBL3 (FCR3), D6 (FCR3) and ID1 2Da (FCR3), as well as the interaction between avidity and Ab level for the same domains was generated. A backward elimination was used to reach a model with all remaining variables that significantly (at level of 0.05) contributed the model as shown on Table 8. The results from a logistic model and the ROC (receiver operating curve) generated are presented below.

- After adjusting for the avidities to DBL1 (3D7) and DBL2 (FCR3) and Ab level to DBL1 (3D7), the odds ratio of being PM+ is 0.78 (95% CI: 0.61 to 0.99) with 1 increase of gravidity
- After adjusting for gravidity, avidity to DBL2 (FCR3) and Ab level to DBL1 (3D7), the odds ratio of being PM+ is 0.58 (95% CI: 0.36 to 0.93) with 10% increase of avidity to DBL1 (3D7)
- After adjusting for the gravidity, avidity and antibody level to DBL1 (3D7), the odds of being PM+ is 0.56 (95% CI: 0.33 to 0.93) with 10% increase of avidity to DBL2 (FCR3)
- After adjusting for gravidity, avidities to DBL1 (3D7) and DBL2 (FCR3), the odds of being PM+ was doubled i.e., 2.03 (95%CI: 1.16 to 3.55) with 4,784 (interquartile range) MFI increase of Ab level to DBL1 (3D7). The model had the area under the curve of 0.749 i.e., the probability

of correctly distinguishing a PM+ from a PM- subject is 0.749 based on the model. These results show that avidity to DBL1-3D7, DBL2-FCR3 and Ab levels to DBL1-3D7 are associated with the presence of PM.

Table 8. Results from a multivariable logistic regression model predicted presence of PM

	ORs	95% CI	P-value
Gravidity, continuous	0.78	(0.61, 0.99)	<b>0.040</b>
Avidity to DBL1 (3D7)	0.58	(0.36, 0.93)	<b>0.025</b>
Avidity to DBL2 (FCR3)	0.56	(0.33, 0.93)	<b>0.027</b>
Antibody level to DBL1 (3D7)	2.03	(1.16, 3.55)	<b>0.013</b>

## Discussion

In this study, a modest correlation was found between Ab avidity and Ab level to FV2 in Cameroonian women living in a low transmission setting. Looking closely at the relationship between the quantity and quality of Ab among the women with and without PM, Ab avidity increased with MFI in both cases. Observing only a minimal increase in the proportion of high avidity Ab per pregnancy and a significant increase from G1 to G2 suggested that affinity maturation of the Ab produced during G1, hence the significant increase to G2 and subsequent pregnancies. However, the women in the PM- avidity group had an overall higher average avidity. The difference in average avidity observed between the G1 and multigravidae was expected, as the primigravidae have been exposed to VAR2CSA only once and the Ab produced during this primary response are yet to undergo affinity maturation. In addition, during the G2 response, all the Ab to other regular malaria antigens are in the body helps to boost the effect of Abs to VAR2CSA, hence increasing the level of protection in the women. It was also observed that the Ab levels also peaked at G2 and remained almost stationary thereafter as seen for Ab avidity with respect to gravidity.

Studies have reported that IgG Ab to VAR2CSA to be mainly responsible for protection from PM. The mechanisms available in evaluating the effector function of the Ab have not fully been established. None the less, current procedures include phagocytosis (Ataide *et al.*, 2011, Lambert *et al.*, 2014), inhibition of binding (O'Neil-Dune *et al.*, 2001, Fried *et al.*, 1998) and surface binding of Ab to surface of IE (Avril *et al.*, 2010). Our lab has previously reported that the proportion of high avidity Ab to FV2 in PM- was significantly higher compared to PM+ women (Tutterrow *et al.*, 2012) in a high transmission. In our current study, we are reporting the same trend, but this time in a low transmission setting. A 0.9% increase in the average avidity

per pregnancy was significant ( $P=0.0001$ ), but not enough to have a biological effect (Fig. 7). The biggest impact of the proportion of high avidity Ab to FV2 is found in G2 results where PM+ women have not acquired as high a level of immune responses as the women in the PM- group (Fig. 8).

Information on Ab avidity has been described for other pathogens. But the idea of Ab avidity regarding VAR2CSA is still at its dawn, with very limited data available. However, the concept of the importance of Ab avidity for protection had earlier been proposed in malaria in general (Bouharoun-Tayoun *et al.*, 1992) with a limited amount of studies available for regular malaria antigens (Iboson *et al.*, 2012, Reddy *et al.*, 2012, Olotu *et al.*, 2014).

The role of Ab avidity to FV2 in protection is still unclear. In a high transmission setting such as the village, women are exposed to at least 250 infectious mosquito bites per year as compared to only about 12 mosquito bites per year in the city. The question is, does it take multiple bites to develop high quality Ab or just a few bites? Previous studies in our lab reported the average of high avidity Ab to FV2 in the village (MG) (a high transmission setting) at delivery to be 45% (Tutterrow *et al.*, 2012) compared to only 25% in the current study in the city of Yaoundé which is a low transmission setting. These results suggest that the level of exposure to infectious mosquito bites, may have a role to play in the generation of high avidity Ab in pregnant women.

Tutterrow *et al.*, 2012 tried to elucidate if anti-FV2 Ab quality is important in parasite clearance, the proportion of high avidity Ab to FV2 was determined in plasma from women in Ngali II (high transmission setting) and women in Yaoundé (low transmission setting) who were seropositive to FV2. Her data set showed that very few women in Yaoundé developed high

avidity Ab, preventing further analysis. But by comparison, in Ngali II during the first trimester none of the Ab in primigravidae were of high avidity. However, by delivery, about 17.8% of anti-FV2 Ab were found to be high avidity. Furthermore, during the first trimester, about 28.4% of anti-FV2 Ab in multigravidae were high avidity, and this increased to about 40% during the third trimester. The proportion of high avidity Ab was higher throughout pregnancy in multigravidae compared to primigravidae implying the development of high avidity anti-FV2 Ab is gravidity-related. Importantly, they found that proportion of high avidity Ab was significantly higher in PM- compared to PM+ women throughout pregnancy ( $P=0.0009$ ). That is, the proportion of high avidity Ab in PM- women rose from  $34\pm 12\%$  at 3 months to  $42\pm 15\%$  at 5 months, and plateaued at 43% until term. This trend was also seen in the low transmission setting, but for different gravidity groups, and not across a single pregnancy as in Yeung's study. In the current study, the proportion of high avidity antibodies peaked during the second pregnancy and stayed constant thereafter. Although the proportion of high avidity Ab was generally higher in multigravid compared to primigravid women, some multigravidae still had placental malaria in both the low and high transmission setting, suggesting high avidity Ab may be needed early in pregnancy for parasite clearance by delivery or subsequent pregnancies. The sample size representing the low transmission setting (Yaoundé) in Tutterrow's study was limiting ( $n=25$ ), so valuable comparison could not be done with respect to the two sites. It is based on these findings that we thought it will be important to study a low transmission setting and see the patterns of antibodies produced across different pregnancies.

To further explore the specificity of Ab avidity with respect different VAR2CSA strains and their domains in our current study, we found in this study that FCR3 seemed to be the most immunogenic compared to its 3D7 and 7G8 counterparts (Fig. 5). Focusing on the FCR3-FV2

and its corresponding DBL domains, our study reports the N-terminal domain (DBL1-3D7, DBL2, DBL3 and ID1-2a) of the FV2 molecule as having an important role in the generation of the quality of Ab they produce (Table 6). This corroborates the findings of Dahlback and his collaborators (2011) recognizing the N-terminus of FV2 molecule as the most immunogenic. Dechavanne *et al.* (2015) in their Senegalese cohort study recently suggested DBL1X-3X region as having an important role in the development of humoral response in high avidity CSA-binding of VAR2CSA. DBL5 tend to be the most immunogenic, producing high quality Abs irrespective of the PM malaria status. This could be due to the presence of cross-reactive Ab in plasma (Avril *et al.*, 2010) suggesting that FCR3-DBL5 may not play an important role in PM.

This study was done with samples collected at delivery, it would have been better to do a longitudinal study in the course of different pregnancies to assess the variation of Ab avidity in the course of pregnancy at different trimesters and to better classify women into specific groups based on their outcome of their clinical history. The study focuses on the role of Ab quality in protection, but doesn't explain how this protection comes about. Functional assays such as inhibition of binding of IE and opsonic phagocytosis are necessary to elucidate the mechanism of protection.

The prevalence of women in the study population with Ab to FV2 was 16.5%. The cut-off value for avidity used in this study was 35%, which was the value used for the previous study in a high transmission setting. In that study, the average %high avidity was 45% while it was 25% for this study. Therefore, applying 35% as a cut-off for a low transmission setting may be too stringent or a little bit unfair leading to an underestimation of the proportion of the population of the population that has high avidity antibodies. The value of 16.5% could be higher

than the actual value if a cut-off value less 35% high avidity previously used for the high transmission setting.

The fact that there is no association between high avidity Ab and gravidity in primigravidae in the high and low transmission settings respectively, is not surprising as it is a first time exposure, and these Ab have not yet undergone affinity maturation.

In secundigravid women in a high transmission setting, there was a consistent increase in percent high avidity with gestation, with all of the women having at least 35% high avidity or more from the 30<sup>th</sup> week of gestation. This is probably because these women had had a previous exposure before during their first pregnancy, so during a second exposure, the response is very rapid and increases with increase in weeks of gestation during pregnancy.

Unlike in primi- and secundigravidae, the multigravida women had an average avidity of about 40% which is higher implying that affinity maturation of the B-cells had probably occurred and reached a plateau, with no change in avidity with gestation and everyone generally being high in a high transmission setting.

Generally, the average avidity was significantly higher in the village across all gravidity groups compared to the city ( $P < 0.0001$ ). This was expected as women are exposed to multiple infectious bites (at least 1 infectious bite per night) as opposed to the city where women are exposed to just one infectious bite per month. Continuous exposure allows for the affinity maturation of B-cells to occur. It should be noted that only one study till date has been able to report the relationship between avidity as a correlate of placental malaria in pregnancy, which was done in a high transmission setting, and now this one in a low transmission. The average percent of high avidity in each of the gravidity groups was generally higher in the high

transmission setting compared to the low transmission setting;  $P < 0.0003$  for primigravidae,  $P < 0.0001$  for secundigravidae and  $P < 0.0004$  for multigravidae. This observation was quite opposing to the notion that in the presence of high antigen concentration (case of high transmission setting) the antigen is in excess, while the Ab concentration is limiting, hence there will be less completion for the antigen by the Ab, the outcome being Ab with less affinity for the antigen. On the contrary, in a case where the antigen is limiting such as a low transmission setting, there will be a high competition of the Ab for the antigen, the results being the production of Ab with relatively high avidity. This also corroborates Tutterrow's observation that transmission force influences the acquisition of Ab (Tutterrow *et al.*, 2012) to VAR2CSA.

Based on the univariate analysis, because a correlation was found between MFI and avidity, which differed by malaria status, multivariate analyses was done. The odds of PM+ is 0.78 which was less than 1, implying a lesser chance of being PM+ .i.e. a lesser likelihood of being PM+. In summary, antibody avidity to DBL1 (3D7) and DBL2 (FCR3) as well as antibody levels to DBL1 (3D7) are associated with the absence of placental malaria in pregnant women in a low transmission setting.

## **Conclusion**

We have observed the relationship between high avidity Ab and Ab levels to VAR2CSA in different pregnancies in a low transmission setting. In response to the study questions we raised in our specific aims, we show that-

- a) *Do high avidity antibodies to FV2 increase with gravidity in women living in Yaoundé, a low malaria transmission setting?*

- Ab quality increased minimally with gravidity, with a major difference between PM+ and PM- women in the G2 group. Affinity maturation peaked at G2 and tended to be almost stable with subsequent pregnancies. The average percent high avidity Ab to FV2 in multigravidae in the city of Yaoundé is 25%, compared to 45% previously reported for women in the village.
- The Ab specificity of the different DBL domains suggests that the N-terminal domain of the FV2 molecule may have a role in the production of high avidity Ab.

*b) Is there an association between Ab avidity and the presence or absence of PM in women living in a low transmission setting?*

- Yes, there was a weak association, with the presence or absence of PM for FV2. However, there was a strong association with the DBL5 domain of the FV2 Molecule and the N-terminal domain of the molecule was found to be associated with the absence of PM and high avidity Ab.

*c) Are high avidity Ab to the full-length VAR2CSA directed against a specific region of the molecule, i.e., one DBL domain, or multiple domains?*

- Yes, our data suggest that DBL1-3D7, DBL2-FCR3, DBL3-FCR3, and ID1-2a had significantly higher avidity Ab in the PM- women when compared to the PM+ women. In general, a modest correlation between high avidity Ab to FV2 and Ab levels.

### **Significance of the study**

If VAR2CSA, or any of its domains has to be used as a vaccine, it may be absolutely necessary to know if the vaccine will be implemented in all regions affected by malaria, regardless of whether it is in a low or a high transmission setting. In addition, it will also be necessary to know

if it is the whole molecule that will be included in the vaccine, or only some parts of the molecule i.e., subunit vaccine. To tackle these issues, the use of important correlates of protection such as Ab avidity to make some of the required decisions involved in evaluating the efficacy of vaccine candidate, VAR2CSA has to come into play. Our data suggest that Ab avidity can serve as an important correlate of protection, hence can be considered an important marker for the assessment of the efficacy of VAR2CSA vaccine candidate, and that the N-terminal part of the molecule may be more associated with the absence of PM in a low transmission setting.

### **Future perspectives**

This study leads to important questions that need to be addressed in future. It will be interesting to look at the avidity of regular malaria antigens such as MSP1, 2, 3 and AMA1 to these women and compare the patterns to that of FV2. Our study could not be used to determine the association between avidity and age, as pregnant women have a limited age range (16-45 years). In addition, very few studies are currently available that have looked at the association between avidity and age in malaria endemic areas. So we hope to investigate the relationship between antibody avidity and age using plasma samples from individuals aged 5 to 85 years of age living in Simbok, Cameroon.

## References

1. Ampomah P, Stevenson L, Ofori MF, Barfod L, and Hviid L. B-cell responses to pregnancy-restricted and -unrestricted *Plasmodium falciparum* erythrocyte membrane protein 1 antigens in Ghanaian women naturally exposed to malaria parasites: *Infection and Immunity*. 2014, **82**:1860-1871.
2. Andersen P, Nielsen MA, Resende M, Rask TS, Dahlbäck M, Theander T, Lund O, and Salantiet A. Structural Insight into Epitopes in the Pregnancy-Associated Malaria Protein VAR2CSA: *PLoS Pathogens*. 2008, **4**:1-8.
3. Barfod L, Bernasconi NL, Dahlbäck M, Jarrossay D, Andersen PH, A. Salanti A, Ofori MF, Turner L, Resende M, Nielsen MA, Theander TG, Sallusto F, Lanzavecchia A, Hviid L. Human pregnancy-associated malaria-specific B cells target polymorphic, conformational epitopes in VAR2CSA: *Molecular Microbiology*. 2007, **63**:335-347.
4. Barfod L, Dobrilovic T, Magistrado P, Khunrae P, Viwami F, Bruun FJ, Dahlbäck M, Bernasconi NL, M. Fried M, John D, P. E. Duffy PE, Salanti A, Lanzavecchia A, Lim CT, Ndam NT, Higgins MK, and Hviid L. Chondroitin Sulfate A-Adhering *Plasmodium falciparum*-Infected Erythrocytes Express Functionally Important Antibody Epitopes Shared by Multiple Variants: *The Journal of Immunology*. 2010, **185**:7553-7561.
5. Beeson JG, Rogerson SJ, Cook BM, Reeder JC, Chai W, Lawson AM, M. E. Molyneux ME, Brown GV. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria: *Nature Medicine*. 2000, **6**:86-90.
6. Benoît G, Smith JD, Viebig NK, Gysin J, and Scherf A. Pregnancy-associated malaria: Parasite binding, natural immunity and vaccine development: *International Journal for Parasitol*. 2007, **37**:273-283.

7. Bockhorst J, Lu F, Janes JH, Keebler J, Gamain JB, Awadalla P, Su XZ, Samudrala R, Jovic N, and Smith JD. Structural polymorphism and diversifying selection on the pregnancy malaria vaccine candidate VAR2CSA: *Molecular Biochemistry and Parasitology* 2007, **155**:103-112.
8. Brabin BJ, Premji Z, Verhoeff F. An analysis of anemia and child mortality: *J Nutr.* 2001, **131**(2S-2):636S-645S.
9. Brabina BJ, Romagosab C, Abdelgalila S, Mene´ndezc C, F. H. Verhoeffa FH, R. McGreadye R, Fletcherera KA, Owensa S, Alessandroh U, Nostene F, Fischerj PR, and Ordib J. The Sick Placenta-The Role of Malaria: *Placenta.* 2004, **25**:359-78.
10. Brolin KJ, Kristina EM, Persson KE, Rogerson SJ, and Chen Q. Differential Recognition of *Plasmodium falciparum* VAR2CSA Domains by Naturally Acquired Antibodies in Pregnant Women from a Malaria Endemic Area: *PLoS One.* 2010, **5**:1-8.
11. Cancro MP, Hao Y, Scholz JL, Riley RL, Frasca D, Dunn-Walters DK, and Blomberg BB. B cells and aging: molecules and mechanisms: *Trends in Immunology.* 2009, **30**:313-318.
12. Celada F, and Seiden PE. Affinity maturation and hypermutation in a simulation of the humoral immune response: *European Journal of Immunology.* 1996, **26**:1350-1358.
13. Childs LM, Baskerville EB, and Cobey S. Trade-offs in antibody repertoires to complex antigens. *Philos Trans R Soc Lond B Biol Sci.* 2015, **370**:1-10.
14. Dechavanne S, Srivastava A, Gangnard S, Nunes-Silva S, Dechavanne C, Fievet N, Deloron P, Chêne A, Benoit G. Parity-dependent recognition of DBL1X-3X suggests an important role of the VAR2CSA high-affinity CSA-binding region in the development of the humoral response against placental malaria: *Infect Immun.* 2015, **83**:2466-2474.
15. Duffy MF, Maier AG, Byrne TJ, Marty AJ, S. R. Elliott SR, M. T. O’Neill MT, P. D. Payne PD, Rogerson SJ, Cowman AF, Crabb BS, Brownet GV. VAR2CSA is the principal ligand for

- chondroitin sulfate A in two allogeneic isolates of *Plasmodium falciparum*: *Molecular and Biochemical Parasitology*. 2006, **148**:117-124.
16. Duffy PE and Fried M. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns: *Infection and Immunity*. 2003, **71**:6620-6623.
17. Elias SC, Choudhary P, Cassan SC, Biswas S, Collins KA, Halstead FD, Bliss CM, Ewer KJ, Hodgson SH, Duncan CJ, Hill AV, Draper SJ. Analysis of human B-cell responses following ChAd63-MVA MSP1 and AMA1 immunization and controlled malaria infection. *Immunology*. 2014, **141**:628-644.
18. Feng G, Simpson JA, Chaluluka E, Molyneux ME, Rogerson SJ. Decreasing burden of malaria in pregnancy in malawian women and its relationship to use of intermittent preventive therapy or bed nets: *PLoS One*. 2010, **5**:1-7.
19. Feng G, Aitken E, Yosaatmadja F, Kalilani L, S. R. Meshnick SR, Jaworowski A, Simpson JA, and Rogerson SJ. Antibodies to variant surface antigens of *Plasmodium falciparum* infected erythrocytes associated with protection from treatment failure and development of anemia in pregnancy: *Journal Infectious Diseases*. 2009, **200**:299-306.
20. Fouda GG, Leke RF, Long C, Druilhe P, Zhou A, Taylor DW, Johnson AH. Multiplex assay for simultaneous measurement of antibodies to multiple *Plasmodium falciparum* antigens: *Clin Vaccine Immunol*. 2006, **13**:1307-1313.
21. Frasca D, Landin AM, Riley RL and Blomberg BB. Mechanisms for decreased function of B cells in aged mice and humans: *Journal of Immunology*. 2008, **180**:2741-2746.
22. Frasca D, Landin AM, Riley RL and Blomberg BB. Mechanisms for decreased function of B cells in aged mice and humans: *Journal of Immunology*. 2008, **180**:2741-2746.

23. Fried M, Domingo GJ, Gowda CD, Mutabingwa TK, Duffy PE. *Plasmodium falciparum*: Chondroitin sulfate A is the major receptor for adhesion of parasitized erythrocytes in the placenta: *Experimental Parasitology*. 2006, **113**:36-42.
24. Gutman J, Mwandama D, Wiegand RE, Abdallah J, Iriemenam NC, Shi YP, Mathanga DP, Skarbinski JV. In vivo efficacy of sulphadoxine-pyrimethamine for the treatment of asymptomatic parasitaemia in pregnant women in Machinga District, Malawi: *Malaria Journal*. 2015, **14**:1-9.
25. Harrington WE, Morrison R, Fried D, and Duffy PE. Intermittent preventive treatment in pregnant women is associated with increased risk of severe malaria in their offspring: *PLoS One* 2013, **8**:1-6.
26. Harrington WE, Mutabingwa TK, Kabyemela E, Fried M, and Duffy PE. Intermittent treatment to prevent pregnancy malaria does not confer benefit in an area of widespread drug resistance: *Clinical Infectious Diseases*. 2011, **53**:224-230.
27. Khunrae P, Dahlbäck M, Nielsen MA, Andersen G, Ditlev SB, Resende M, V. V. Pinto VV, Theander TG, Higgins MK, and Salanti A. Full-Length Recombinant *Plasmodium falciparum* VAR2CSA Binds Specifically to CSPG and Induces Potent Parasite Adhesion-Blocking Antibodies: *Journal of Molecular Biology*. 2010, **397**:826-834.
28. Leke RGF and Taylor DW. The use of intermittent preventive treatment with sulfadoxine-pyrimethamine for preventing malaria in pregnant women: *Clinical Infectious Diseases*. 2011, **53**:231-233.
29. Mace KE, Chalwe V, Katalenich BL, Nambozi M, Mubikayi ML, Mulele CK, Wiegand RE, Filler SJ, Kamuliwo M, Craig AS, Tan KR. . Evaluation of sulphadoxine-pyrimethamine for

- intermittent preventive treatment of malaria in pregnancy: a retrospective birth outcomes study in Mansa, Zambia: *Malaria Journal*. 2015, **14**:1-9.
30. Matondo SI, Temba GS, Kavishe AA, Kauki JS, Kalinga A, Zwetselaar M, Reyburn H, Kavishe RA. High levels of sulphadoxine-pyrimethamine resistance Pfdhfr-Pfdhps quintuple mutations: a cross sectional survey of six regions in Tanzania: *Malaria Journal*. 2014, **152**:1-8.
31. Menéndez C, Bardají A, Sigauque B, Sanz S, Aponte JJ, Mabunda S, and Alonso PL. Malaria prevention with IPTp during pregnancy reduces neonatal mortality: *PLoS One*. 2010, **5**:1-6.
32. Nahrendorf W, Scholzen A, Bijker EM, Teirlinck AC, Bastiaens GJ, Schats R, Hermsen CC, Visser LG, Langhorne J, Sauerwein RW. Memory B-cell and antibody responses induced by *Plasmodium falciparum* sporozoite immunization: *Journal of Infectious Diseases*. 2014, **210**:1981-1990.
33. Ndam NT, Denoed-Ndam L, Doritchamou J, Viwami F, Fievet N, Massougbodji A, Luty J, Deloron P.  
Protective antibodies against placental malaria and poor outcomes during pregnancy, Benin: *Emerging Infectious Diseases*. 2015, **21**:813-23.
34. Oleinikov VA, Rossnagle E, Francis S, Mutabingwa TK, Fried M, and Duffy PE. Effects of sex, parity, and sequence variation on seroreactivity to candidate pregnancy malaria vaccine antigens: *The Journal of Infectious Diseases*. 2007, **196**:155-164.
35. O'Neil-Dunne I, Achur RN, Agbor-Enoh ST, Valiyaveetil M, Valiyaveetil M, Naik RS, Ockenhouse CF, Zhou A, Megnekou R, Leke R, Taylor DW, Gowda DC . Gravidity-dependent production of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy: *Infection and Immunity*. 2001, **69**:7487-7492.

36. Salanti A, Dahlbäck M, Turner L, Nielsen MA, L. Barfod L, Magistrado P, A. Jensen ATR, Lavstsen T, Ofori F, Hviid L and Theander TG,. Evidence for the Involvement of VAR2CSA in Pregnancy-associated Malaria: *Journal of Experimental Medicine*. 2004, **200**:1197-1203.
37. Saveria T, Oleinikov AV, Wiliamson K, Chaturvedi R, Lograsso J, Keitany GJ, Fried M, Duffy PE. 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: *Infection and Immunity*. 2013, **81**:1031-1039.
38. Staalsoe T, Megnekou R, Fievét N, Ricke CH, Zornig HD, Leke R, D. W. Taylor DW, Deloron P, and Hviid L. Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes that protect against placental parasitemia: *The Journal of Infectious Diseases*. 2001, **184**:618-626.
39. Tagbor H, Cairns M, Bojang K, Coulibaly SO, Kayentao K, Williams J and Greenwood B. A non-inferiority, individually randomized trial of intermittent screening and treatment versus intermittent preventive treatment in the control of malaria in pregnancy: *PLoS One*. 2015, **10**:1-17.
40. Tako EA, Zhou A, Lohoue J, Leke R, Taylor DW, and Leke RF. Risk factors for placental malaria and its effect on pregnancy outcome in Yaoundé, Cameroon: *Am J Trop Med Hyg*. 2005, **72**:236-242.
41. Taylor DW, Zhou A, Marsillio LE, Thuita LW, Leke EB, Branch O, Long C, and Leke RFG. Antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A and to the C terminus of merozoite surface protein 1 correlate with reduced placental malaria in Cameroonian women: *Infection and Immunity*. 2004, **72**:1603-1607.

42. Tiono AB, Ouedraogo A, Bougouma EC, Diarra A, Konaté AT, Nébié L, Sirima SB. Placental malaria and low birth weight in pregnant women living in a rural area of Burkina Faso following the use of three preventive treatment regimens: *Malaria Journal*. 2009, **224**:1-8.
43. Tonga C, Kimbi HK, Anchang-Kimbi JK, Nyabeyeu HN, Z. B. Bissemou ZB, and Lehman LG. Malaria risk factors in women on intermittent preventive treatment at delivery and their effects on pregnancy outcome in Sanaga-Maritime, Cameroon: *PLoS One*. 2013, **8**:1-11.
44. Travassos MA, Coulibaly D, Bailey JA, Niangaly A, Adams M, Nyunt MM, Ouattara A, Lyke KE, Laurens MB, Pablo J, Jasinskas A, Nakajima R, Berry AA, Takala-Harrison S, Kone AK, Kouriba B, Row JA, Doumbo OK, Thera MA, Laufer MK, Felgner PL, Plowe CV. Differential recognition of terminal extracellular *Plasmodium falciparum* VAR2CSA domains by sera from multigravid, malaria-exposed Malian women: *American Journal of Tropical Medicine Hygiene*. 2015, **92**:1190-1194.
45. Tutterrow YL, Avril M, Singh K, Long CA, Leke RJ, Sama G, Salanti A, Smith JD, Leke RG, Taylor DW. 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: *Infection and Immunity*. 2012, **80**:1479-1490.
46. Tutterrow YL, Avril M, Singh K, Long CA, Leke RJ, Sama G, Salanti A, Smith JD, Leke RG, Taylor DW. 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**:1479-1490.
47. Tutterrow YL, Salanti A, Avril M, Smith JD, I. S. Pagano IS, S. Ako, J. Fogako J, Leke RGF, and Taylor DW. High Avidity Antibodies to Full-Length VAR2CSA Correlate with Absence of Placental Malaria. *PLoS One*. 2012, **7**:1-8.

48. Wipasa J, Suphavitai, Okell LC, Cook J, Corran PH, Thaikla K, Liewsaree W, Riley EM, Hafalla JC. Long-lived antibody and B-Cell memory responses to the human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*: *PLoS Pathogens*. 2010, **6**:1-15.
49. World Health Organisation. Updated WHO policy recommendation: intermittent preventive treatment of malaria in pregnancy using sulfadoxine-pyrimethamine (IPTp-SP). Geneva: World Health Organisation; 2012.
50. Zakeri S, Babaeekhou L, Mehrizi AA, Abbasi M, Abassi M, and Djadid. Antibody responses and avidity of naturally acquired anti-*Plasmodium vivax* Duffy binding protein (PvDBP) antibodies in individuals from an area with unstable malaria transmission: *American Journal of Tropical Medicine and Hygiene*. 2011, **84**:944-950.