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RESEARCH ARTICLE

The Quantity, Quality and Two Major Effector Functions of Antibodies to VAR2CSA and their Association with Pregnancy Outcomes in a Low Malaria Transmission Area

Yukie M. Lloyd[1], Naveen Bobbili[1], Ali Salanti[2], Philomina Gwanmesia[3], Josephine Fogako[3], Rose F.G. Leke[3], and Diane W. Taylor[1]*

[1] Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A. Burns School of Medicine, University of Hawaii at Manoa, Hawaii, USA
[2] Centre for Medical Parasitology, Department of Immunology and Microbiology, University of Copenhagen, Denmark; Department of Infectious Diseases, Copenhagen University Hospital, Denmark
[3] Faculty of Medicine and Biomedical Sciences, Biotechnology Centre, University of Yaoundé I, Yaoundé, Cameroon

*Corresponding author: dwtaylor@hawaii.edu

ABSTRACT

Background: Women produce antibodies to VAR2CSA when infected with *Plasmodium falciparum* during pregnancy that reduce disease severity in the current and subsequent pregnancies. In addition to antibody quantity, antibody quality (e.g., avidity) and function (e.g., inhibition of binding and opsonic phagocytosis) are immunologically important. Studies comparing the quantity, avidity and effector mechanisms of antibodies to VAR2CSA in the same group of women with pregnancy outcomes, especially in low transmission areas, are limited.

Aims: The purpose of this study was to characterize antibodies to VAR2CSA using four assays, determine the correlation among the assays, and relate this to pregnancy outcome.

Methods: A panel of 310 plasma samples from women in Yaoundé (a city with low malaria transmission) who had antibodies to full-length VAR2CSA were screened in assays that measured i) level of antibodies to VAR2CSA, ii) antibody avidity, iii) reduction in binding (RiB) of fluorescent VAR2CSA-coupled beads to fluorescent-CSA-coupled beads, and iv) opsonic phagocytosis using VAR2CSA-coupled beads and human THP1 cells. Results from the assays were compared with clinical information from 614 women who were Ab-negative.

Results: A modest association was found among the 4 assays, i.e., as the amount of antibodies increased, a small increase in avidity, RiB and phagocytosis was observed; however, the association between RiB and avidity was poor. When results were dichotomized to above and below the median, antibody avidity, but not antibodies in the other assays, was associated with a significant reduction in prevalence of placental malaria and lower placental parasitemia. However, women who had antibody values above the median in amount (p=0.03), avidity (p=0.006), reduction in binding (p=0.018) and probably phagocytosis (p=0.065) had significantly lower placental parasitemia than women who lacked Abs to VAR2CSA.

Conclusions: In this urban setting, women with the highest (above the median) antibody levels in the four assays had a lower prevalence of placental malaria and placental parasitemia than women who lacked anti-VAR2CSA antibodies. Thus, VAR2CA-based vaccine trials in low transmission areas should consider using all four assays before and after vaccination.

Introduction

Plasmodium falciparum infections during pregnancy can have a severe effect on both the mother and developing fetus. In malaria endemic areas, individuals acquire immunity to malaria early in life after repeated infections; however, in pregnant women *P. falciparum* parasites express a unique ligand called VAR2CSA on the surface of infected erythrocytes (IE) that mediates binding to chondroitin sulfate A (CSA) on trophoblasts lining the intervillous space (IVS).[1,2] As a result, IE accumulate in the IVS causing placental malaria (PM), a condition characterized by inflammation and dysregulation of the chemokine balance.[3–5] This makes pregnant women at an increased risk of maternal anaemia, spontaneous abortions, premature delivery and giving birth to low birthweight (LBW) babies.[5–7] In areas of high malaria transmission, women become infected multiple times during pregnancy and generally develop antibodies (Abs) to VAR2CSA. As a result, after several pregnancies, substantial immunity is obtained against PM that reduces the severity of disease and improves pregnancy outcomes (reviewed in Ref. 7).[8–12] In low transmission areas; however, women may be infected only a few times during pregnancy and many women fail to seroconvert to VAR2CSA. For example, in the urban city of Yaoundé, Cameroon, approximately half of the women, who were infected during the first six months of pregnancy, were both Ab-negative and placental-malaria negative at delivery.[13] Thus, Abs to other malarial antigens, in conjunction with Abs to VAR2CSA, contribute to immunity.[9,14]

Many studies have measured the amount of Abs to VAR2CSA by ELISA or Luminex-based multiplex assays. In addition, studies have measured antibody (Ab) avidity, inhibition of binding (IoB) of IE to CSA, and opsonic phagocytosis of VAR2CSA-expressing IE and found associations between Abs to VAR2CSA and reduced prevalence of PM and improved pregnancy outcomes. For example, under high transmission conditions, the presence of ≥35% high avidity Abs at 5-6 months of pregnancy was associated with absence of PM at delivery.[13] A number of studies have found an association of IoB activity and improved pregnancy outcomes, e.g., longer length of gestation, higher birthweight, and reduced risk of premature deliveries and LBW babies.[8,11,12,15] Opsonic phagocytosis of VAR2CSA-expressing IE has also been associated with clearance of placental-type IE and reduced maternal anemia.[12,16–18] Most of the above studies were conducted in areas in Africa where pregnant women receive multiple infections per pregnancy. To our knowledge, no study has directly compared the amount, quality, avidity, IoB and opsonic-phagocytosis activity of anti-VAR2CSA Abs in African women who receive only a few infections per pregnancy and compared the results with pregnancy outcomes.

Traditional assays for measuring IoB and opsonic phagocytosis use *ex vivo* models with biological reagents and are technically challenging. As a result, data are subject to variability from day-day-to-day variation and between laboratories. Recently, flow-cytometry-based assays using non-biological reagents have been developed, two of which were used in this study. To measure Ab-mediated reduction of VAR2CSA-binding to CSA, fluorescent-yellow beads were coupled to VAR2CSA, incubated with plasma, combined with CSA-coupled Nile blue beads, and the percentage reduction in binding (RiB) was determined by flow cytometry. Several flow-cytometry-based assays have been developed for opsonic phagocytosis.[19–21] The assay used in this study employed full-length VAR2CSA-coupled to fluorescent beads that were treated with plasma, combined with THP-1 monocytes, and percent phagocytosis was determined fluorometrically.[21]

The current study used plasma and clinical information from women living in Yaounda, Cameroon, where the estimated infection rate was approximately 1 infectious bite per month.[22] Based on prior studies, 310 women who had Abs to VAR2CSA were identified and their plasma screened in serological assays for anti-VAR2CSA Abs that measured i) levels (amount), ii) Ab avidity, iii) reduction in VAR2CSA binding to CSA, and, iii) Ab-negative mediated phagocytosis. First, assay results were compared with each other. Then, assay results were tested to determine if having Ab values above the median in each of the assays was associated with absence of PM at delivery. Finally, presence/absence of PM and placental parasitemia were compared between women with above median Ab activity in each of the assays with 614 Ab- women. Results showed that the presence of high avidity Abs to VAR2CSA was associated with lower prevalence of PM and that placental parasitemia were significantly lower in mothers with high amounts of Ab, avidity, RiB, and probably opsonic-phagocytosis than Ab-negative women. These results contribute to our understanding of the role of Abs to VAR2CSA in immunity to PM in urban areas.

Methods

Ethical approval The original study was conducted between 1996-2001 in Yaoundé, Cameroon. Clinical information and plasma samples were obtained with the approval of the National Ethics Committee, Cameroon and the Institutional Review

Board of Georgetown University (1994-158). All participants gave written informed consent and all aspects of the study were conducted according to all relevant guidelines and regulations. The use of the deidentified, archival information and samples in the current study was approved by the Committee on Human Subjects, University of Hawai'i (2017-00059).

Study sites and populations The archival plasma samples were collected at delivery from women who lived in Yaoundé, an area with relatively low *P. falciparum* transmission. A standardized questionnaire was used to collect information on demographics of the women, their clinical histories, and the birth outcome. Detailed information on the original study has been previously described.[23] The advantage of using clinical material obtained prior to 2004 is that the women were recruited before implementation of intermittent preventive treatment (IPT); therefore, the women developed natural immunity to placental malaria that helped clear their placental infections. Post-IPT, placental parasitemia were either prevented or eliminated by sulfadoxine– pyrimethamine treatment, making it impossible to assess the role of Abs to VAR2CSA in naturally-acquired immunity. For the serological assays, plasma from sympatric males and nulligravidae were used as negative controls.

Diagnosis of malaria Thick and thin blood smears of peripheral and placental blood were prepared, stained with Diff-Quick (Polysciences, Warrington, PA) and examined for malaria IE. Hematocrits were measured by transferring an aliquot of maternal blood to heparinized micro-hematocrit tubes and centrifugation. Whole blood was centrifuged at 400 x *g* for 10 minutes; plasma was isolated and stored in -80°C until used. Placental biopsies were used to make impression smears and were examined for IE or fixed in buffered formalin for histology. A woman was identified as PM-positive (PM+) if IE were detected in the placental blood smear, impression smear, or histological section; absence of parasites in both peripheral blood and placenta were considered PM-negative (PM-). Women who had IE in peripheral blood, but not in placenta were excluded from the study.

Sample selection Previously, plasma from 1,377 women in the original study were screened for Abs to VAR2CSA using a Luminex multiplex bead-based assay.[9,14] Based on median fluorescence intensity (MFI) units, women were divided into 3 categories: i) Ab+ women (values above the cut-off for positivity (3,709 MFI) that was the mean + 2SD of sympatric males and nulligravidae), ii) women with borderline values near the cut-off (1,500-3,709 MFI); and iii) Ab- women (below 1,500 MFI). All Ab+ women with adequate remaining plasma were selected (n=310). Information from Ab-negative women (n=614) (i.e., those with <1,500 MFI) was used for comparison in data analysis.

IgG antibody levels to full-length VAR2CSA (FV2) The multiplex immunoassay used to determine IgG Abs levels to VAR2CSA has been described previously.[13,24] In brief, saturating amounts of recombinant FV2 (FCR3 line) was covalently-coupled to SeroMAP beads using the method provided by the manufacturer. Fifty microliters containing 2,000 FV2-coupled beads were combined with 50µl of plasma diluted 1:200 in PBS-1% BSA (final plasma dilution 1:400) for one hour on a horizontal shaker. Then, beads were washed twice with PBS with 0.05% Tween (PBS-Tween) and once with PBS with 1% BSA (PBS-1% BSA). Beads were then incubated with 100µl of 2µg/ml R-phycoerythrin-conjugated goat anti-human IgG (Jackson ImmunoResearch) for 1 hour, washed twice, and the level of fluorescence was measured using a LiquiChip 200 analyzer (Luminex Corp.). Results were expressed in median fluorescence intensity (MFI) units.

Percentage of high avidity IgG to VAR2CSA To conform with earlier studies, high avidity Abs were defined as those that remained bound to FV2 after treatment for 30 minutes with 3M NH_4SCN.[13,25] The protocol has been detailed previously.[13] In brief, 2,500 VAR2CSA-coupled beads were incubated with 50 µl of plasma diluted 1:300 with PBS-BSA for 1 hr. After washing twice with PBS-tween and once with PBS-BSA, bead-Ab complexes were incubated for 30 min with 100µl of PBS or 3M NH_4SCN. Following another washing step, beads were incubated with 100µl of 2µg/ml R-phycoerythrin-conjugated goat anti-human IgG for one hour, washed and MFI was measured on LiquiChip 200 analyzer. Percentage of high avidity Ab was calculated by [MFI of beads incubated with NH_4SCN] / [MFI of beads incubated with PBS] x100.[13]

Preparation of fluorescent beads for the reduction in binding and phagocytosis assays FV2 and an irrelevant antigen (keyhole limpet hemocyanin - KLH) were coupled to 5.0-5.9µm carboxylated, yellow fluorescent beads (CFL-5052-2; Spherotech) and CSA (#C9818, Sigma) was coupled to smaller (0.95µm) carboxylated, Nile Blue (NB) fluorescent beads (AFP-0865-2, Spherotech) as follows. Beads were resuspended by vortexing and sonicating for 20 seconds before each step unless indicated

otherwise. Beads were transferred to a low-adhesion microcentrifuge tube, pelleted and resuspended in 100µl of ddH_2O. Beads were again pelleted and the supernatant was replaced with 80µl of activation buffer (0.1M NaH_2PO_4, pH 6.2). Freshly-prepared Sulfo-N-hyroxysulfosuc-cinimide (Sulfo-NHS) and 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce) solutions were added to the tube, gently mixed and incubated for 20 minutes in the dark, with gentle mixing every 10 minutes. Activated beads were pelleted, supernatant was removed and beads were resuspended in 100µl of coupling buffer (0.05M MES buffer, pH 7.0). After two washes with the coupling buffer, beads were resuspended with the coupling buffer. One million yellow beads were incubated with 200ng of FV2 or KLH for the Ab-mediated phagocytosis assays, and 1µg for FV2-CSA binding assays. After gentle vortexing, the coupling reaction was incubated at 4°C overnight on a horizontal shaker. Following incubation, coupled beads were pelleted and supernatant was replaced with 1 ml of blocking/storage buffer (50 ml PBS-1%BSA, 10µl Tween 20, 25mg or 125µl of a 20% solution sodium azide). After two washes, coupled beads were finally resuspended in blocking/storage buffer at 25,000 beads /µl and stored at 4°C until used.

Flow-cytometry-based phagocytosis assay Detailed steps of the assay have been published previously.[21] In brief, 100,000 VAR2CSA-coupled beads were incubated for 1 hour with 30µl of diluted, heat-inactivated plasma in a foil-sealed, 96-well V-bottom plate (Corning). Following two washes with PBS, beads were incubated with 50µl of 2 x 10^4 THP-1 cells, to reach a beads-to-monocyte ratio of 5:1, for 45 minutes in a 37°C incubator (5% v/v CO_2). After incubation, the 96-well plate was placed on ice and chilled PBS was added to each well to stop phagocytosis. Samples were immediately read on Attune®NxT Flow Cytometer (Fisher Scientific). THP-1 cells were gated by size and granularity on FSC and SSC plots.[21] Data for at least 5,000 THP-1 cells were recorded for each sample and were analyzed using the FlowJo (version 10.6.0) software. Percent phagocytosis was the proportion of THP-1 cells that were positive for fluorescence in the BL-1 channel.

Flow-cytometry-based assay for antibody-mediated reduction in binding of VAR2CSA to CSA. First, 50,000 fluorescent yellow FV2-coupled beads were incubated for 1 hour with 20µl of heat-inactivated plasma diluted 1:10 in a foil-sealed, 96-well V-bottom microtiter plate (Corning). Following one wash with PBS-tween and one wash with PBS, beads were incubated with fluorescent Nile-blue CSA-coupled beads at a ratio of 1:10 for 30 minutes in a 37°C incubator. After incubation, PBS was added to each well and samples were immediately read on Attune®NxT Flow Cytometer (Fisher Scientific). Single FV2-beads were gated by size and granularity on FSC and SSC plots (Fig. 1a, b). Data for at least 10,000 FV2-beads were recorded for each sample and were analyzed using the FlowJo (version 10.6.0) software. The percentage of yellow FV2-beads bound to Nile Blue-CSA-beads (FV2+CSA complexes) was determined by the proportion of FV2-beads that were positive for fluorescence in the RL-1 (i.e., far red) channel (Fig. 1c). Percent RiB was determined by comparing samples treated with test plasma with beads treated with plasma from Cameroonian males and nulligravidae and subtracting from 100. Representative results from beads treated with PBS (Fig. 1 D) and plasma from a multigravida with high Ab levels to FV2 (Fig. 1E) is provided.

Statistical analysis Characteristics of the women and newborns (age, gravidity, primigravidae, length of gestation, birthweight, hematocrit) were described in mean and standard deviations (SD) or median and 25% Interquartile ranges for continuous variable and percentages for categorical variables. Based on the Kolmogorov-Smirnov test, differences between groups (e.g., PM+ and PM-) were compared by paired t-test or Mann-Whitney. Simple linear regression correlation coefficients were used to examine associations between the different assays. Median and interquartile ranges were reported for parasitemia. All statistical analyses were performed on Microsoft Excel and Prism version 7.04 (Graph Pad Software Inc.). Women who had less than 30% hematocrits were classified as anemic, singletons weighing <2,500 gm were considered LBW, detection of IE in the IVS was defined as PM+. P-values less than 0.05 were considered significant.

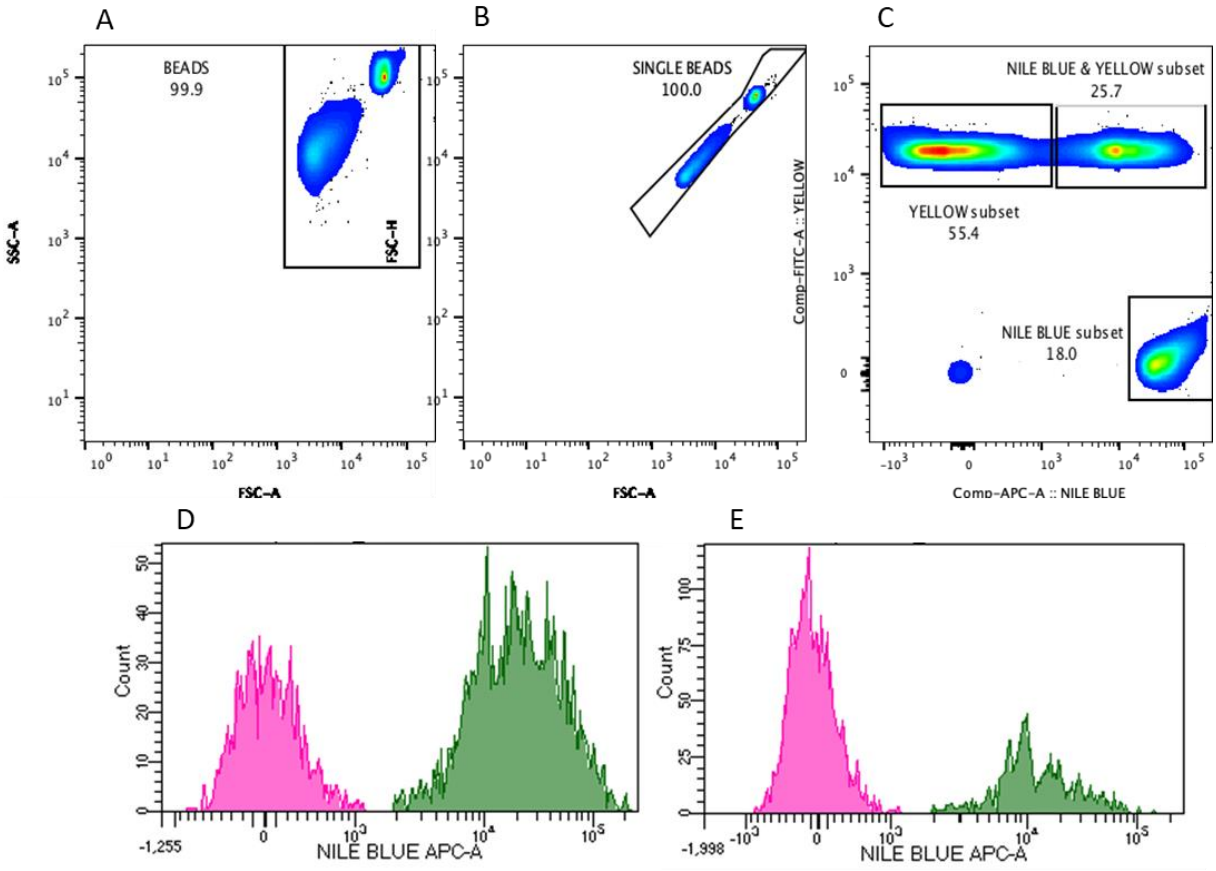

Figure 1. Flow-cytometric parameters for VAR2CSA-yellow beads and CSA-Nile blue beads. To determine the percentage of FV2 (yellow bead) CSA (Nile blue-(NB) beads) complexes, FV2-yellow beads were gated by size (A) and on singlets (B). Three distinct groups of beads were identified (C), i.e., the i) yellow subset (FV2), ii) Nile blue & yellow subset (FV2+CSA complexes), and iii) CSA-Nile blue subset. Representative profiles for beads incubated with PBS (D) and plasma with high Ab levels to VAR2CSA (E) are show as histograms. The pink histogram contains FV2-yellow beads alone, the green histogram shows the FV2+CSA complexes.

Results

Women with Ab to VAR2CSA were more likely to be older and multigravidae. Characteristics of the 310 women who had Ab to FV2 are shown in Table 1 along with comparative information for 614 Ab-negative (Ab-) women (Table 1: first 3 columns). Compared to Ab-negative women, Ab+ women had more pregnancies (median G3 (p=0.0016); 54.7% multigravidae), slightly lower hematocrits (p=0.0003), were more likely to have PM (34% vs 14.5%; p<0.0001), but with significantly lower placental parasitemia at delivery (p<0.0001). Abs to FV2 did not have a significant influence on length of gestation, infant birthweight, or percentage of LBW or premature deliveries (PTD). In this low transmission area, some Ab-negative women may not have been i) infected, ii) been infected but failed to produce Ab, or iii) produced a rapid humoral response that quickly waned prior to delivery.

Many women with antibodies to VAR2CSA still had placental malaria at delivery. Among the 310 Ab+ women, 105 had PM at delivery and 205 women did not (Table 1-last 3 columns). Ab+ women with PM tended to be younger (23.7 vs 25.6 years; p=0.0016), first-time mothers or had fewer pregnancies (p values <0.0001), and a higher risk of anaemia (p=0.02). PM in Ab+ women did not have an effect on length of gestation, baby birthweight, premature deliveries or LBW babies. Although the 310 women had Ab to FV2, clearly not all women had sufficient immunity to prevent or eliminate *P. falciparum* infections by the end of pregnancy. Thus, the overarching question became, are Abs detected by any of the 4 assays associated with lower prevalence of PM or reduced placental parasitemia at delivery?

Table 1: Characteristics of the pregnant women in the study

	Status of Abs to VAR2CSA			Ab-Positive women (n=310)		
	Ab- women	All Ab+ women	P values[a]	PM+	PM-	P value[a]
number	614	310		105	205	
Age in years: mean ± SD (range)	25.9±5.6 (15-42)	24.9 ± 5.5 (14-39)	0.015	23.7 ± 5.2 (14-35)	25.6 ± 5.0 (16-39)	0.0016
Gravidity: median (range)	2 (1-12)	3 (1-6)	0.0016	2 (1-6)	3 (1-6)	<0.0001
% primigravidae	31.9	23.6	0.009	37.1	16.7	0.0001
% secundigravidae	22.3	21.7	0.86	21.0	22.1	0.885
% Multigravidae	45.8	54.7	0.012	41.9	61.3	0.0016
Hematocrit mean ± SD (range)	34.9±6.1 (10-51)	33.3 ± 5.5 (10–45)	0.0003	31.4 ± 5.7 (12-44)	34.3 ± 5.1 (10-45)	0.0001
% anaemia	16.7	20.7	0.18	28.1	16.0	0.026
Placental Malaria positive	14.5	34.0	<0.0001	100 (NA)	0 (NA)	
Median % placental parasitemia of PM+ women (range)	1.7 (0.001-82)	0.60 (0.003–44)	0.001	0.60 (0.003-44)	0	
Weeks of delivery (mean)	38.5±5.2	38.5±5.2	0.86	38.5 ± 3.6	38.7 ± 3.2	0.418
Birthweight (mean ± SD grams)	3,034±703	3,068 ± 613	0.47	2,986 ± 577	3,117 ± 628	0.096
Percent Low Birthweight	18.9	14.8	0.14	15.4	14.4	0.865
Percent preterm deliveries	25.4	23.6	0.57	22.7	23.5	0.888

[A] p values: unpaired t test (age, hematocrit, weeks of delivery, birthweight) and Mann-Whitney (gravidity); Fisher's exact test (percent primi-, secundi-, multigravidae; percent anaemia, percent preterm delivery). Anaemia <30% hematocrit; PM, placental malaria positive

A wide range of antibody values were observed among the 310 women. Results from the 4 assays revealed a wide range of results, with some women having high levels; whereas, others did not.

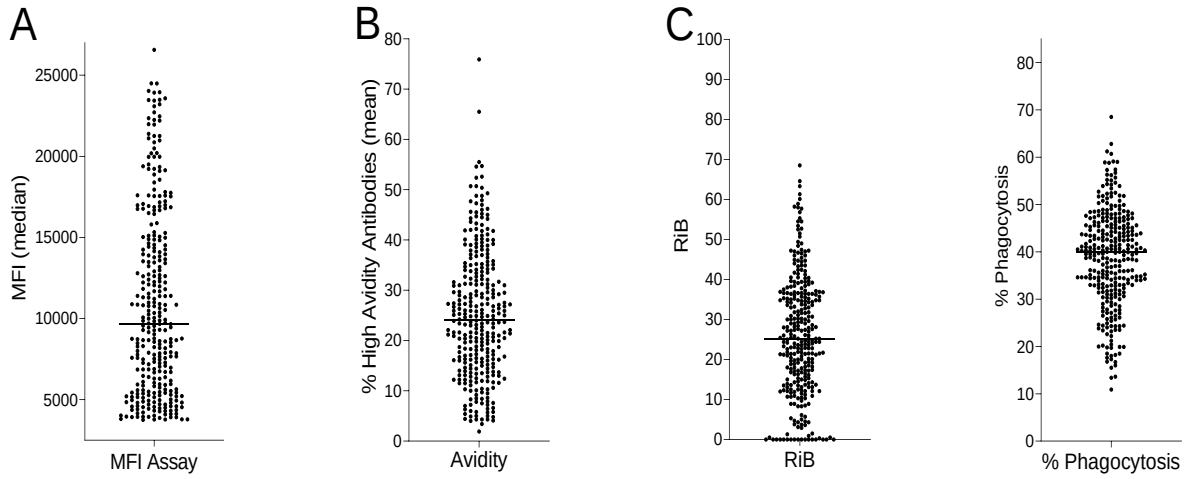

Figure 2. Distribution of Abs to VAR2CSA detected in the different assays. The assays were: A) MFI, bead-based multiplex assay for Ab levels, B) Ab avidity (% of Abs remaining bound); C) RiB, percent reduction in number of FV2-CSA complexes; and D) percent phagocytosis of FV2-fluorescent beads. Since only results for avidity passed the test for normality, medians are shown for the other assays. N=310 women for MFI, avidity, % phagocytosis, and n=306 for RiB.

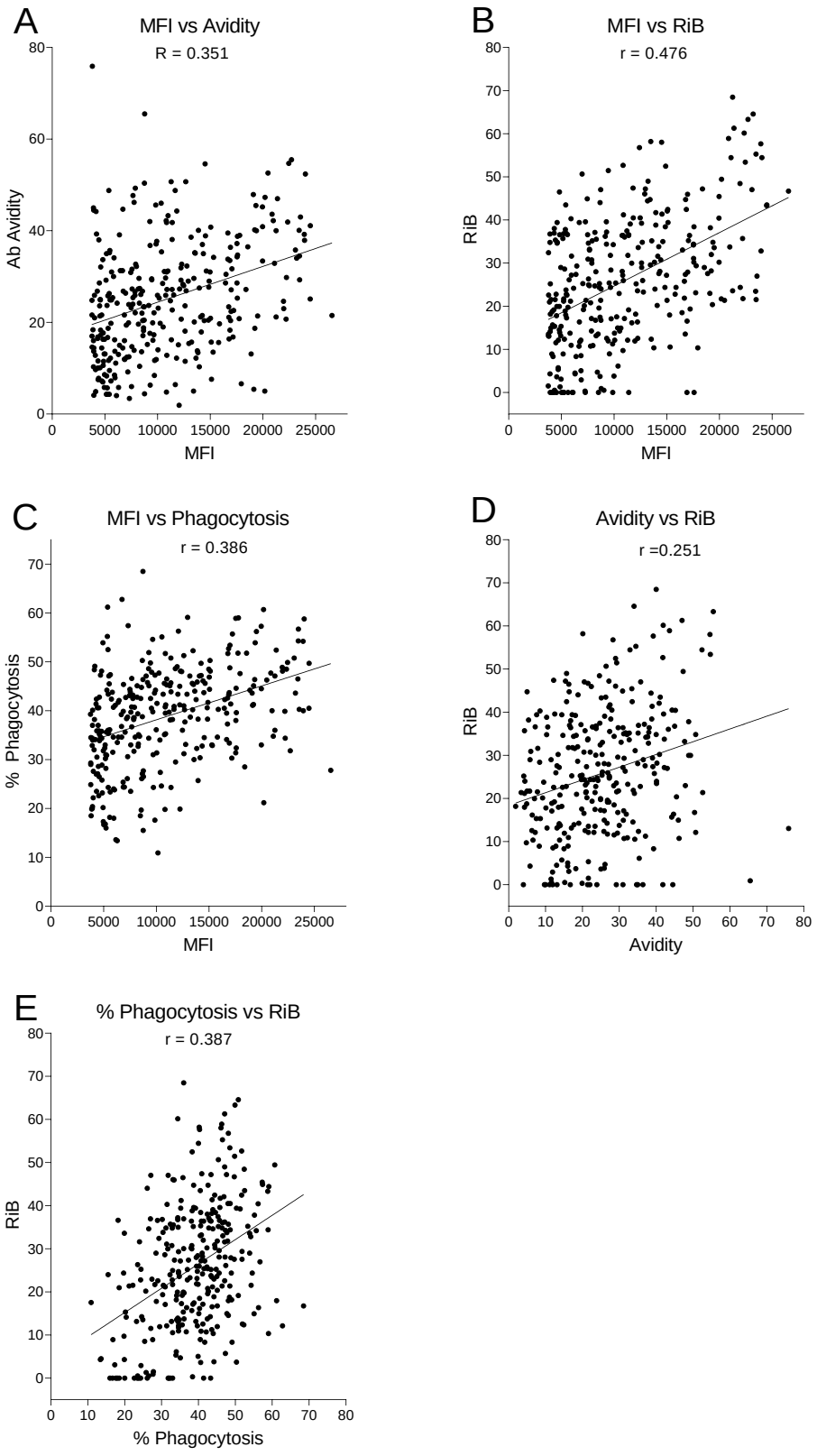

Figure 3. Comparison of four the assays using 310 plasma samples collected in Yaoundé. Simple linear regression was used to determine the association of A) Ab level (MFI) vs. antibody avidity, B) MFI vs. RiB of FV2+CSA complexed beads, C) MFI vs percent phagocytosis of FV2-coupled beads, D) Ab avidity and RiB, and E) percent phagocytosis vs. RiB. P values for all comparisons were significant (all $p < 0.0001$).

Only a moderate association was detected among the results of the four assays. Linear regression was used to assess the relatedness of the assays (Fig. 3 A-E). Only a moderate correlation was found. That is, as the amount (MFI) of Ab to FV2 increased, a small increase in Ab avidity (r=0.351), percentage RiB (r=0.476), and percent opsonic phagocytosis (r = 0.386) was observed. Likewise, a moderate correlation was found between the two functional assays, RiB and percent phagocytosis (r=0.387). Interestingly, however, the association between Ab avidity and RiB was very weak (r=0.251), suggesting that the two parameters were marginally related.

Ab levels were higher and avidity lower in women with placental malaria. Results (Fig. 2) were stratified into PM+ and PM-negative (Fig. 4). Overall, Ab MFI were higher in PM+ women than PM-negative women (p=0.043); percent high avidity Abs was lower in PM+ women (p=0.02); whereas, there was no difference between the women in the other two assays. Thus, in Yaoundé, having higher Ab amounts was related to infection and not protection.

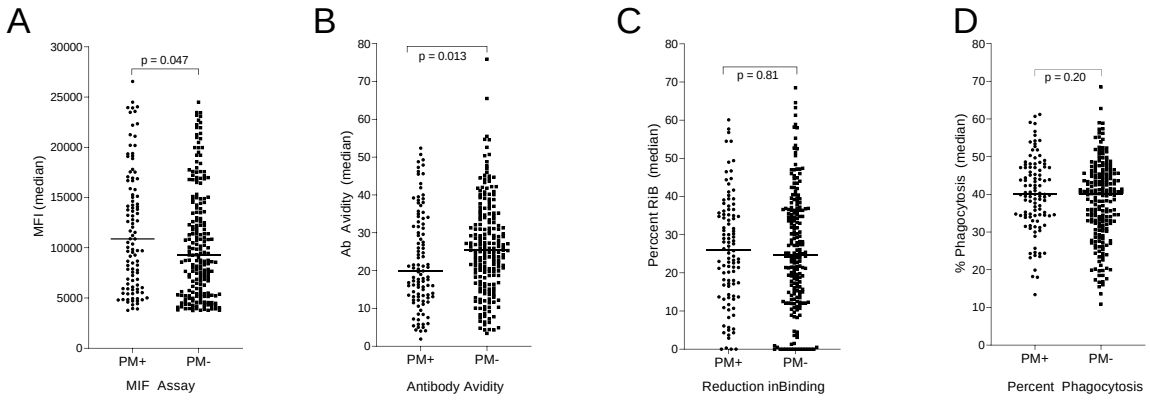

Figure 4: Comparison of Ab values in women with and without PM. Women were separated based on PM status (n=205 PM-negative; n=105 PM+). A. MFI Assay (levels); B. Ab avidity; C. RiB, and D. percent Phagocytosis of FV2-coated beads. MFI values were significantly higher in PM+ women (p=0.047) and Ab avidity were low in PM+ women (p=0.013). Horizontal lines represent medians. Mann-Whitney test.

Only high avidity Abs were associated with lower prevalence of PM. In each assay, the 310 women were divided into two groups, i.e., those with Ab values above or below the median (n=155 women per group). Then, the prevalence of PM was determined and compared between the high and low Ab groups (Table 2). The prevalence of PM was significantly lower in women with above the median Ab avidities compared to those in the lower half (p=0.008). Whereas, no significant differences were seen in the other assays. Thus, having a high proportion of high avidity Abs to VAR2CSA was associated with lower prevalence of PM, but not the other assays.

Table 2: Percent placental malaria in women with high (above) and low (below) the median Ab values in the different assays

	High Ab (Above median)[A] (n=155)	**Low Ab (Below median)[A] (n=155)**	**P value[B]**
Ab levels in MFI	38.1	29.7	0.15
Ab avidity	19.5	41.9	0.008
RiB	33.5	34.2	0.99
% Phagocytosis	31.8	35.3	0.55

[A]_Median values for the assays were: Ab level, 9,500MFI; Ab avidity, 24%; FV2+CSA, 75%; % opsonic-phagocytosis, 40%.
[B]_Fisher exact Test

Next, placental parasitemia was compared between women with above (high) and below (low) the median Ab values PM (Fig. 5). No significant reduction of placental parasitemia was found in women with above compared to below the median in any assay. Although having a high Ab avidity was associated with lower prevalence (Table 2), it was not associated with reduced placental parasitemia (Fig. 5).

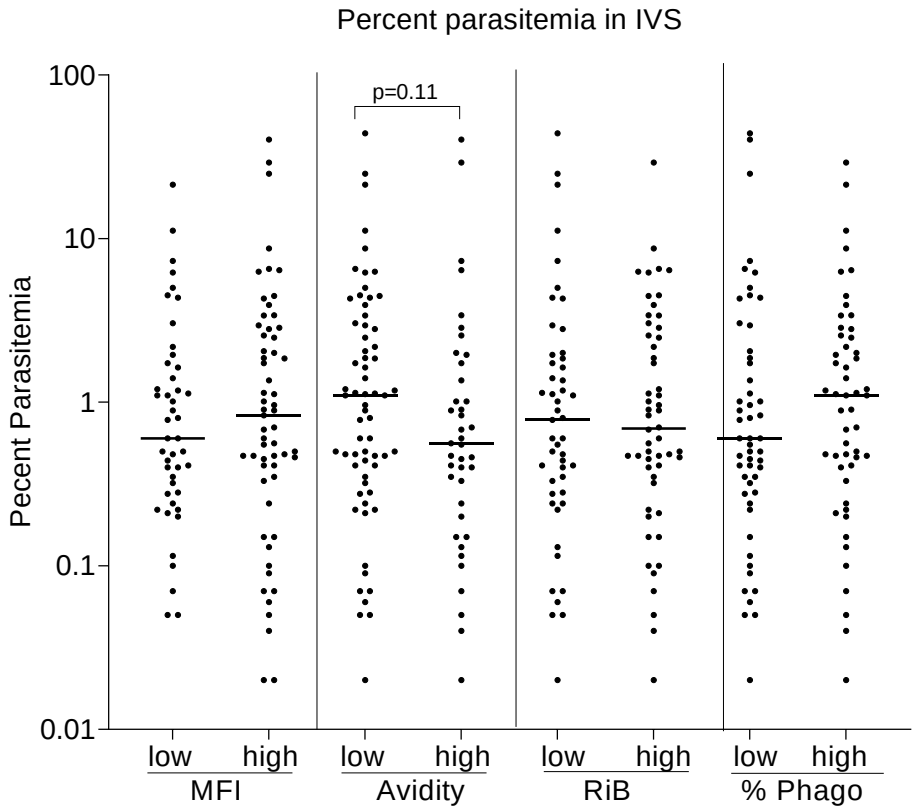

Figure 5: Percent infected erythrocytes in the IVS at delivery. Placental parasitemia was compared between PM+ women with high (above median) and lower (below median) Ab levels in each of the assays. None of the comparisons between low and high levels of Ab were significant (Mann-Whitney: all p >0.5; p value for avidity: p= 0.10). Median Ab values were: MFI 9700 MFI; Avidity 24%; RiB 25%; % phagocytosis, 40%. Horizontal lines represent group medians. n=155 women per group.

Women with above median antibody values had lower placental parasitemia than antibody-negative women. Placental parasitemia of women with above the median Ab values were compared with those of Ab-negative women (n=614) who had placental malaria (n=89) (Fig. 6). Women with high Ab amount (MFI, p=0.03), avidity (p=0.006); RiB (p=0.018) and possibly opsonic-phagocytosis (p=0.065) had significantly lower placental parasitemia. These results suggest that having high Ab values in all 4 assays aid in reducing placental parasitemia when compared to Ab-negative women.

Discussion

Currently, VAR2CSA-based vaccines are under development for use in pregnant women. The vaccines contain the region of VAR2CSA that interacts with CSA on trophoblasts, i.e., the CSA-binding site.[26–29] It is envisioned that vaccine-induced Abs will bind to the CSA-binding site and block attachment of IE in the placenta. As a result, IE would be eliminated either by opsonic phagocytosis in the IVS or by removal in the spleen. The primary assay being used in vaccine trials in Africa is the inhibition of binding assay; however, it remains unclear if this is the best indicator of protection or if other assays should be used that would provide additional information. The results of this study suggest that if women were screened both before and after vaccination for Ab avidity to VAR2CSA and opsonic phagocytosis, in addition to inhibition of binding, additional insight into the immune status of pregnant women would be obtained.

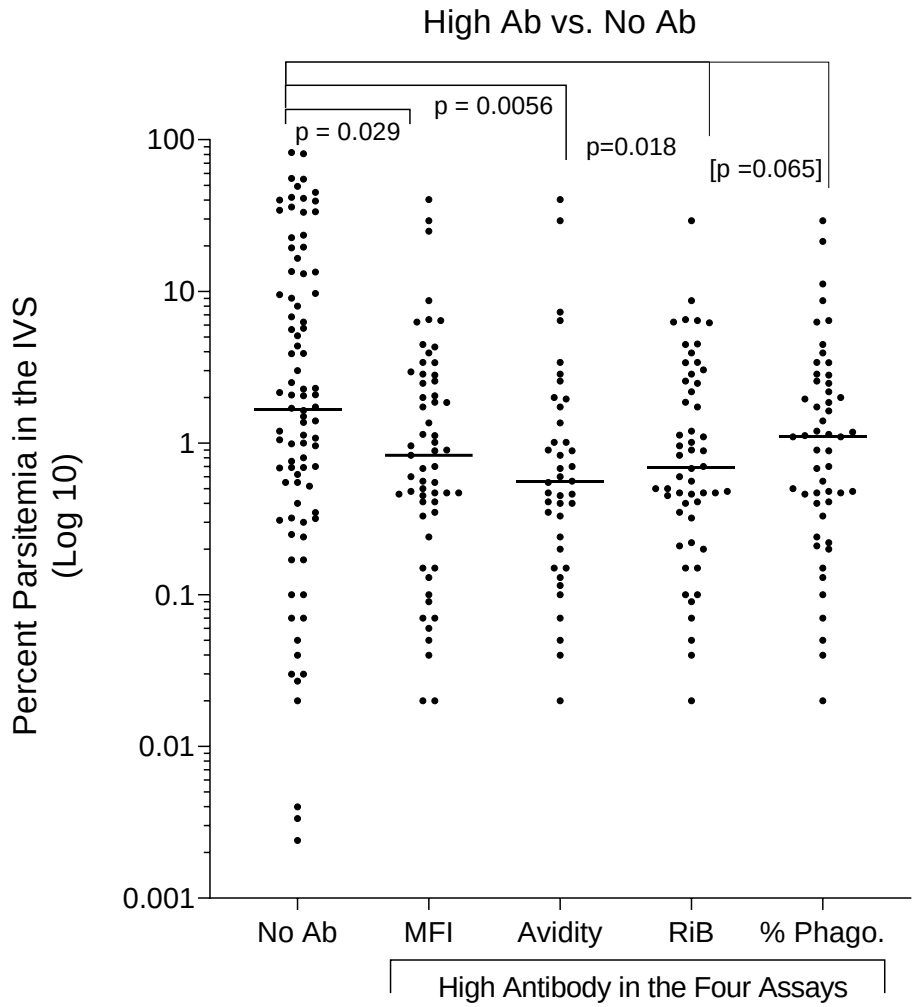

Figure. 6: Comparison of placental parasitemia at delivery by antibody status. Comparison of placental parasitemia in women who lack Abs to VAR2CA (No Ab) with women who had Ab values above the median. The number of PM+ were: No Ab n=89; MIF assay, n=59; Avidity assay, n=40; RiB n=54; and % phagocytosis, n=52). Horizontal lines: medians. Mann-Whitney test.

The importance of Abs to VAR2CSA in immunity to PM is well established. The role of blocking Ab was highlighted in a comprehensive study that evaluated 169 features of anti-VAR2CSA Abs at mid-pregnancy and their association with protection from PM at delivery.[30] Six of the features predicted with 86% accuracy absence of PM at delivery, and included 5 factors related to inhibition of binding and one to phagocytosis. However, different combinations of these immune responses were found among "protected" women, leading to the conclusion that different combinations of Ab characteristic to VAR2CSA contribute to protection from PM. Similarly, in an area where pregnant women were bitten 2-3 times per night by infected mosquitoes, the presence of high Ab levels to i) FV2, ii) multiple DBL domains, and iii) high avidity Abs in the second trimester were associated with absence of PM at delivery.[13,31] In these situations, the immune system is continuously exposed to *P. falciparum* parasites and a combination of Abs to VAR2CSA variants plays a critical role in control and elimination of IE sequestered in the IVS. In contrast, Abs to VAR2CSA seem to play a lesser, although still important, role in urban areas. In this case, pregnant women had been exposed to *P. falciparum* throughout their lives and developed a wide repertoire of Abs to malarial antigens. Thus, when bitten by an infected mosquito, their immunity might be adequate to eliminate IE, possibly before the parasitized IE expressed VAR2CSA, and the women would remain malaria-free for an extended period of time before they became infected again. However, if IE were not cleared, IE expressing VAR2CSA would be produced, stimulate an Ab response, and anti-VAR2CSA Abs would work in conjunction with Abs to other malarial antigens, to reduce the parasite burden in the placenta.

We initially hypothesized a correlation would exist between Ab levels, Ab avidity and RiB. That is, large amounts of Ab with high avidity to the CSA-binding site on VAR2CSA would be required to efficiently block attachment of IE to the placenta; whereas, relatively small amount of Abs to a variety of epitopes on VAR2CSA would be needed to mediate opsonic phagocytosis. However, only a weak association was observed when comparing results from the Ab avidity and RiB assays (Fig. 3). These data agree well with those reported previously that the majority of high avidity Abs are directed against the DBL5 domain and not the CSA-binding site.[32] The combined results support the conclusion that high avidity Abs are a surrogate marker of the overall maturity of the immune response and not associated with better inhibition of binding.

In Yaoundé, Ab levels (MFI) were higher to VAR2CSA in PM+ compared to PM-negative women, showing that high Ab levels at delivery are not always a marker of protection, but rather an indication of placental infection (Fig. 4). In contrast, previous studies have reported that high VAR2CSA Ab levels in women living in rural Cameroonian villages, where transmission is high, are associated with protection.[31] Thus, the intensity of malaria transmission has an impact on immunity to PM.

All women in the study were selected because they were seropositive for VAR2CSA in the multiplex-immunoassay and had a wide range of Ab levels (Fig. 2). Thus, the design allowed us to evaluate the effect of "better performing Abs" and "poorer performing Abs" on PM status in a large group of women. Avidity was the only parameter that showed a difference between having high or low Ab values in the various assays (Table 2). Women with above the median high avidity Abs were less likely to have PM than those below the median (19.5% vs 41.9% PM+, p=0.008) and also lower median placental parasitemia (median (IQR) 0.55% (0.21,1,6) vs. 1.1% (0.4 -3.0), although the p value was slightly above significance (p= 0.10) (Table 2, Fig. 4). The importance of high avidity Abs in this urban setting has being associated with reduce prevalence of PM, placental parasitemia, and higher birthweights.[25,32] For example, in multigravidae a logistic regression model found high avidity Abs to VAR2CSA were associated with reduced likelihood of PM, with a 5% increase in the proportion of high avidity Abs associated with nearly a 15% lower likelihood of PM.[25] In addition, women with high avidity Abs had babies that averaged 104 gm heavier than babies born to mothers with below the median avidity (p= 0.045).[32] Thus, finding an association between high avidity Abs and lower placental parasitemia and improved birthweight has been a consistent finding.

Women with high Abs in each of the four assays also had lower placental parasitemia compared to Ab-negative women (Fig. 6). Accordingly, it appears that high amounts, avidity, and RiB of FV2+CSA complexes, and probably opsonic phagocytosis, all contribute to reduction of parasite burden in PM+ women. These results agree with an early study showing that women who had high amounts of anti-adhesion Abs in a traditional IoB assay (i.e., Abs that blocking of VAR2CSA-expressing IE from binding to placental chondroitin sulfate proteoglycans) had lower placental parasitemia (coefficient values (r^2 -0.37; p=0.02).[15] Although results from this study did not provide a better correlate of protection, they show that high amounts, quality and functional Abs collaborate, with Abs to other malarial antigens, in reducing PM.

There are several benefits as well as limitations to the study. As for advantages, the samples were collected before IPT became the governmental policy for pregnant women in Cameroon. Thus, the impact of VAR2CSA Abs on PM could be assessed. Today, it is not possible to conduct similar studies since preventive treatment with sulfadoxine-pyrimethamine is the standard of care. Secondly, the original panel of >2,500 samples has been used in prior studies that provided information about different aspects of PM and established an association between Ab levels and improved pregnancy outcomes.[13,15,25,31] A limitation is that the RiB assay is a new assay for measuring Ab reduction of VAR2CSA binding to CSA. The assay was called RiB to distinguish it from the traditional IoB assays that use viable IE. In its current form, the RiB assay is highly stringent since Abs must prevent *all* Nile-blue-CSA beads from attaching to each yellow-FV2 bead in order to move a bead from the "yellow+blue" channel to the "yellow" only channel (Fig. 1 C-D). Further studies are needed to determine if the sensitivity can be improved by measuring reduction in Nile-blue fluorescence in the "yellow-blue" (FV2-CSA) channel (Fig. 1 D, E). Finally, the RiB assay needs to be validated by other investigators and under different epidemiological conductions. In the current study, RiB increased with increasing amounts of Ab and precent phagocytosis, suggesting the strong potential of this relatively simple assay (Fig 3 B, D). In summary, in situations where pregnant women have acquired immunity to *P. falciparum* and are exposed to a limited number of infections during pregnancy, the best indicator of partial immunity to PM is having a high percentage of high avidity Abs to VAR2CSA, since they are associated with lower

prevalence of PM and lower placental parasitemia. The overall results further support the hypothesis that high avidity Abs are a marker of the overall maturity of the immune response to PM and they are not directly associated with higher avidity Abs that block binding. Unfortunately, in Yaoundé most women do not have high avidity Abs.[13] Thus, a VAR2CSA vaccine that induces a higher, more-rapid level of humoral responsiveness would be beneficial to pregnant women who are exposed to *P. falciparum*.

Conclusions

Yaoundé, Cameroon, where transmission of *P. falciparum* is low, high Ab levels to VAR2CSA are a marker of infection, and not protection. However, women with high avidity Abs had a significant reduction in prevalence of PM and placental parasitemia compared to women with low Ab levels. Importantly, compared to women who lacked Abs to VAR2CSA, women with above the median values in all four assays, namely, Ab quantity, quality, and the functional assays for inhibition of binding and opsonic phagocytosis, had a significant reduction in prevalence of PM and placental parasitemia. Thus, Abs to VAR2CSA play an important role in improving pregnancy outcomes in this urban area of Africa.

Conflict of Interest Statement. The authors have no conflict of interest to declare.

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