Positive Selection of *Plasmodium falciparum* Parasites With Multiple *var2csa*-Type PfEMP1 Genes During the Course of Infection in Pregnant Women

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Placental malaria infections are caused by *Plasmodium falciparum*-infected red blood cells sequestering in the placenta by binding to chondroitin sulfate A, mediated by VAR2CSA, a variant of the PfEMP1 family of adhesion antigens. Recent studies have shown that many *P. falciparum* genomes have multiple genes coding for different VAR2CSA proteins, and parasites with >1 *var2csa* gene appear to be more common in pregnant women with placental malaria than in nonpregnant individuals. We present evidence that, in pregnant women, parasites containing multiple *var2csa*-type genes possess a selective advantage over parasites with a single *var2csa* gene. Accumulation of parasites with multiple copies of the *var2csa* gene during the course of pregnancy was also correlated with the development of antibodies involved in blocking VAR2CSA adhesion. The data suggest that multiplicity of *var2csa*-type genes enables *P. falciparum* parasites to persist for a longer period of time during placental infections, probably because of their greater capacity for antigenic variation and evasion of variant-specific immune responses.

Malaria in pregnancy is associated with *Plasmodium falciparum*-infected red blood cells (iRBCs) accumulating in the placenta, which severely increases the risks of miscarriage, maternal anemia, and low infant birth weight [1]. The iRBCs bind to chondroitin sulfate A (CSA), an abundant proteoglycan on the surface of the placental trophoblast [2]. The parasite ligand mediating binding is VAR2CSA [3], a 350-kD protein that is

a member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of surface adhesion antigens [4]. During a blood-stage infection, PfEMP1 molecules on iRBCs enable parasites to bind to host endothelial receptors, thus avoiding passage through the spleen [5]. The PfEMP1 proteins are encoded by ~60 members of the *var* multigene family [4], and the expression of PfEMP1 appears to be controlled by a form of allelic exclusion, whereby each parasite expresses a single variant on the iRBC surface [6]. In addition, parasites can perform antigenic variation by switching to expression of alternative PfEMP1 variants, thereby avoiding variant-specific antibodies (reviewed by Scherf A et al [7]).

A recent study showed that several *P. falciparum* isolates harbor at least 2 *var2csa* genes, which have been mapped to loci on chr. 1, chr. 5–9, and chr. 12, the latter locus being conserved in all examined *P. falciparum* isolates [8]. Of interest, quantitative real-time polymerase chain reaction (PCR) data suggest that these

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multi-*var2csa* copy parasites are more frequent in pregnant women than in nonpregnant individuals. Sequencing data [8] and transcription analysis [8, 9] have provided evidence that the *var2csa*-type genes are functional rather than pseudogenes, and phylogenetic analysis indicates that, in most cases, *var2csa*-type sequences from the same parasite genome are not more closely related to each other than to any other *var2csa*-type sequence [8], probably indicating that VAR2CSA proteins encoded from these genes are recognized as distinct antigens.

This study further examined the in vivo occurrence and consequences of placental infection, with *P. falciparum* isolates harboring multiple *var2csa*-type genes by comparing the mean *var2csa* copy number per parasite genome in a large panel of longitudinally collected blood samples from pregnant women and nonpregnant individuals living in 2 Cameroonian sites with different intensities of *P. falciparum* transmission [10].

MATERIALS AND METHOD

Collection of Samples

During 2001–2005, longitudinal studies were conducted in pregnant Cameroonian women residing in Yaoundé, where transmission is low (~13 infectious bites/year) and in the village of Ngali II, where *P. falciparum* infection is hyperendemic (362 infectious bites/year) [10]. Nonpregnant women were also recruited. Women ≤15 weeks pregnant were enrolled and followed up monthly until delivery. Each month, a sample of peripheral blood was collected for detection of malaria parasites. If parasites were detected by blood smear, the information was reported to the attending physician, who prescribed treatment. At delivery, infant birth weight was recorded; samples of maternal peripheral and intervillous space (IVS) blood were collected, and a biopsy of the placenta was obtained. Thick and thin blood smears of peripheral and IVS blood were prepared, and impression smears of placenta tissue were made, stained with Diff-Quick (Baxter Scientific), and examined for parasites. Histological sections were also prepared. A woman was considered to have placental malaria if parasites were found in the smear of IVS blood, impression smears, or histological sections. All deliveries were full-term (≥38 weeks), and all infants had normal birth weights (≥2500 g).

After completing the study, blood samples were screened for *P. falciparum* with use of a nested PCR [11]. All PCR- and slide-positive samples with sufficient volume of cryopreserved RBCs were used in the current study, including 250 samples collected during pregnancy, 64 peripheral and placental samples at delivery, and 31 samples from nonpregnant women. A description of the women included in the study is shown in Table 1.

Quantitative Real-time PCR

Quantitative real-time PCR was performed using the Rotorgene 6000 (Corbett Research). PCR amplification was done in 20-μL

reaction volumes with use of Quantitec SYBER Green PCR master mix (Qiagen) and primer concentrations of 1 μM. The cycle threshold (Ct) was manually set at 0.025.

Estimation of the *var2csa* Copy Number Per Parasite Genome Using the $2^{-\Delta\Delta Ct}$ Method

Parasite genomic DNA was extracted from samples. The $2^{-\Delta\Delta Ct}$ method of relative quantification [12] was adapted to estimate the mean *var2csa* gene copy number per *P. falciparum* genome in blood samples that contained polyclonal populations of parasites. An evaluation of the method, including a complete description of the used primers, has been published previously [8]. In short, the copy number was estimated by normalizing the amount of target gene DNA against a house-keeping gene that has a constant copy number. With use of the below equations [1, 2], the copy number of the target gene was calculated by comparing its amount in relation to the amount of DNA of the *fructose-bisphosphate aldolase* gene (PF14_0425_v5.5, PlasmoDB). The FCR3/It4 clone that has 1 copy of both the *var2csa* and the *aldolase* gene [8] was used as the calibrating parasite genome.

(1) $\Delta\Delta Ct = (Ct \text{ var2csa} - Ct \text{ aldolase})\chi - (Ct \text{ var2csa} - Ct \text{ aldolase})y$, where χ = unknown sample and y = FCR3/It4. Z

(2) $2^{-\Delta\Delta Ct}$, the copy number of the *var2csa* is expressed as N-fold changes, compared with the *aldolase* gene number in the calibrating genome.

Each sample was run in triplicate in 2 separate experiments, and the *var2csa* copy number was expressed as the geometric mean between the 2 experiments. Because low concentrations of template DNA resulted in greater variation (data not shown), 2 exclusion criteria were used for all samples: (1) Ct value >26 and (2) triplicate samples with coefficient of variation >1%.

Antibodies to VAR2CSA

Antibody levels were determined using a multiplex assay as described elsewhere [13]. In brief, 5 μg of recombinant DBL4ε, DBL5ε, and full-length VAR2CSA (FCR3/It4 strain) were coupled to 5×10^6 microspheres (Luminex). Plasma was diluted 1:100 in phosphate-buffered saline containing 1% bovine serum albumin, and equal volumes (50 μL) of plasma and antigen-coated microspheres were combined for 1 h. Subsequently, the microspheres were incubated with phycoerythrin-conjugated goat-antihuman IgG Fcγ specific antibody (Jackson Immuno Research) for 1 h prior. The beads were washed and analyzed using a Luminex M100 Workstation (Luminex). In each assay, pooled plasma samples from multigravidae was used as the positive control, and pooled plasma samples from American adults who had not been exposed to malaria served as the negative control. Results were expressed as median fluorescent intensity units (MFIs), and individual MFIs for the plasma samples were used for subsequent statistical analyses.

Table 1. Characteristics of Study Participants

Characteristic	Pregnant women		Nonpregnant women*
	Ngali II	Yaoundé	
Number of women	63	50	22
Number of total samples	134	116	31
Primigravidae women (%)	40	41	
Maternal age (mean $\pm$ SD in years)	23,4 $\pm$ 5.9	23,8 $\pm$ 4.8	27.0 $\pm$ 7.9
Number of samples – collected at 1 st ; 2 nd ; 3 rd trimesters	0;55;79	6;47;63	
Number of samples (placenta IVS; peripheral blood) collected at term	19;25	7;13	

NOTE. *Includes women from both sites (Ngali II, $n = 12$ and Yaoundé, $n = 10$ women).

Statistical Analyses

All statistical analyses were performed using Stata MP, version 10 (StataCorp), and statistical significance was set at $P = .05$. Paired Student t test and Spearman's rank sum test were used to assess the level of variation between *var2csa* copy number estimates of paired samples collected from peripheral and placental blood from the same woman at delivery. The influence of site of residence on *var2csa* copy number was assessed using a nonparametric Mann Whitney U analysis testing if the median *var2csa* copy number was significantly different between control samples from Yaoundé and Ngali II. The *var2csa* copy number estimates were subsequently divided into 3 groups: (1) samples from pregnant women in Ngali II, (2) samples from pregnant women in Yaoundé, and (3) control samples from nonpregnant individuals living in both Yaoundé and Ngali II.

To test whether *P. falciparum* parasites containing multiple *var2csa*-type genes were more common in blood samples from pregnant than in nonpregnant women, the nonparametric Kruskal Wallis equality of population rank test was conducted to evaluate whether the median *var2csa* copy number differed between the 2 populations. After this analysis, the 2-sample Mann Whitney U analysis was used for 2-by-2 analysis to determine which of the data sets differed from the others with regard to the median *var2csa* copy number. Multiple linear regression was used to test the association between the *var2csa* copy number of infecting parasites and gravidity (primi- or multigravidae), gestation week, maternal age, parasite load (Log[iRBCs/ μ l]), hemoglobin levels (g/dL), and reactivity of plasma samples against recombinant proteins (MFI). The Xtset command was used to reflect the longitudinal data sampling. Individual woman's identification was used as panel variable and gestation week as the time variable. Birth weights of newborn babies were modeled using multiple linear regression analysis using data from the time of birth.

RESULTS

Characteristics of Patients

Patient groups and study components from Ngali II (site where disease is hyperendemic) and Yaoundé (low transmission site)

were similar with respect to number of women, proportion of primigravidae, and number of samples collected (Table 1). To validate the assay, gene copy number variation between paired peripheral and placental blood samples ($n = 22$) from the same woman was assessed under the assumption that the genetic composition of parasites from each compartment was likely to be the same. The measured *var2csa* copy number did not, in fact, differ significantly between the 2 compartments (mean difference, .125; 95% confidence interval [CI], $-.184$ to $.834$; $P = .769$, by Student t test), and the *var2csa* gene copy number of parasites in placental and peripheral blood samples were strongly correlated ($k_s, .936$; $P < .001$, by Spearman's rank order test). These results validate the use of the assay for measuring *var2csa* copy numbers and the use of samples originating from placental and peripheral blood. No statistically significant difference in the *var2csa* copy number of infecting parasites was found between nonpregnant women living at the 2 sites ($P = .384$, by Mann Whitney U test). Thus, results from these women were combined in subsequent analyses.

Comparison of *var2csa* Copy Number per Parasite Genome in Parasites From Pregnant and Nonpregnant Women

The estimated median *var2csa* copy number was found to be significantly different among pregnant women living in Ngali II (2.34) and Yaoundé (1.84) and the control nonpregnant women (1.45; $P = .001$; 144 observations, 2 degrees of freedom, Kruskal-Wallis equality of population rank test) (Figure 1). Subsequent 2-sample Mann Whitney U analysis showed that samples from pregnant women in Ngali II had a significantly higher median *var2csa* copy number per parasite genome, compared with those from pregnant women in Yaoundé or nonpregnant women. No statistically significant difference in mean *var2csa* copy number was found between pregnant women in Yaoundé and nonpregnant women (Figure 1).

The Influence of Gravidity, Weeks of Pregnancy, Maternal Age, and Parasite Load on the *var2csa* Copy Number

The effect of gravidity, gestation week, maternal age, and parasite load on the *var2csa* copy number was assessed using multiple linear regression analysis. Gestation week was positively associated with the *var2csa* copy number of infecting parasites in

Figure 1. Median *var2csa* gene copy number per parasite genome in pregnant women in Ngali II and Yaoundé, and in nonpregnant women residing in both locations. Bars indicate the 25th and 75th percentiles.

samples from Ngali II ($P = .033$) (Table 2), although *var2csa* copy number showed a tendency to decrease with increasing weeks of pregnancy in women in Yaoundé ($P = .068$) (Table 2). A significant positive association was found between parasite load and the estimated *var2csa* copy number in women in Ngali II, indicating that women infected by parasites with multiple copies of *var2csa*-type genes tended to have a higher parasite load than did women infected with single-copy parasites (Table 2).

The Effect of the Level of Anti-VAR2CSA IgG on the Estimated *var2csa* Copy Number

Estimated *var2csa* copy number was compared with the levels of specific antibodies present in donor plasma at the time that the sample was collected. These antibodies recognize various recombinant constructs of domains and subregions of the VAR2CSA antigen. The level of IgG antibodies to the VAR2CSA DBL4 ϵ , but not to the DBL5 ϵ or the full-length ectodomain, was positively associated with the *var2csa* copy number of parasites in pregnant women in Ngali II ($P = .001$, by multiple linear regression analysis) (Table 3). The positive association shows

Table 2. Multiple Linear Regression Analysis Testing the Association Between *var2csa* Gene Copy Number and Gravidity, Weeks of Pregnancy, Maternal Age, and Parasitemia

Tested parameters	Coeff.	[95% CI]	P value
Ngali II ($n = 134$ samples)			
Primi-multigravidae	0.90	-.084 to 1.89	.073
Weeks of pregnancy	0.036	.0028 to .068	.033
Maternal age	-.063	-.14 to .017	.123
Parasitemia	0.12	.016 to .22	.024
Cons-*	2.00	.084 to 3.84	.041
Yaoundé ($n = 114$ samples)			
Primi-multigravidae	-.25	-1.24 to .74	.622
Weeks of pregnancy	-.038	-.078 to .0028	.068
Maternal age	0.011	-.091 to .11	.836
Parasitemia	-.027	-.16 to .10	.686
Cons-	3.70	1.17 to 6.23	.004

NOTE. CI, confidence interval.

*Cons- (constant alpha) is the value that the dependent variable is predicted to have when all the independent variables (parameters) are equal to zero.

that, in infections with multi-*var2csa* copy parasites, women tend to have higher levels of particular types of anti-VAR2CSA antibodies.

Effect of High Copy Numbers of *var2csa* on Anemia and Birth Weight

In samples from Ngali II, increasing *var2csa* copy number was also associated with higher hemoglobin levels ($P = .009$, multiple linear regression) (Table 4). This association was not seen in Yaoundé, where only gestation week was positively associated with hemoglobin level (Table 4). The *var2csa* copy number was negatively associated with birth weight in Yaoundé ($P = .027$, by multiple linear regression) (Table 5), but not in Ngali II.

DISCUSSION

During placental infections due to *P. falciparum*, VAR2CSA is the parasite erythrocyte surface adhesin that mediates binding to placental trophoblast surface CSA. It has been demonstrated that there is relatively limited antigenic diversity among placental parasites, because protective IgG antibodies that can block the binding of placental parasites to CSA [14] are acquired rapidly during the first 1–2 pregnancies [1, 14], and such antibodies can recognize placental parasites originating from the tropical malaria zone [14].

The haploid genome of some *P. falciparum* field isolates can contain multiple, unlinked *var2csa* genes [8, 15]. Previously published data, collected on a small number of field isolates, suggest that multi-*var2csa* copy parasites are more prevalent in pregnant women than in nonpregnant individuals and that there may be selective advantage of the multi-*var2csa* copy genotype during placental infections [8]. We therefore compared *var2csa*

Table 3. Multiple Linear Regression Analysis Testing the Association Between *var2csa* Gene Copy Number and Gravidity, Weeks of Pregnancy, Age, Parasitemia, and Anti-*VAR2CSA* IgG Antibody Levels^H

Tested parameters	Coeff.	[95% CI]	P value
Ngali II <i>n</i> = 55			
Primi-multigravidae	-.50	-1.68 to .78	.474
Weeks of pregnancy	0.027	-.013 to .068	.181
Maternal age	0.023	-.079 to .12	.662
Parasitemia	0.18	.050 to .31	.007
Anti- <i>VAR2CSA</i> DBL4 ϵ IgG	0.38	.16 to .60	.001
Cons-*	-.20	-2.17 to 1.77	.843
Yaoundé (<i>n</i> =43)			
Primi-multigravidae	0.58	-.62 to 1.78	.341
Weeks of pregnancy	-.037	-.080 to .0059	.091
Maternal age	-.0057	-.13 to .12	.928
Parasitemia	0.026	-.098 to .15	.682
Anti- <i>VAR2CSA</i> DBL4 ϵ IgG	-.070	-.48 to .34	.739
Cons-	3.12	.076 to 6.17	.045

NOTE. CI, confidence interval.

^H IgG antibody levels of individual plasma samples were recorded as the Median Fluorescent Intensity (MFI) using a multiplex bead-based assay.

* Cons- (constant alpha) is the value that the dependent variable is predicted to have when all the independent variables (parameters) are equal to zero.

copy numbers in parasites from pregnant and nonpregnant individuals collected as part of 2 parallel longitudinal studies in areas of different intensity of malaria transmission in Cameroon.

Parasites isolated from nonpregnant women living in the 2 different transmission intensity study sites had essentially the same number of *var2csa* genes ($P = .384$), implying that the

Table 4. Multiple Linear Regression Analysis Testing the Association Between Haemoglobin Level and *var2csa* Copy Number, Gravidity, Weeks of Pregnancy, Maternal Age, and Parasitemia

Tested parameters	Coeff.	[95% CI]	P value
Ngali II (<i>N</i> = 131)			
<i>Var2csa</i> copy number	0.22	.053 to .38	.009
Primi-multigravidae	-.12	-1.08 to .83	.799
Weeks of pregnancy	0.016	-.016 to .047	.326
Maternal age	0.069	-.0076 to .15	.077
Parasitemia	-.071	-.17 to .028	.161
Cons-*	8.73	6.92 to 10.55	.000
Yaoundé (<i>n</i> =110)			
<i>Var2csa</i> copy number	0.016	-.12 to .15	.811
Primi-multigravidae	0.014	-.72 to .74	.970
Weeks of pregnancy	0.033	.0034 to .062	.029
Maternal age	0.040	-.036 to .12	.301
Parasitemia	0.043	-.050 to .14	.363
Cons-	8.84	6.89 to 10.80	.000

NOTE. CI, confidence interval.

* Cons- (constant alpha) is the value that the dependent variable is predicted to have when all the independent variables are equal to zero.

Table 5. Multiple Linear Regression Analysis Testing the Association Between Infant Birth Weight and *var2csa* Copy Number, Gravidity, and Parasitemia

Tested parameters	Coeff.	[95% CI]	P value
Ngali II (<i>n</i> = 25)			
<i>Var2csa</i> copy number	-24.97	-128.78 to 78.84	.622
Primi-multigravidae	464.51	-21.034 to 950.056	.060
Parasitemia	2.583	-69.017 to 74.18	.941
Cons-*	2810.97	2182.40 to 3439.54	.000
Yaoundé (<i>n</i> = 13)			
<i>Var2csa</i> copy number	-194.84	-362.18 to 27.50	.027
Primi-multigravidae	366.16	-50.70 to 783.017	.078
Parasitemia	-34.76	-93.58 to 24.067	.214
Cons-	3461.062	2915.27 to 4006.85	.000

NOTE. CI, confidence interval.

* Cons- (constant alpha) is the value that the dependent variable is predicted to have when all the independent variables are equal to zero.

prevalence of multi-*var2csa* copy parasites in the population is not influenced by transmission intensity per se. However, the median *var2csa* copy number was significantly higher in parasites collected from pregnant women in Ngali II than in parasites from nonpregnant women ($P = .0002$) or from pregnant women in Yaoundé ($P = .037$). The results of a linear regression analysis demonstrated that the *var2csa* copy number of infecting parasites increased during pregnancy in women living in the high-transmission area (Table 2). This is consistent with multi-*var2csa* copy parasites being at a selective advantage during malaria in pregnancy.

In the Yaoundé low-transmission site, *P. falciparum* parasites did not have significantly higher *var2csa* copy numbers in pregnant women, compared with parasites from nonpregnant individuals ($P = .13$). The median *var2csa* copy number per parasite genome tended to decrease during pregnancy in the Yaoundé samples, although the decrease was not statistically significant. The lack of difference in median *var2csa* copy number of parasites from pregnant women in Yaoundé and nonpregnant women could be attributable to the fact that pregnant women in Yaoundé, but not in the rural setting of Ngali II, received monthly prenatal care [10]; thus, their infections may have been cured by antimalarial drugs before selection of multi-copy parasites could occur. Furthermore, women living in Ngali II were more likely to have pre-existing anti-*VAR2CSA* antibodies, compared with women from Yaoundé, and this may be the reason why parasites in women living in Ngali II had higher *var2cas* copy numbers than parasites from nonpregnant individuals.

To test whether host immunity is exerting selection pressure favoring parasites with multiple *var2csa*-type genes, a linear regression analysis was used to test the relationship between the *var2csa*-copy number and anti-*VAR2CSA* antibody levels. The analysis showed that women living in the area with high malaria

transmission who had high plasma levels of anti-VAR2CSA DBL4 ϵ antibodies were more likely to be infected with multi-*var2csa* copy parasites (Table 3). No association was seen between *var2csa* copy numbers and antibody levels to DBL5 ϵ or a full-length VAR2CSA ectodomain construct. Previously, antibodies to DBL4 ϵ were shown to have a high capacity to inhibit placental-type parasites from binding to CSA [16, 17], whereas other domains (eg, DBL5 ϵ that are well recognized by naturally acquired IgG) do not seem to be involved in a protective response [17, 18]. This could be the reason why no association was found between the *var2cas*-copy number and anti-VAR2CSA DBL5 ϵ antibodies. The IgG response toward the VAR2CSA full-length ectodomain could likewise be dominated by antibodies targeting immunodominant regions not involved in the adhesion process. These results suggest that parasites with multiple VAR2CSA variants are better able to circumvent adhesion blocking by protective anti-VAR2CSA IgG antibodies.

Because of the evidence that multi-*var2csa* copy parasites are more prevalent in infections in pregnant women in Ngali II, compared with nonpregnant individuals and the fact that the *var2csa* copy number of infecting parasites was shown to be positively associated with both the measured parasite load and the levels of anti-VAR2CSA DBL4 ϵ antibodies (Tables 2 and 3), consideration should be given to the possibility that this genotype causes more prolonged and, therefore, more virulent infections.

However, a full outcome analysis of whether high copy numbers of *var2csa*-type genes are associated with anemia and low infant birth weight was compromised by the fact that all the enrolled women were treated with antimalarial drugs when parasites were found in blood smears [10]. Nevertheless, sample sets from women with malaria in pregnancy are very rare, and some efforts to investigate outcomes were made. In Ngali II, a positive association between the *var2csa* copy number of infecting parasites and hemoglobin levels was found in pregnant women. This finding is consistent with the notion that multi-*var2csa* copy parasites accumulate during pregnancy, because these parasites are better at surviving in women with high levels of anti-VAR2CSA antibodies, which protect the women from anemia. In the high-transmission area, there was no association between the *var2csa* copy number and infant birth weight. By contrast, women in the low-transmission area delivered babies with significantly lower birth weight if they were infected with parasites with multiple *var2csa*-type genes (Table 5). However, this analysis was based on a small number of observations ($n = 13$).

Our results demonstrate that parasites carrying multiple *var2csa*-type genes are selected for during *P. falciparum* infection in pregnant women and that there is a complex interaction between *var2csa* copy number, malaria endemicity, and acquisition of antibodies to VAR2CSA. Our data do not reveal how

these interactions are controlled, but several mechanisms could be operating.

At the onset of the blood stage infection, several PfEMP1 adhesins are expressed on the surface of iRBCs during the first wave of parasites released from the liver [19]. Selection will tend to favor those parasites expressing the strongest binding PfEMP1 adhesin not recognized by pre-existing antibodies [20]. Therefore, it is possible that parasites harboring multiple *var2csa*-type genes have a better chance of establishing an infection because of a higher probability of expressing a VAR2CSA variant not recognized by pre-existing antibodies. Alternatively, the ability to switch between antigenically different VAR2CSA adhesins during a placental infection would increase the length of infection as a result of antigenic variation. Both mechanisms would be expected to result in an increased frequency of multicopy parasites as host humoral immunity develops during pregnancy.

It is not known whether the multiple *var2csa*-type genes contained by single parasite genomes represent functionally distinct variants with respect to adhesion phenotype and antigenic properties. However, on the basis of the findings presented in this study, development of a VAR2CSA-based pregnancy malaria vaccine should take into account the possibility of multicopy *P. falciparum* parasites with the ability to switch expression of different variants.

In conclusion, we found that *P. falciparum* isolates with multiple *var2csa*-type genes accumulate during the course of pregnancy in women exposed to high levels of malaria transmission. Selection of multi-*var2csa* copy parasites may be driven by host immunity, because the level of anti-VAR2CSA antibodies correlated with the *var2csa* copy number.

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References

1. Brabin BJ. An analysis of malaria in pregnancy in Africa. *Bull World Health Organ* **1983**; 61:1005–16.
2. Fried M, Duffy PE. Adherence of plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science* **1996**; 272:1502–4.
3. Salanti A, Staalsoe T, Lavstsen T, et al. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. *Mol Microbiol* **2003**; 49:179–91.
4. Su XZ, Heatwole VM, Wertheimer SP, et al. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic

- variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **1995**; 82:89–100.
5. Kraemer SM, Smith JD. A family affair: var genes, PfEMP1 binding, and malaria disease. *Curr Opin Microbiol* **2006**; 9:374–80.
 6. Chen Q, Fernandez V, Sundstrom A, et al. Developmental selection of var gene expression in *Plasmodium falciparum*. *Nature* **1998**; 394:392–5.
 7. Scherf A, Lopez-Rubio JJ, Riviere Lc. Antigenic variation in *Plasmodium falciparum*. *Annu Rev Microbiol* **2008**; 62:445–70.
 8. Sander AF, Salanti A, Lavstsen T, et al. Multiple var2csa-type PfEMP1 genes Located at different Chromosomal loci occur in many *Plasmodium falciparum* isolates. *PLoS One* **2009**; 4:e6667.
 9. Brolin K, Ribacke U, Nilsson S, et al. Simultaneous transcription of duplicated var2csa gene copies in individual *Plasmodium falciparum* parasites. *Genome Biol* **2009**; 10:R117.
 10. Leke RFG, Bioga JD, Zhou J, et al. Longitudinal studies of *Plasmodium falciparum* malaria in pregnant women living in a rural Cameroonian village with high Perennial transmission. *Am J Trop Med Hyg* **2010**; 83:996–1004.
 11. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* **1993**; 58:283–92.
 12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $^{-\Delta\Delta CT}$ method. *Methods* **2001**; 25:402–8.
 13. Fouda GG, Leke RFG, Long C, et al. Multiplex assay for Simultaneous Measurement of antibodies to multiple *Plasmodium falciparum* antigens. *Clin Vaccine Immunol* **2006**; 13:1307–13.
 14. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. *Nature* **1998**; 395:851–2.
 15. Kraemer SM, Kyes SA, Aggarwal G, et al. Patterns of gene recombination shape var gene repertoires in *Plasmodium falciparum*: comparisons of geographically diverse isolates. *BMC Genomics* **2007**; 8:45.
 16. Magistrado PA, Minja D, Doritchamou J, et al. High efficacy of anti DBL4[var epsilon]-VAR2CSA antibodies in inhibition of CSA-binding *Plasmodium falciparum*-infected erythrocytes from pregnant women. *Vaccine* **2011**; 29:437–43.
 17. Nielsen MA, Pinto VV, Resende M, et al. Induction of adhesion-Inhibitory antibodies against placental *Plasmodium falciparum* parasites by using single domains of VAR2CSA. *Infect Immun* **2009**; 77:2482–7.
 18. Barfod L, Nielsen MA, Turner L, et al. Baculovirus-Expressed constructs Induce Immunoglobulin G that recognizes VAR2CSA on *Plasmodium falciparum*-infected erythrocytes. *Infect Immun* **2006**; 74:4357–60.
 19. Wang CW, Hermesen CC, Sauerwein RW, Arnot DE, Theander TG, Lavstsen T. The *Plasmodium falciparum* var gene transcription strategy at the onset of blood stage infection in a human volunteer. *Parasitol Int* **2009**; 58:478–80.
 20. Pasternak ND, Dzikowski R. PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite *Plasmodium falciparum*. *Int J Biochem Cell Biol* **2009**; 41:1463–6.