Antibodies to Chondroitin Sulfate A–Binding Infected Erythrocytes: Dynamics and Protection during Pregnancy in Women Receiving Intermittent Preventive Treatment


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Background. *Plasmodium falciparum* parasites that cause malaria in pregnancy express unique variant surface antigens (VSAs). Levels of immunoglobulin G (IgG) antibody to pregnancy-associated VSAs measured at delivery are gravidity dependent, and they have been associated with protection from disease. It is not known how these IgG responses develop in pregnant women receiving intermittent preventive treatment during pregnancy (IPTp) or whether IgG levels in early pregnancy predict pregnancy outcomes.

Methods. We performed longitudinal measurements of IgG antibody to VSAs by flow cytometric analysis of serum samples obtained from 549 Malawian women receiving IPTp. We examined fluctuations in IgG levels over time and associated the IgG levels noted at study enrollment with clinical outcomes.

Results. Levels of IgG antibody to pregnancy-associated VSAs were gravidity dependent. Overall, levels decreased while women were receiving IPTp, but the levels of the individuals were highly dynamic. Primigravidae (ie, women in their first pregnancy) often lack such antibodies [5], which explains, in part, the susceptibility of such women to malaria [6]. In cross-sectional studies, antibodies to pregnancy-associated VSAs have been associated with protection from maternal anemia, premature birth, or LBW infants among subsets of women at delivery [7, 8]. However, prospective longitudinal studies associating antibody levels with protection are lacking.

Conclusion. Levels of IgG antibody to pregnancy-specific VSAs decrease during receipt of IPTp. Antibody levels in early pregnancy did not predict clinical outcome. IPTp and decreasing malaria prevalence pose challenges for the evaluation of novel interventions for malaria during pregnancy.

Trial registration. Clinicaltrials.gov identifier NCT00131235.
regardless of infection status—reduces the clinical effect of malaria in pregnancy [9], but it might also interfere with the acquisition and maintenance of pregnancy-specific malaria immunity [10]. We prospectively measured levels of immunoglobulin G (IgG) antibodies to the VSAs of 2 placental type (CSA-binding) parasite lines and of 1 parasite line that binds to intercellular adhesion molecule–1 (ICAM-1) and expresses different VSAs. To investigate pregnancy-associated changes in antibody acquisition, levels of IgG antibody to VSAs were measured during pregnancy up until 6 months postpartum and, when possible, during successive pregnancies. Using one of the CSA-binding lines, we examined the association between pregnancy-specific VSA antibodies and adverse clinical outcomes of malaria during pregnancy in women receiving IPTp.

METHODS

Study population. The study population was a subset of women randomly selected from the Lungwena Antenatal Intervention Study (LAIS) cohort. Pregnant women who were between their 14th and 26th gestational weeks (gw) were enrolled at antenatal clinics in Lungwena, Malawi, between December 2003 and October 2006, after they provided written, informed consent. Women received IPTp in the form of sulfadoxine-pyrimethamine (1500 mg/75 mg) on ≥2 occasions, starting at the time of the enrollment visit and finishing at or before 37 gw; some women also received 2 doses of azithromycin (1000 mg) on 2 occasions.

Serum was separated from venous or finger prick blood samples obtained at enrollment (ie, at 14–26 gw); at 28–34 gw; and at 1, 3, and 6 months postpartum (mpp). Serum samples were frozen and shipped to Melbourne for IgG analysis. Giemsa-stained peripheral blood smears were produced for malaria microscopy performed at enrollment and at 28–34 gw. The hemoglobin level was measured at enrollment, at 28–34 gw, and at 1 mpp, by use of a HemoCue instrument. Pregnancy duration was calculated using an ultrasound assessment at enrollment.

Ethics approval was provided by the human research ethics committee at the Walter and Eliza Hall Institute of Medical Research and by the College of Medicine Research Ethics Committee at the University of Malawi.

P. falciparum lines. The P. falciparum lines that were used were the genetically distinct HCS3 and CS2 lines, which adhere to CSA [11, 12] and express var2csa as the dominant transcript [13]. We also used the parent line of CS2, E8B-ICAM (which adheres to ICAM-1 but not to CSA) [14]. Parasites were grown in group O red blood cells provided by the Australian Red Cross Blood Service. The PRBCs were cultured, as described elsewhere [15], in Roswell Park Memorial Institute (RPMI)–N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) medium with 0.2% w/vol NaHCO₃ and were supplemented with 0.5% Albumax II (GIBCO), for CS2 and E8B-ICAM, or 10% pooled heat inactivated non-immune human serum, for HCS3.

Measurement of IgG antibody levels. Flow cytometry was used to measure levels of IgG antibody to VSAs on the PRBCs, as described by Mount et al [16], with minor modifications. In summary, in a 96-well plate, heat-inactivated human serum samples (1:20 dilution; in duplicate) were coincubated with PRBCs at 0.1% hematocrit in a total volume of 50 μL of phosphate-buffered saline (PBS) with 1% newborn calf serum (PBS/NCS) for 30 min. The PRBCs were washed 3 times with PBS/NCS and were coincubated with 25 μL of polyclonal rabbit anti–human IgG antibody (DakoCytomation) that was diluted 1:100 in PBS/NCS for 30 min, washed again, and then incubated with Alexa Fluor 488 donkey anti–rabbit IgG (Invitrogen) diluted 1:500 in PBS/NCS with 10 μg/mL ethidium bromide for 30 min. PRBCs were then washed again, resuspended in PBS, and passed through a FACSCalibur flow cytometer (Becton-Dickinson). Cells were gated based on forward and side scatter characteristics, and they were plotted by fluorescence in channel 1 (Alexa Fluor 488) and channel 2 (ethidium bromide).

The adjusted geometric mean fluorescence intensity (MFI) for each sample was calculated by subtracting the MFI of non-infected red blood cells from that of gated ethidium bromide–positive cells. Using this result, a relative MFI between 0 and 100 was then calculated using negative and positive controls. Individual serum samples obtained from 6 adults who had not been exposed to malaria were used as negative controls. The positive control was pooled serum from women with known high levels of IgG antibody to CS2-VSA. Disagreeing duplicates, which were defined as having an adjusted MFI variance of >20% from their mean value and a relative MFI variance >10, were rerun (if possible) or excluded. Serial samples obtained from individual patients were run in the same assay. A total of 1139 samples obtained from 549 women were assayed for IgG antibody to CS2-VSA, 272 samples from 67 women were assayed for IgG antibody to HCS3-VSA, and 174 samples from 52 women were assayed for IgG antibody to E8B-VSA.

Levels of IgG antibody to schizont extract were measured using enzyme-linked immunosorbent assays, as described elsewhere [17], on 274 samples obtained from 65 women. In brief, schizont protein was prepared from 3D7 line parasite cultures, samples were tested in duplicate, and positive and negative controls from each plate were used to standardize results.

Data analysis. Samples were classified as being positive for IgG antibody to VSAs of a particular line, if the adjusted MFI was greater than the mean value plus 2 standard deviations of negative controls and if the relative MFI was >3. High or low IgG levels at enrollment were defined as being in the lower or top quartile, respectively, of all samples measured toward that parasite line. Trends in IgG antibody to VSA over time or
Table 1. Demographic and Clinical Characteristics of Participants in the Lungwena Antenatal Intervention Study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Primigravidae</th>
<th>Secundigravidae</th>
<th>Multigravidae</th>
<th>All</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravidity, % of women</td>
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<td>19.7</td>
<td>56.5</td>
<td>549</td>
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</tr>
<tr>
<td>Age, mean ± SD, years</td>
<td>18 ± 1.9</td>
<td>21.4 ± 3.3</td>
<td>28.9 ± 5.8</td>
<td>24.9 ± 6.7</td>
<td>549</td>
</tr>
<tr>
<td>Received SP</td>
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<td></td>
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<tr>
<td>2 doses</td>
<td>30.5</td>
<td>31.5</td>
<td>31.9</td>
<td>31.5</td>
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</tr>
<tr>
<td>Monthly</td>
<td>31.3</td>
<td>35.2</td>
<td>32.3</td>
<td>32.6</td>
<td>179</td>
</tr>
<tr>
<td>Monthly plus 2 doses of AZT</td>
<td>38.2</td>
<td>33.3</td>
<td>35.8</td>
<td>35.9</td>
<td>197</td>
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<td>HIV positive</td>
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<td>12.9</td>
<td>17.7</td>
<td>14.3</td>
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<td>Completed gestation at enrollment, mean ± SD, gw</td>
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<td>19.7 ± 3.1</td>
<td>20.1 ± 3.1</td>
<td>20 ± 3.0</td>
<td>549</td>
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<td>Hemoglobin level, mean ± SD, g/L</td>
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<td>At 14–26 gw</td>
<td>106.3 ± 17.4</td>
<td>114.8 ± 18.3</td>
<td>114 ± 19.1</td>
<td>111.2 ± 15.5</td>
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<td>At 28–34 gw</td>
<td>112.2 ± 14.6</td>
<td>112.8 ± 14.5</td>
<td>110.3 ± 16.1</td>
<td>112.3 ± 18.8</td>
<td>508</td>
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<tr>
<td>At 1 mpp</td>
<td>124.4 ± 17.0</td>
<td>123.5 ± 17.5</td>
<td>121.5 ± 19.0</td>
<td>122.6 ± 18.3</td>
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<td>At 14–26 gw</td>
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<td>42.6</td>
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<td>At 28–34 gw</td>
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<td>508</td>
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<tr>
<td>At 1 mpp</td>
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<td>14.4</td>
<td>22.6</td>
<td>19.7</td>
<td>497</td>
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<td>At 14–26 gw</td>
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<td>5.2</td>
<td>8.6</td>
<td>549</td>
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<tr>
<td>At 28–34 gw</td>
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<td>2.4</td>
<td>499</td>
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<td>At delivery</td>
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<td>1.9</td>
<td>4.4</td>
<td>114</td>
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<td>3.0 ± 0.4</td>
<td>3.1 ± 0.4</td>
<td>3.0 ± 3.4</td>
<td>494</td>
</tr>
<tr>
<td>Low birth weight</td>
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<td>9.4</td>
<td>1.8</td>
<td>6.3</td>
<td>494</td>
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<tr>
<td>Gestation duration at birth, mean ± SD, gw</td>
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<td>38.3 ± 2.4</td>
<td>38.9 ± 2.0</td>
<td>38.6 ± 2.3</td>
<td>548</td>
</tr>
<tr>
<td>Premature birth</td>
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<td>22.4</td>
<td>9.7</td>
<td>13.7</td>
<td>548</td>
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</tbody>
</table>

NOTE. Data are the percentage of women, unless otherwise indicated. AZT, azithromycin; gw, gestational week; HIV, human immunodeficiency virus; mpp, months postpartum; SD, standard deviation.

a Sulfadoxine-pyrimethamine (1500 mg/75 mg) was given once at enrollment (at 14–26 gw) and then once at 28–34 gw, or monthly starting at enrollment, or monthly starting at enrollment but with AZT (1000 mg) also given at enrollment and at 28–34 gw.
b Defined as a hemoglobin level <110 g/L.
c Parasitemia at 14–26 gw and at 28–34 gw was diagnosed as the presence of parasites in peripheral, cord, and/or placental blood at delivery.
d Included single births only.
e Defined as <2.5 kg.
f Defined as <37 gw completed.

Gravidity were determined using Cuzick’s test. Correlations of IgG levels were determined using Spearman’s ρ. Associations between the IgG antibody level and outcome were examined using multivariate linear regression. Comparison of high or low antibody levels with outcome was done using Student’s t test, and comparison of IgG levels between 2 populations was done using the Mann-Whitney U test. Women were grouped based on the level IgG antibody to CS2-VSA noted over the first 3 time points, by Ward’s linkage cluster analysis, which uses analysis of variance. Dendrograms were formed of clusters to determine the appropriate number of groups for Ward’s linkage analysis. Independence of antibody dynamics and gravidity categories was determined by χ² test. Maternal anemia was defined as a hemoglobin level <110 g/L, LBW was defined as a birth weight <2.5 kg, and prematurity was defined as birth before completion of 37 gw. Data were analyzed using Stata software (release 9.2; Stata).

RESULTS

IgG acquisition and dynamics. To investigate pregnancy-related changes in acquisition of malaria-specific antibody, IgG responses to VSAs and schizont extract were measured longitudinally in pregnant Malawian women (see Table 1 for a summary of study participants). Levels of IgG antibody to VSAs of parasite lines CS2, HCS3, and E8B, as well as levels of IgG antibody to schizont extract, were measured at enrollment (14–26 gw); at 28–34 gw; and at 1, 3, and 6 mpp. IgG antibody to CSA-binding HCS3 and CS2 PRBCs increased with gravidity at each time point, but responses to both ICAM-1–binding E8B and schizont extract did not (Figure 1). Levels of IgG antibody to HCS3 and CS2 were highly correlated with each other (r = 0.86; P < .001), IgG antibody to VSAs of HCS3 and CS2 were only weakly correlated with those against E8B (r = 0.27 and r = 0.34, respectively; P ≤ .001), and none of the
Figure 1. Levels of immunoglobulin G (IgG) antibody (expressed as the median value [center line], interquartile range [boxes], and smallest and largest observations [whiskers]) to variant surface antigens (VSAs) of chondroitin sulfate A (CSA)–binding parasite lines CS2 (A) and HCS3 (B), intercellular adhesion molecule–1 (ICAM-1)–binding parasite line E8B–ICAM-1 (C) and schizont extract (D), as categorized by gravidity (with “M” denoting multigravidae; “P,” primigravidae; and “S,” secundigravidae) at 5 time points (with “gw” denoting completed gestational weeks and “mpp” denoting months postpartum). Gravidity-dependent IgG levels are seen at each time point for both CSA-binding lines (x) but not at any time point for the ICAM-1–binding line (y) or for the schizont extract (z) (as determined by Cuzick’s trend test across ordered groups). Adjusted OD, optical density value standardized using positive and negative controls; relative MFI, mean fluorescence index relative to positive and negative controls.

responses to VSAs correlated with levels of antibody to schizont extract (x). Median levels of IgG antibody to CS2-VSA decreased over pregnancy, from enrollment to 1 mpp (y). Analysis of changes in IgG antibody to VSAs during pregnancy focused on CS2, because a smaller sample set was tested against HCS3-VSA. A similar downward trend was observed for levels of IgG antibody to HCS3-VSA from enrollment to 1 mpp, but this trend was not significant (P = .076). Interestingly, levels of antibody to schizont extract also decreased significantly during pregnancy (z = −2.27; P = .023), whereas levels of antibody to E8B VSAs did not (z = 1.05; P = .3).

Of note, the majority (71.3%) of primigravidae had detectable IgG antibody to CS2-VSA at enrollment, from as early as 14 gw (Figure 2). The levels of these antibodies increased with the gestational week at enrollment (r = 0.29; P = .001). High levels of IgG antibody (defined as the top quartile of all responses) were found in 9.9% of primigravidae at enrollment, and they were detectable by 17 gw.

IgG antibody responses in individuals were often highly dynamic over follow-up. To further investigate dynamics of IgG antibody responses to CS2-VSA, we performed Ward’s linkage cluster analysis with the use of data for 82 women who had IgG levels measured at enrollment, at 28–34 weeks, and at 1 mpp. The analysis separated women into 2 groups (Table 2). One group (which included most of the primigravidae) had consistently low levels of IgG antibody to CS2-VSA. This group was known as “stable-low responders,” because levels were generally low, and levels of IgG antibody to CS2-VSA noted at enrollment correlated with levels noted at 1 mpp (ρ = 0.54; P < .001) (Figure 3A). The other group had higher responses overall, but IgG antibody responses in individual women
Levels of immunoglobulin G (IgG) antibody to CS2–variant surface antigen (VSA) in individual primigravidae, according to their gestational week at enrollment. Levels of IgG antibody to CS2-VSA at enrollment correlate with gestational week at enrollment ($p = 0.29; P = .0011$, by Spearman’s $\rho$). The unbroken line denotes the cutoff for high IgG levels (relative MFI [ie, mean fluorescence index relative to positive and negative controls], $\geq 32.8$ [top 75th percentile of the total IgG level of the cohort]), and the broken line denotes the cutoff for positive IgG levels (an adjusted MFI $\geq 2$ standard deviations from the negative control and a relative MFI $\geq 3$). The majority of primigravidae had detectable IgG antibody to CS2-VSA, and high IgG antibody CS2-VSA levels were detected at as early as 17 gestational weeks.

**Figure 2.** Levels of immunoglobulin G (IgG) antibody to CS2–variant surface antigen (VSA) in individual primigravidae, according to their gestational week at enrollment. Levels of IgG antibody to CS2-VSA at enrollment correlate with gestational week at enrollment ($p = 0.29; P = .0011$, by Spearman’s $\rho$). The unbroken line denotes the cutoff for high IgG levels (relative MFI [ie, mean fluorescence index relative to positive and negative controls], $\geq 32.8$ [top 75th percentile of the total IgG level of the cohort]), and the broken line denotes the cutoff for positive IgG levels (an adjusted MFI $\geq 2$ standard deviations from the negative control and a relative MFI $\geq 3$). The majority of primigravidae had detectable IgG antibody to CS2-VSA, and high IgG antibody CS2-VSA levels were detected at as early as 17 gestational weeks.

showed large fluctuations between estimations; this group was known as “dynamic-high responders” (Figure 3B). In this group, IgG antibody to CS2-VSA at enrollment did not correlate with IgG antibody at 1 mpp ($p = 0.21; P = .2$).

IgG antibodies to CS2-VSA decreased postpartum, but they were still detectable in many women 6 months later. The decrease was greater in dynamic-high responders than in stable-low responders (Figure 3C), probably reflecting the higher levels of IgG antibody to CS2-VSA noted in dynamic-high responders at 1 mpp. At 6 mpp, however, IgG antibody to CS2-VSA was still detectable in 72.3% of women, and it was present at high levels in 18.4%.

**IgG levels over successive pregnancies.** Twenty-two women were enrolled over 2 successive pregnancies and had $\geq 1$ sample available for determination of levels of IgG antibody to CS2-VSA for each pregnancy. Overall, median levels of IgG antibody to CS2-VSA did not change significantly at any time over the 2 pregnancies ($P = .75$, by Kruskal-Wallis test). Secundigravidae who were also in the study as primigravidae (with a known IPTp history) ($n = 11$) were compared with secundigravidae who were not in the study as primigravidae (with an unknown IPTp history; $n = 88$). Secundigravidae who received IPTp as primigravidae had similar levels of IgG antibody to CS2-VSA at enrollment, compared with secundigravidae with an unknown IPTp history (median MFI [interquartile range [IQR]], 6.3 [1.4–10.4] and 8.2 [2.7–41.7], respectively ($P = .25$, by Mann-Whitney $U$ test), and there was no evidence that the groups differed in their ability to mount an immune response during the second pregnancy. The proportion of women who were high-dynamic responders was similar in secundigravidae who participated in the study during their previous pregnancy (3 of 9 women) and those who did not (5 of 9 women) ($P = .34$, by $\chi^2$ test).

**The association of IgG antibody to pregnancy-associated VSA with clinical outcomes.** After adjusting for gravidity, levels of IgG antibody to CS2-VSA did not correlate with maternal hemoglobin levels at 28–34 gw (regression coefficient [95% confidence interval [CI]], 0.018 [−0.03 to 0.066]) ($P = .45$), birth weight (−0.00023 [−0.0016 to 0.0011]) ($P = .74$), or gestational week at birth (−0.0029 [−0.0097 to 0.004]) ($P = .41$). For primigravidae, secundigravidae, and multigravidae, clinical outcomes, maternal hemoglobin level, birth weight, and gestational week at birth did not differ significantly between women who had high or low antibody levels at enrollment (Table 3). Levels of IgG antibody to CS2-VSA at enrollment were lower among human immunodeficiency virus–infected women (median MFI [IQR], 7.2 [3.6–33.1]) than among human immunodeficiency virus–uninfected women (median MFI [IQR], 13.9 [4.9–40.7]), but this difference did not reach significance ($P = .15$).

**DISCUSSION**

The recommendation that all pregnant women in malaria-endemic regions of Africa receive IPTp [18] makes it important to understand the development of naturally acquired immunity to malaria in pregnancy in the context of IPTp. We measured levels of IgG antibody to the VSAs of up to 3 parasite lines at different time points during and after pregnancy in Malawian women exposed to malaria who were receiving IPTp; in some cases, we were able to monitor women over successive pregnancies.

In keeping with studies published elsewhere [5, 15, 19, 20], levels of IgG antibody to the VSAs of the 2 CSA-binding lines showed gravidity dependence, whereas levels of IgG antibody to VSAs of E8B-ICAM (which binds to CD36 and ICAM-1) and to schizont extract did not vary with gravidity (Figure 1). The high correlation between levels of IgG antibody to VSAs of the 2 CSA-binding lines tested suggests either that cross-reactive epitopes are commonly recognized by this population or that women rapidly develop a breadth of antibody responses that allow them to recognize a variety of CSA-binding isolates [21].

The use of ultrasound at enrollment to date pregnancy accurately allowed us to examine the timing of acquisition of pregnancy-associated IgG antibody. Most primigravid women had detectable IgG antibody to CS2-VSAs at enrollment, sometimes at as early as 14 gw (Figure 2), the lower limit for enrollment. This was not caused by previous pregnancies, because...
primigravid women had no previous history of miscarriage or stillbirth. Our results suggest that IgG antibody to pregnancy-associated VSA develops soon after the placental blood spaces develop (ie, as early as 8–9 gw [22]), as suggested by Cox et al [23], and rather earlier than was first reported by Staalsoe et al (at 20 gw) [20].

Levels of IgG antibody to CSA-binding parasites decreased over pregnancy, probably because effective suppression of infection by IPTp resulted in a low prevalence of parasitemia during follow-up and, therefore, in a restricted boosting of antibody responses. This observation contrasts with the findings of previous studies, in which women received no or inadequate prophylaxis and in which antibody levels increased over the course of pregnancy [20, 23]. Our prospective data are consistent with previous retrospective observations that increasing numbers of IPTp doses were associated with lower levels of IgG antibody to pregnancy-associated VSA in the third trimester [10]. Despite these overall reductions in levels of IgG antibody to CS2–variant surface antigen (VSA) during follow-up and delivery, some women experienced large fluctuations in responses (Figure 3). Similarly, dynamic patterns of individuals’ levels of antibody to VSA have been seen elsewhere in children [24], and this finding, together with our observations, counsels against overinterpretation of data from single time points.

Many women acquired new or increasing levels of IgG antibody to CS2-VSA at ≥1 time point under study, despite the low prevalence of parasites observed during follow-up. This finding suggests that the overall rates of exposure to pregnancy-associated parasites were higher than those recorded by our infrequent blood slide determinations. Careful estimation of the burden of malaria in pregnancy may require frequent screening throughout pregnancy (as is currently practiced in some low-transmission settings [25]). Also of use may be more-sensitive techniques, such as examination of placental histologic findings (which frequently reveals infections not seen in peripheral blood, as well as past infections [26]) and polymerase chain reaction (PCR). PCR can detect low-density infections that are postulated to occur in association with suppression of parasitemia in the context of IPTp and that may assist in the development of immunity [27].

In this study, almost all primigravidae (and a number of secundigravidae and multigravidae) had consistently low levels of IgG antibody to CS2-VSA. The consequences of a lack of pregnancy-specific immunity at the end of a first pregnancy on the susceptibility to malaria during a second pregnancy are unknown, but one study suggests that chemoprophylaxis during a first pregnancy does not increase rates of malaria or complications of the disease in a second pregnancy [28]. When we were able to monitor antibody levels over 2 pregnancies, we found no evidence that IPTp during the first pregnancy affected the development of antibody responses during the second pregnancy; however, the number of women studied was small, and larger longitudinal studies conducted over successive pregnancies would be of great value in evaluating the effects of IPTp on pregnancy-specific immunity. If IPTp does interfere with developing antibody responses, it may render women susceptible to malaria in subsequent pregnancy, and therefore protective measures should not be restricted to primigravidae. Decreasing malaria transmission and, therefore, exposure during pregnancy may further increase the proportion of potentially susceptible women.

Levels of pregnancy-specific IgG antibody have previously been noted to decrease in the postpartum period [20], and our study agrees with this finding, which is consistent with the hypothesis that exposure to these antigens is largely limited to pregnancy. Nevertheless, our data suggest that these responses persist until at least 6 months postpartum in many women. It is unknown whether antibody responses in early pregnancy predict protection from malaria-associated adverse pregnancy outcomes. The lack of association between IgG antibody to CS2-VSA at enrollment and birth weight, maternal hemoglobin level, and gestational week at birth suggests that these early IgG levels did not have an important role in protection from disease in this cohort. This may be because participants received ad-
Figure 3. Levels of immunoglobulin G (IgG) antibody to CS2–variant surface antigen (VSA) over and after a single pregnancy of 2 Ward’s linkage cluster analysis groups, with formation based on individual pregnancy-associated IgG levels measured from 14–26 gestational weeks (gw) to 1 month postpartum (mpp) (A), individual levels of IgG antibody to CS2-VSA in stable-low responders (women whose levels of IgG antibody to CS2-VSA were generally low and in whom levels of IgG antibody to CS2-VSA noted at enrollment correlated with levels noted at 1 mpp) (B), and individual levels of IgG antibody to CS2-VSA in dynamic-high responders (the group of women who had higher responses overall, although the IgG antibody responses in individual women showed large fluctuations between estimations) (C). The median (interquartile range) IgG antibody level of the stable-low responders (black) and dynamic-high responders (gray) is shown. IgG antibody levels in dynamic-high responders decreased postpartum (, Cuzick’s trend test across ordered groups) and were higher at 6 mpp, compared with those of the stable-low responders (, by the Mann-Whitney U test). IgG antibody levels of stable-low responders did not change postpartum (, by Cuzick’s trend test across ordered groups). Relative MFI, mean fluorescence index relative to positive and negative controls.

In conclusion, this study has several important findings of relevance to understanding the relationship between pregnancy-specific antibodies, malaria interventions, and pregnancy outcomes. Pregnancy-associated antibody levels may be detectable early in a first pregnancy, but they remain low among women receiving IPTp, potentially increasing their susceptibility to malaria during pregnancy with IPTp or because few women had parasitemia during follow-up, suggesting that the malaria-attributable fractions of LBW, anemia, and premature birth may be low. Alternatively, the highly dynamic nature of the antibody response may make identification of correlations difficult. We observed that, among women who developed significant antibody responses to CS2-VSA at ≥1 time points, the IgG levels measured at enrollment were not correlated with the same responses measured shortly after delivery.

In this data set, the lack of correlations between IgG antibody against pregnancy-associated VSAs and clinical outcomes do not encourage hope that simple measures of antibody at booking might identify women who are at risk of malaria-related complications. It remains to be seen whether functional measures of immunity, such as prevention of adhesion to CSA [8] or opsonizing antibodies that facilitate phagocytic clearance of PRBCs [29, 30], are more relevant measures of immunity. Among women receiving IPTp, many of whom also used insecticide-treated nets, few experienced further documented episodes of parasitemia, and factors other than malaria may be playing a major role in causing adverse outcomes, such as anemia and LBW. Although antibody levels in later pregnancy could be a better indication of protection [7, 8], prospective identification of women at high risk remains an elusive goal.

The decreasing prevalence of malaria reported from a number of sites in Africa [31, 32], combined with the lower-than-expected incidence of infection during follow-up in our study, has important implications for future study designs. The prevalence of parasites among our participants was significantly lower than that previously reported from this area of Malawi [33, 34–36]. Enrollment of larger numbers of women in intervention studies [37] may be necessary when women receive insecticide-treated nets and IPTp as the standard of care, and this may be very relevant to future studies of malaria drugs or vaccines for pregnant women.

The present study faced a number of limitations. The unexpectedly low prevalence of malaria in this cohort diminished the power of the study. Being a community-based study, it was rarely possible to collect data at delivery, and so we were unable to investigate placental infection. Limited samples and logistic constraints prevented us from examining immune responses to a broader range of parasite isolates and from testing responses to all 3 parasite lines in every sample. Nevertheless, we were able to make a number of important findings.
## Table 3. Comparison of Clinical Outcomes in Women with High or Low Levels of Antibody to CS2–Variant Surface Antigen (VSA) at Enrollment, as Classified by Gravidity

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>Primigravidae</th>
<th>Secundigravidae</th>
<th>Multigravidae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With a low antibody level&lt;sup&gt;a&lt;/sup&gt;</td>
<td>With a high antibody level&lt;sup&gt;a&lt;/sup&gt;</td>
<td>With a low antibody level&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Value</td>
<td>n</td>
</tr>
<tr>
<td>Hemoglobin level, mean ± SD, g/L</td>
<td>41</td>
<td>113.2 ± 15.3</td>
<td>10</td>
</tr>
<tr>
<td>Birth weight, mean ± SD, kg</td>
<td>36</td>
<td>2.95 ± 0.42</td>
<td>9</td>
</tr>
<tr>
<td>Gestational weeks at birth, mean ± SD, no.</td>
<td>43</td>
<td>38.7 ± 2.7</td>
<td>11</td>
</tr>
</tbody>
</table>

### NOTE.
- SD, standard deviation.
- <sup>a</sup> Defined as the lower/higher quartile of all samples measured against CS2-VSA.
- <sup>b</sup> *P* values were obtained by use of Student’s *t* test, to compare clinical outcomes between women classified as having high and low antibody levels.
laria in subsequent pregnancies. Levels of antibody to pregnancy-associated CS2 parasites are highly dynamic throughout pregnancy, suggesting that isolated antibody determinations should be treated with caution. The lower-than-expected parasite prevalence and the lack of association between pregnancy-specific IgG levels and clinical outcomes suggest that evaluating the protective efficiency of new interventions, whether drugs or vaccines, may be increasingly challenging in the context of insecticide-treated nets and IPTp use and decreasing prevalence of malaria.

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References