Variable Adhesion Abilities and Overlapping Antigenic Properties in Placental Plasmodium falciparum Isolates

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Background. Pregnancy-associated malaria is characterized by selection and multiplication, in the placenta, of a distinct population of Plasmodium falciparum expressing particular variant surface antigens (VSAs) that adhere to chondroitin sulfate A (CSA).

Methods. The adhesion of 40 freshly collected placental parasite isolates to bovine CSA and human placental low-sulfated chondroitin proteoglycans (CSPGs) was investigated. Plasma samples from 30 pregnant women were used to test, by flow cytometry, their recognition of and their adhesion-inhibition capacity toward 6 of these isolates.

Results. Adhesion to CSA and CSPGs varied between isolates but was strongly correlated between receptors ($P<.001$). Adhesion of isolates to receptors strongly and negatively correlated with low birth weight (LBW) of the neonate (odds ratio [95% confidence interval], 5.2 [1.1–25.1]). In plasma samples from pregnant women, the level of specific immunoglobulin G against each placental isolate (anti-VSAPAP) strongly correlated with the level of anti-VSA PAP antibodies against all other isolates ($P<.01$) and increased with parity in all isolates ($P<.05$). Conversely, adhesion-inhibitory antibodies did not correlate with isolates or with the level of anti-VSAPAP antibodies.

Conclusion. The level of adhesion of placental parasites to chondroitin sulfate receptors is an important risk factor for LBW. Parasite heterogeneity suggests that they are composed of mixed adhesion phenotypes capable of inducing immune responses to a range of different and overlapping targets.

Much of the pathological process associated with infection with the human malaria parasite Plasmodium falciparum occurs as a consequence of adhesion-related phenomena [1]. Infected red blood cells (IRBCs) containing the mature stages of the parasite adhere to endothelial cells (sequestration) and to uninfected red blood cells (RBCs) (rosetting). Deep-tissue sequestration is believed to favor survival of the parasite by preventing its clearance during passage through the spleen [2]. In areas where malaria is endemic, P. falciparum infection is more common in pregnant women than in other adults [3]. Women are more susceptible to malaria during their first pregnancy, even if they have been previously immune. The prevalence rates of peripheral and placental parasitemias, as well as parasite densities, are highest in primigravid women; women become less susceptible to infection after their second pregnancy [4–6]. Pregnancy-associated malaria (PAM) is characterized by the selection and multiplication of P. falciparum parasites presenting with a peculiar adhesion phenotype. Such parasites adhere in the intervillous space of the placenta, where specific adhesion of IRBCs is observed on the surface of syncytiotrophoblasts [7, 8]. Although it has proven to be difficult to determine whether PAM results in impaired placental development and function, the accumulation of IRBCs in this anatomical site correlates with low birth weight (LBW), abortion, and maternal mortality [9, 10]. The syncytiotrophoblast expresses a large amount of chondroitin sulfate A (CSA) [8], and placental parasite isolates preferentially adhere to CSA in vitro, whereas parasites from men and nonpregnant women usually do not [7, 11]. Accumulated evidence shows that CSA mediates the
adhesion of IRBCs in the human placenta [7], and low-sulfated chondroitin proteoglycans (CSPGs) in the placenta represent the major factor mediating sequestration there [12].

This adhesion phenotype is associated with the expression of distinct variant surface antigens (VSAs) on the surface of IRBCs. The major P. falciparum antigen involved in adhesion is the variant P. falciparum erythrocyte membrane protein–1 (PfEMP1), which is expressed on the surface of IRBCs [13, 14]. Each variant of PfEMP1 can mediate the adhesion of IRBCs to a specific host molecule, including CSA [15–17]. Pregnancy-associated parasites (PAPs) do express a conserved family of PfEMP1. The difference in susceptibility to PAM, between primigravid and multigravid women, is attributed to the lack, in primigravid women, of antibodies against this particular VSA, which inhibit the adhesion of IRBCs to CSA [18]. Primigravid women with noninfected placentas are associated with fewer antibodies agglutinating either CSA-selected or peripheral blood isolates from pregnant women [19]. Studies have also reported increasing levels of antibodies specific to VSAs expressed by CSA-adhering IRBCs, with increasing gravidity, suggesting gravidity-dependent increased protection against PAM [20–22]. Although most evidence concerns the biological relevance of PfEMP1, a few other variant proteins expressed on the surface of IRBCs—including the rifins, stevor, clag, sequestrin, and modified band 3 protein—could also contribute to the induction of an immune response (reviewed in [23]), which may have different implications in the adhesion process in the placenta and in the evolution of the infection. The relationship between plasma level of anti-VSAPAP antibodies (as assessed by flow cytometry) and inhibition of sequestration in the placenta is still unclear. Several epidemiological studies have reported an association between the level of anti-VSAPAP or adhesion-inhibition antibodies and protection against PAM [21, 24]. Other studies reported the ability of VSAPAP-specific IgG to interfere with in vitro adhesion to CSA [18, 20, 21, 25], but convincing evidence has not been fully documented. In the present study, we analyzed the adhesion of freshly collected placental P. falciparum isolates to CSA, using bovine CSA and purified human placental CSPGs. We also used plasma samples from pregnant women, to measure their levels of anti-VSAPAP IgG and their ability to inhibit the adhesion of placental isolates to chondroitin sulfate (CS) receptors.

SUBJECTS, MATERIALS, AND METHODS

Collection of parasites. Samples were collected in November 2003 at 2 health centers in Guediawaye, a suburb of Dakar, Senegal. Women attending the maternity wards were explained the nature of the project, and informed, verbal consent was obtained before delivery. The human-experimentation guidelines of both the French and the Senegalese governments were followed in the conduct of clinical research. The study was approved by the ethical committee of the Ministry of Health, Senegal. Women were screened for P. falciparum infection by use of the immunochromatography Core Malaria Pf test (Core Diagnostics), and 40 women presenting with an infection were enrolled. Clinical data were collected, as well as 10 mL of peripheral blood in Vacutainers (Becton Dickinson) containing sodium heparin. The placenta was collected, and a placental thick blood smear was made for microscopic examination. The mean ± SD age of these women was 24.1 ± 6.1 years; 15 were primigravid, 6 were secundigravid, and 19 were multigravid. At birth, neonates were weighed by use of an electronic balance—16 presented with an LBW (<2500 g).

P. falciparum IRBCs were collected from infected placentas (parasite density: range, 0.1%–50%; mean ± SD, 12.8 ± 12.7) by flushing the placenta with 0.1% sodium heparin in PBS, as described elsewhere [26]. Monocytes were removed by incubation for 1 h at 37°C in petri dishes, in 50% fetal calf serum–RPMI 1640 medium. In selected cases, parasite densities were enriched by magnetic separation using Macs columns (Miltenyi Biotec).

Collection of plasma samples. Plasma samples from 30 women were selected from a plasma bank of a cohort study of pregnant women conducted 2 years earlier in Thiadiaye, Senegal, where malaria is seasonally transmitted during the rainy season, from September to December, with ~15 infected bites/person/year. These samples were previously selected on the basis of their variable reactivity (low or high) against placental isolates. Six plasma samples from nulligravid women living in the same area were also included and were used as controls. For all experiments, these samples were coded and tested blindly. For these plasma donors, peripheral and placental thick and thin blood smears were examined by microscopy. Active placental malaria infection was defined according to placental histologic evaluation [27] and/or to examination of peripheral and placental blood smears and species-specific P. falciparum polymerase chain reaction. ABO-RBC typing was performed.

Purification of CSPGs from the placental intervillus space. The placental intervillous CSPGs were purified from a naive European placenta immediately after delivery. CSPGs were isolated by the combination of ion-exchange salt-gradient chromatography (DEAE-Sephalcel), CsBr density-gradient ultracentrifugation, and sepharose Cl-6-B gel–filtration chromatography, as described elsewhere [12].

Adhesion and adhesion-inhibition assays. The bovine CSA (Sigma) and the human CSPGs were coated as circular spots onto untreated Falcon petri dishes (Becton Dickinson). A Dako pen for immunochemistry was used to draw several circles of ~4 mm diameter on the petri dishes. Twenty microliters of CSA (10 µg/mL in PBS) or CSPGs (2.5 µg/mL in PBS) was spotted into each circle and incubated overnight at 4°C in a humidified chamber [7]. Spots were inhibited with 20 µL of 2% bovine
serum albumin in PBS (PBS-BSA) for 2 h at room temperature. All samples were tested in duplicate, and the mean values were used. Mean variation for replicates was 15% with CSA and 22% with CSPGs.

Isolates freshly collected from infected placentas were washed in RPMI 1640–HEPES (pH 6.8) and resuspended in binding buffer (RPMI without NaHCO₃, 10% human serum [pH 6.8]). Twenty microliters of IRBC suspension (mean ± SEM parasite density, 31.5 ± 1.5; 2% hematocrit) were layered onto CSA- or CSPG-coated spots. After 40 min of incubation at room temperature, the unbound cells were removed by 4 gentle washes with RPMI medium, and bound cells were fixed with 2% glutaraldehyde and were stained with Giemsa. Adhesion was quantified as the number of IRBCs bound per millimeter squared, estimated on 20 high-power fields.

RBCs from peripheral blood were washed 3 times and incubated in RPMI medium for 18–20 h at 37°C in a candle jar, allowing maturation of ring stages to trophozoites [28]. The suspension was enriched by passage over a Macs column and was allowed to adhere to CSA and CSPGs spots. All matched pairs (peripheral and placental) were tested together on the same plate, in duplicate.

For adhesion-inhibition assays, only parasite isolates from donors with O-type RBCs were used. Freshly collected isolates from infected placenta were adjusted to 40% parasite density (2% hematocrit) in RPMI medium and preincubated with human plasma (diluted 1:5) in 96-well round-bottom microtiter plates. After 60 min of incubation at 37°C with intermittent mixing, the suspension was allowed to adhere to immobilized CSA or CSPGs, as described above. Antiadhesion activity was expressed as the percentage of adhesion, compared with the control for adhesion, defined by the mean adhesion occurring with plasma samples from 6 nulligravid Senegalese women. This adhesion did not significantly differ from that of PBS. Mean variation for replicates was 29% with CSA and 28% with CSPGs.

Flow cytometry. Antibodies (IgG) against VSAs were measured by flow cytometry, using the same isolates that were used for adhesion-inhibition assays, as described elsewhere [29]. In brief, IRBCs containing late stages from infected placentas consisting of O-type RBCs (40% parasite density and 2.5% hematocrit) were washed 3 times in PBS-BSA. Cells were labeled (1 μL/10⁵ IRBCs) with ethidium bromide (0.1 mg/mL) and were preincubated with test plasma samples (diluted 1:20 in PBS-BSA) in 96-well round-bottom plates. After 30 min of incubation at 4°C in the dark, cells were sequentially incubated, by use of the same conditions, with goat anti–human IgG (A473; Dako; diluted 1:250) and fluorescein isothiocyanate (FITC)–conjugated rabbit anti–goat IgG (F250; Dako; diluted 1:100). Between each labeling step, samples were washed 3 times in PBS-BSA buffer and were analyzed by use of a FACSCalibur (Becton Dickinson) cytometer. For each sample, the level of IgG recognizing VSA was expressed as the FITC median fluorescence intensity (MFI) on the labeled IRBCs, gated according to ethidium-bromide fluorescence. For each isolate, the threshold for positivity was previously defined as 2 SDs above the mean of MFI obtained with plasma samples from 30 nulligravid Senegalese women. The proportion of labeled IRBCs was also calculated, as described by Kinyanjui et al. [30]. For each isolate, this proportion was strongly correlated with MFI (r = 0.84; P < .0001), demonstrating that MFI reflects the proportion of cells labeled. Given the level of correlation of both variables, only data on MFI are presented here.

Statistical analysis. Adhesion of IRBCs (per millimeter squared) between isolates was compared by use of analysis of variance. Differences between groups were tested by use of the appropriate Student’s paired or unpaired t test or a nonparametric test (Wilcoxon or signed rank test). Correlations were examined by use of Pearson’s test or nonparametric Spearman’s rank test. Analysis of possible confounding factors was performed by use of multivariate linear regression. Logistic regression analysis was used for dependent binary variables, and odds ratios for expressing effects of adhesion activity were derived. The significant limit was P < .05. Statistical analysis was performed by use of SAS software (version 8.2; SAS Institute).

RESULTS

Adhesion of P. falciparum IRBCs collected from human placentas. To determine the variability of the adhesion capacity of the isolates, we first analyzed the adhesion of 40 freshly
collected isolates to bovine CSA and purified human CSPGs. The CSPG2 fraction from placental intervillous space was successfully isolated by successive DEAE-sephacel chromatography [12]. The adhesion assay was previously optimized by use of CSA-selected and -unselected laboratory strains (PA<sup>CSA</sup> and PA) maintained in the laboratory. The CSPGs supported significant levels of adhesion for IRBCs, and optimal adhesion (reaching 5000 parasites/mm<sup>2</sup>) was exhibited at a coating concentration of 2.5 µg/mL in PBS, whereas adhesion of unselected strains was generally <10 parasites/mm<sup>2</sup>. Isolates from all women yielded variable adhesion abilities; the number of IRBCs that bound ranged from 46 to 3700 parasites/mm<sup>2</sup> for bovine CSA and from 37 to 3450 parasites/mm<sup>2</sup> for human CSPGs (figure 1). Adhesion was strongly related between bovine CSA and human CSPGs ($r = 0.88; P < .0001$), thus establishing their functional similarities. Microscopic examination of placental samples revealed that they were essentially composed of trophozoite and schizont stages (ring stages were rare). The level of adhesion did not differ according to the distribution of the parasite maturation stages present in the samples, as assessed by the trophozoite/schizont ratio for each sample ($P = .26$). Comparison of the adhesion properties from peripheral and placental parasites collected at birth was performed with 10 pairs of matched samples that originated from the same women. The mean level of adhesion to CSA ($P = .84$) and CSPGs ($P = .69$) was similar for both types of parasites. The adhesion of peripheral and placental parasites to both CSA and CSPGs was strongly correlated (figure 2; $r = 0.66; P = .04$), suggesting that peripheral ring-stage parasites are likely composed, in a large majority, of the progeny of mature forms sequestered in the placenta.

### Table 1. Risk factors for low birth weight related to the adhesion of parasites to chondroitin sulfate receptors.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>AOR (95% CI)</th>
<th>$P$</th>
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<tr>
<td>High level of adhesion to CSPGs&lt;sup&gt;a&lt;/sup&gt; ($\geq 780$ parasites/mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>5.22 (1.08–25.13)</td>
<td>.04</td>
</tr>
<tr>
<td>Primigravid and secundigravid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.28 (0.69–15.65)</td>
<td>.13</td>
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<tr>
<td>Placental parasitemia</td>
<td>1.03 (0.98–1.09)</td>
<td>.2</td>
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**NOTE.** AOR, adjusted odds ratio; CI, confidence interval; CSPGs, placental low-sulfated chondroitin proteoglycans.<br><br><sup>a</sup> Reference class: low level of adhesion to CSPGs ($< 780$ parasites/mm<sup>2</sup>).

<sup>b</sup> Reference class: multigravid ($n > 2$).
Table 2. Levels of 6 Plasmodium falciparum placental isolates in the presence of plasma samples from pregnant women.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>55</th>
<th>53</th>
<th>51</th>
<th>49</th>
<th>48</th>
<th>47</th>
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<tr>
<td>A</td>
<td>1.9 (1.9, 1.9)</td>
<td>5.0 (3.5, 6.5)</td>
<td>13.2 (13.2, 13.2)</td>
<td>82.8 (76.6, 89.4)</td>
<td>89.2 (71.0, 110.53)</td>
<td>145.7 (104.5, 187.4)</td>
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<tr>
<td>B</td>
<td>23.9 (19.4, 28.4)</td>
<td>81.4 (77.1, 85.5)</td>
<td>122.4 (78.9, 165.8)</td>
<td>37.2 (31.9, 42.5)</td>
<td>142.2 (105.3, 157.9)</td>
<td>21.6 (4.5, 38.7)</td>
</tr>
<tr>
<td>C</td>
<td>23.5 (13.4, 33.6)</td>
<td>9.0 (6.9, 11.0)</td>
<td>35.5 (21.0, 50.0)</td>
<td>57.4 (51.1, 63.8)</td>
<td>29.7 (26.3, 34.2)</td>
<td>14.4 (8.1, 20.7)</td>
</tr>
<tr>
<td>D</td>
<td>123.7 (97.0, 150.7)</td>
<td>61.8 (42.1, 81.4)</td>
<td>77.0 (76.3, 77.6)</td>
<td>31.9 (14.9, 48.9)</td>
<td>25.9 (26.3, 26.3)</td>
<td>30.1 (27.9, 32.4)</td>
</tr>
<tr>
<td>E</td>
<td>78.3 (66.4, 90.3)</td>
<td>92.5 (67.7, 117.2)</td>
<td>103.3 (72.4, 134.2)</td>
<td>91.8 (74.5, 109.4)</td>
<td>25.9 (26.3, 26.3)</td>
<td>13.0 (8.1, 18.0)</td>
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**NOTE.** Data are mean adhesion level (individual values) percentages of the adhesion obtained with nonparous controls. To illustrate the relation between isolates, 5 plasma samples are shown. Values in bold type indicate mean adhesion levels below the median of the corresponding isolate.
Figure 4. Relationship between the level of surface recognizing antibodies, measured on freshly collected placental infected red blood cells. The level of each antibody was measured in plasma samples from 30 Senegalese women, by use of 6 freshly collected placental isolates. **Top panels:** Levels of anti-variant surface antigen (VSA) antibodies (median fluorescence intensity [MFI], determined by flow cytometry) measured on all 6 isolates significantly increased with parity (illustrated by isolates 51, 53, and 55). **Middle panels:** Levels of anti-adhesion antibodies measured on 5 of 6 isolates were not related to parity (illustrated by isolates 51 and 55). The last isolate (isolate 53) yielded a parity-dependent increase of inhibition pattern (placental low-sulfated chondroitin proteoglycan [CSPG]-coated plate). **Bottom panels:** Levels of anti-VSA antibodies and adhesion-inhibition activity to CSPGs were not significantly correlated between 4 of 6 isolates (isolate 51) but were significantly correlated between 2 isolates (isolates 53 and 55).

**DISCUSSION**

The sequestration of *P. falciparum* IRBCs in the placenta plays a key role in the pathogenesis of PAM. Parasite isolates obtained from infected placentas preferentially adhere to CSA, and sequestration of IRBCs in the placenta appears to be mediated by CSPGs expressed in the intervillous space of the placenta. Adhesion of placental isolates demonstrated that all isolates
naturally sequestering in the placenta adhere in vitro to both CSA and CSPGs. This is consistent with prior reports on the properties of placental parasites. On the basis of the observed adhesion patterns of freshly collected placental isolates to either CSA or CSPGs, we have demonstrated that CSPGs are functionally strongly related to CSA. The strong data correlation between CSA and CSPGs not only validates structural homology of both receptors but also strengthens the observations made in this model. The differences in the adhesion patterns observed between individual isolates demonstrate differential interactions between placental parasite populations and the CS receptors. This variability in the adhesion pattern to CS receptors, which was not related to parasite density, suggests that the accumulation of IRBCs in the placenta does not result from the selection of parasites with a uniform adhesion phenotype. The process may be more complex and may involve host factors such as the underlying immunity background, as well as additional receptors.

Some studies have compared placental parasites to those circulating in the periphery of the same host, mainly by genotyping polymorphic markers, such as merozoite surface protein genes. These studies showed important genetic overlaps between the 2 populations of parasites [31, 32]. Association between the VSAs expressed by peripheral parasites and those expressed by placental parasites was also reported [33]. Our data on 10 matched placental and peripheral blood samples highlight strong positive correlations in the adhesion ability of isolates from the 2 compartments to both CSA and CSPGs. Despite the 24-h difference in the life cycles of the parasites collected in these anatomical compartments, parasite populations in the periphery share adhesive properties with those in the placenta, as far as their adhesion abilities to CS receptors are concerned. Other investigators have reported differences in the adhesion phenotypes of these parasite populations [11]. This could be due to differences in malaria transmission between study areas.

Our major finding is that, in our study population in Senegal, the level of adhesion of placental isolates to CS receptors is associated with significantly reduced birth weight in neonates. To our knowledge, this is the first time that this phenomenon has been clearly demonstrated. LBW is the strongest risk factor for mortality during infancy [34] and has been related to the accumulation of IRBCs in the placenta [4, 10, 35]. No complete understanding of the biological mechanisms by which placental infection causes LBW has been reached so far. Although placental parasitemia was also related to LBW in the present study, the adhesion of isolates to CS receptors was stronger and was independently associated with LBW, demonstrating that the level of adhesion of parasites to CS receptors is an important risk factor for LBW.

Many studies have reported that CSA is the principal receptor for adhesion of parasites in the placenta and have suggested that the relative resistance in multigravid women is due to the acquisition of antibodies against CSA-adhesion parasites [7, 11, 18]. In the present study, the adhesion-inhibition profiles yielded by several plasma samples differed between isolates, highlighting differences in the antigenic constitution of the isolates. Similar differences have been reported for agglutinating antibodies [11]. However, the cross-inhibitory effect of plasma samples against the different isolates demonstrates conserved and shared epitopes among the isolates used in the present study, as well as with those previously encountered by the plasma donors. These observations are in line with those on freshly collected isolates previously reported by Fried et al. [18].

To elucidate the ability of anti-VSAant antibodies to inhibit adhesion of placental isolates to CS receptors, we analyzed plasma samples from pregnant women. We have shown that the plasma level of anti-VSAant IgG among gravid women is not predictive of their inhibitory activity toward adhesion of parasites to CS receptors. Some samples that were either negative or weakly positive by flow cytometry appeared to strongly inhibit adhesion. Such a relation is observed when data on flow cytometry are expressed as MFI or as the proportion of IRBCs labeled (data not shown). The lack of association between antibodies recognizing VSAant and a functional role in preventing adhesion to CS receptors suggests that separate or overlapping epitopes are involved in these 2 phenomena. This observation supports the idea that placental parasites could exhibit mixed adhesive properties consistent with their differential abilities to adhere to CS receptors. Other studies have demonstrated that samples strongly positive by flow cytometry more effectively inhibit adhesion to CSA [20] or have reported significant association between the level of CSA-adhesion inhibitory antibodies and that of specific IgG [25]. These apparent discrepancies might result from the fact that the present study was performed using freshly collected placental isolates without any

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<tr>
<td>55</td>
<td>0.383&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.208</td>
<td>-0.216</td>
<td>-0.252</td>
<td>-0.119</td>
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<tr>
<td>53</td>
<td>0.535&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.154</td>
<td>-0.080</td>
<td>-0.063</td>
<td>-0.217</td>
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<tr>
<td>51</td>
<td>0.614&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.591&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.012</td>
<td>0.059</td>
<td>0.140</td>
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<tr>
<td>49</td>
<td>0.723&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.759&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.703&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.238</td>
<td>0.300</td>
<td></td>
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<tr>
<td>48</td>
<td>0.708&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.760&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.763&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.916&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.195</td>
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<tr>
<td>47</td>
<td>0.461&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.722&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.636&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.645&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.601&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> P < .05.

<sup>b</sup> P < .001.

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in vitro culture, whereas previous studies relied on in vitro–selected *P. falciparum* strains. Such freshly collected isolates are likely to more accurately reflect the parasite populations encountered in vivo in the placenta.

Flow cytometry probably measures the spectrum of antibodies directed against a range of targets on the IRBC surface (mainly PfEMP1), whereas adhesion-inhibition assays measure a specific subset of antibodies interfering with CSA-adhesion sites. This may explain why global measures exhibited dependency on parity in the levels of antibodies against all placental isolates, compared with adhesion-inhibition antibodies, for which this pattern was observed only with a single isolate. Other investigators have found a positive association between antiadhesion or anti-VSA<sub>ava</sub> antibodies and increased birth weight [24, 36]. Although this may be due to a limited sample size, we did not observe such relationships. Antibody-recognition levels yielded by the studied isolates clearly highlight differences and similarities in antigenic targets among placental isolates. Because the levels of anti-VSA and antiadhesion antibodies are not related in most isolates, further study to elucidate the fine specificity of antibodies interfering with parasite sequestration will be of particular value in the development of future vaccines against PAM.

ABO-RBC antigens have been shown to interfere with host-parasite interactions, notably in rosetting ability, with a diminished rosetting potential in O-type RBCs [37]. The ability of parasites to adhere to CSA or CSPGs was not related to the ABO blood group of the IRBCs. However, plasma donors with O-type blood were more likely to present with a high anti-VSA<sub>ava</sub> antibody titer than were donors without O-type blood. Such a phenomenon is often reported in the pathological mechanisms of other diseases [38–40] but remains unclear in malaria.

In conclusion, placenta-associated parasites may phenotypically differ, hence stimulating different host responses. This antigenic polymorphism of parasite populations suggests that future studies analyzing the protective function of serum samples might be better served by adhesion models involving multiple potential receptors, such as human placental sections or human syncytiotrophoblast cultures. Despite their high levels of anti-VSA<sub>ava</sub> antibodies, multigravid women may still remain susceptible to some isolates adhering to other receptors, such as hyaluronic acid and nonimmune immunoglobulin [41, 42]. This may explain why many studies struggle to find associations between adhesion-inhibition antibodies and placental parasitemia. The results of the few studies reporting such an association [22, 24] still have not been reproduced [25]. Further studies are needed to study the prevalence of CSPG-adhesion phenotypes among placental parasites, since these have shown a strong effect on birth weight. The search for other potential receptors and elucidation of whether parasite sequestration in the placenta requires multiple receptors and/or parasite adhesins, or a limited number of the latter, will be of great interest in the development of a protective vaccine.

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