Major Article

Low Antibody Levels to Pregnancy-specific Malaria Antigens and Heightened Cytokine Responses Associated With Severe Malaria in Pregnancy

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Background. Pregnant women living in unstable malaria transmission settings may develop severe malaria (SM). The pathogenesis of SM in pregnancy is poorly understood.

Methods. To determine whether SM in pregnancy is associated with lower malarial antibody responses and higher cytokine responses, plasma samples were collected from 121 Sudanese pregnant women of whom 39 were diagnosed with SM. Antibodies to pregnancy-specific and non-pregnancy-specific Plasmodium falciparum variant surface antigens (VSA) and concentrations of cytokines TNF, IFNγ, IL-1β, IL-6, IL-8 and IL-10 were measured.

Results. Pregnant women with SM demonstrated significantly lower antibody levels to pregnancy-specific VSA (P = .020) and higher plasma IFNγ (P = .020), IL-10 (P = .0002) and IL-6 levels (P < .0001) than uninfected pregnant women. Concentrations of inflammatory cytokines IL-1β (P = .001), IL-6 (P = .004) and IL-8 (P = .020) were inversely correlated with antibodies to VAR2CSA-DBL5 in pregnant women with SM. Lower haemoglobin levels and higher parasite densities were associated with lack of pregnancy-specific antibodies (P = .028) and higher levels of inflammatory cytokines, in particular IL-6 and IL-8.

Conclusions. Pregnant women with SM lack pregnancy-specific malaria immunity, and this correlates with heightened inflammatory cytokine concentrations, low haemoglobin levels and high parasite density, suggesting that failure of antibody to control parasitaemia may contribute to SM pathogenesis.

Keywords. Severe malaria; pregnancy-specific malaria antigens; variant surface antigens; cytokines; VAR2CSA-DBL5; severe anaemia; parasitaemia; cerebral malaria; Sudan; Plasmodium falciparum infection.
P. falciparum [10, 11]. Naturally acquired antibodies to VAR2CSA-related VSA are associated with reduced risk of maternal anaemia [12] and low birth weight [13], but their potential role in SM during pregnancy has never been investigated.

To eliminate malaria infection, a host requires effective cooperation between the adaptive and the innate immune systems. Innate immunity provides the initial defense against infection when pathogens are first encountered, which is followed by development of specific antibody-secreting B lymphocytes. These responses are strongly controlled by cytokines and chemokines, the secretion of which is finely tuned in time and intensity to avoid pathology. It is believed that much of the pathology associated with SM is a consequence of imbalance in cytokine secretion [14], in which pro-inflammatory cytokine responses dominate [15]. Current evidence regarding the associations between cytokines and SM comes from studies conducted in animal models or non-pregnant individuals. Hence it is important to investigate this relationship in the context of pregnancy especially since pregnancy is a natural immune suppression state, where Th2 cytokine responses or anti-inflammatory cytokine responses dominate.

We measured naturally acquired anti-malarial antibodies and circulating cytokines in pregnant Sudanese women from an area of unstable malaria transmission [6]. Women with SM were matched to pregnant women with uncomplicated malaria (UM) or without malaria infection.

**METHODS**

**Study Participants**

Between August and November 2010, 121 pregnant women were enrolled at the maternity hospitals at Kassala, Wad Medani, El Gadarif and Sennar in the central and southern regions of Sudan. All participants were tested for malaria infection by microscopic examination of thick blood smears [5]. In brief, the blood films were prepared and stained with Giemsa and the number of asexual P. falciparum pRBCs were counted per 200 leucocytes. Parasitaemia was determined assuming 8000 leucocytes/μL [5]. Pregnant women who were parasitaemic and diagnosed with one or more of the following clinical manifestations were categorised as having SM (n = 39) [5, 16]. Definitions include severe anaemia, haemoglobin levels <7 g/dL; cerebral malaria, unarousable coma; hypoglycaemia, blood glucose levels <40 mg/dL; hypotension, systolic blood pressure <90 mmHg; jaundice, physical diagnosis or bilirubin levels >3 mg/dL (modified compared to the standard upper reference level of >1 mg/dL) and hyperparasitaemia, >10 000 parasites/μL. Parasitaemic pregnant women without such features (n = 41) were defined as UM. An additional 41 pregnant women with negative blood films and no signs of clinical malaria were enrolled as uninfected controls. The infected participants were treated according to the WHO guidelines with quinine [16]. Following collection of signed consent forms (in the case of comatose patients, a guardian was referred to sign the forms), 7 mL of blood was withdrawn prior to quinine treatment, for biochemical analyses (liver and renal function and blood glucose levels) and plasma was separated for malaria immunity and cytokine/chemokine studies.

The study was approved by the ethical committee of the Faculty of Medicine, University of Khartoum, Sudan with further approval by the Melbourne Health Human Research Ethics Committee.

**Parasites and Cell Culture**

Parasite line CS2 adheres to chondroitin sulphate A (representative of placental-binding malaria parasite isolates), whereas E8B adheres to ICAM-1 and CD36 but not CSA. These lines were grown in group O red blood cells provided by the Australian Red Cross Blood Service. Cultures of parasites were maintained in Roswell Park Memorial Institute (RPMI) 1640 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) medium supplemented with 0.2% w/v NaHCO3 (Walter and Eliza Hall Institute media preparation services, Parkville, VIC, Australia), 0.5% Albumax II (GIBCO, Life technologies, Mulgrave, VIC, Australia) and 5% v/v heat inactivated pooled non-immune human sera.

Pro-monocytic THP-1 cells were obtained from ATCC (Manassas, VA, USA; catalogue number TIB-202) and maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin-Glutamine and 25 mM HEPES (GIBCO). The cells were maintained at 1 to 2 × 10^5/mL in 80 cm² cell culture flasks (Nunc, Thermo Fisher Scientific, Scoresby, VIC Australia) in the upright position in a humidified 37°C incubator with 5% CO₂.

**Measuring IgG to Pregnancy-specific and non-pregnancy-specific Variant Surface Antigens**

Total IgG antibody levels against VSAs were measured by flow cytometry as previously described [17]. The geometric mean fluorescence intensity (MFI) of the samples were measured via HyperCyt flow cytometer with a plate reader adapter (Intellicyt, NM, USA) connected to CyAnADP analyser (Beckman Coulter Inc., CA, USA). The positive controls for the assay included a pool of sera collected from individuals with known high IgG levels against VSA and 8 negative controls were collected from malaria-unexposed adults from Melbourne. The samples were re-run if the variance between the duplicates was greater than 20%. The geometric MFI was adjusted for intra (duplicates) and inter-plate variability (all the samples were assayed on the same day). Total IgG levels to CS2 and E8B were measured for all 121 samples and the antibody levels were reported as geometric MFI of the samples. A participant is seropositive to VSA antibodies if the geometric MFI of the participant’s plasma sample was greater than the sum of the
average and 2 standard deviations of the geometric MFI of the negative controls.

Opsonic Phagocytosis Assay
A high throughput opsonic phagocytosis assay was performed as described [18] with minor modifications (Teo C., unpublished methods). In brief, purified mid to late trophozoite stage pRBCs were stained with ethidium bromide (EtBr) and incubated at 1.65 × 10⁷/mL with 1:10 dilution of patient sera in a neonatal calf serum-coated 96 well U bottom plate (BD Falcon, BD biosciences, North Ryde, NSW, Australia) for 1 hour at room temperature for opsonisation. The opsonised cells were then resuspended in 50 µL of THP-1 culture medium and aliquoted into duplicates, 25 µL each. Phagocytosis was then performed by incubating the cells with THP-1 cells at 5 × 10⁷/mL (2.5 × 10⁵ pRBCs to 2.5 × 10⁷ THP-1 cells) at 37°C in 5% CO₂ in a humidified incubator for 40 minutes.

The samples were acquired the following day using HyperCyt flow cytometer. THP-1 cells alone were gated based on forward and side scatter using the FL-1 channel (EtBr) and the gating was set to less than 5% to eliminate autofluorescence as previously described [18]. Phagocytosis of unopsonised pRBCs, incubated with media and THP-1 cells alone, was subtracted from each sample and the percentage phagocytosis was determined relative to the positive control, 0%–100% (pRBCs incubated with a pool of sera from patients with high IgG against VSA; 100% being the highest concentration of the positive control). In addition, the seropositivity for opsonising antibodies for each sample was determined as described for the VSA antibody assay using the negative controls (pRBCs incubated with pool of sera from malaria-unexposed Melbournians).

Multiplex Cytokine Bead Assay
Peripheral plasma cytokine profiles of the participants were measured using a multiplex human inflammatory cytokine bead array (BD Biosciences, CA, USA) which measures concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF. The assay was optimized while following manufacturer’s instructions. In brief, patient sera were diluted in serum enhancement buffer in 1:4 dilution and 10 µL of this was incubated with 10 µL of mixed beads (containing mixture of equal volumes of each bead type) for 1.5 hours under agitation. In parallel, human inflammatory cytokine standards were serially diluted from a stock of 5000 pg/mL to 20 pg/mL (0 pg/mL of the standard used as the negative control) and incubated with 10 µL of mixed beads for 1.5 hours under agitation. Following incubation the beads were washed and incubated with 10 µL of Phycoerythrin (PE) conjugated antibodies for 1.5 hours under agitation. The bead/sample/antibody sandwich complexes were washed and resuspended in wash buffer and passed through a FACSCalibur (BD biosciences). The standards and all 121 samples were run on the same assay and on the same day to avoid any inter-assay variability. The fluorescence emerging from the FL-2 channel (PE) determined the amount of cytokine bound to each bead while the fluorescence emerging from FL-3 channel (beads) determined the type of cytokine. The concentration of each cytokine in each sample was then quantified from the respective cytokine standard curve, using the FCAP array software version 3.0 (Soft flow Inc. MN, USA).

Enzyme Linked Immunosorbent Assay for Measuring Plasma Interferon Gamma Levels
Plasma IFNγ levels were measured using a human IFNγ ELISA kit (MabTech, Thomastown, VIC, Australia) by following the manufacturer’s protocol.

Enzyme Linked Immunosorbent Assay for Measuring Antibodies to VAR2CSA-DBL5
Plasma antibodies to potential pregnancy malaria vaccine candidate Plasmodium falciparum VAR2CSA, DBL5e domain (kindly provided to us by Prof. Joseph D Smith) were measured by ELISA. The procedure is described in Supplementary methods.

Statistical Analysis
All statistical analysis was performed using STATA 12.0 (StataCorp LP, Texas, USA). Figures were created using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA). Statistical differences in malaria antibody levels or cytokine concentrations across the groups were determined using the Kruskal-Wallis test or if comparing between two groups Mann-Whitney test was used. Spearman rank correlations were performed to determine associations between antibodies and plasma cytokine levels in SM and UM infected groups. Further Spearman rank correlations were performed between antibody and cytokine levels with maternal haemoglobin concentration and parasite density among women with malaria infection. Differences for each statistical analysis will be reported as significant if P < .05.

RESULTS

Patient Characteristics
Age, gravidity and gestational age of the pregnant women were similar across the three groups (Table 1). Pregnant women with SM had lower median haemoglobin concentrations compared to the other two groups as a proportion of women in this group were diagnosed with SA. All the women in the SM group demonstrated the characteristic malaria fever (with chills) and the duration of fever was similar between the two infected groups. Although slightly elevated in the SM group, median creatinine and bilirubin levels were similar between UM and SM groups while the plasma glucose levels were lower in pregnant women with SM compared to women with UM as a proportion of women in SM group were diagnosed with hypoglycaemia.
Antibodies to Pregnancy-specific But Not Non-pregnancy-specific VSA Were Significantly Reduced in Pregnant Women Suffering From Severe Malaria

Analysis of total IgG to *P. falciparum* VSA demonstrated that only 19.0% of the women in the cohort had IgG to pregnancy-specific VSA (Table 2). These antibodies were significantly lower among women suffering from SM than uninfected women (*P* = .020) (Figure 1A) and were not affected by the gravidity or age of the mother (Supplementary Table 1). When compared to UM group, these antibody levels were not significantly different in women with SM. Opsonising antibodies to pregnancy-specific VSA were found only in 23.0% of women with no difference in antibody levels between the groups (Figure 1C).

The levels of total IgG against VSA of non-pregnancy-specific *P. falciparum* were similar across the three groups (Figure 1B). Similarly, the levels of opsonising antibodies to these antigens did not differ between the groups (Figure 1D). Immunoglobulin G to VAR2CSA-DBL5 were detected in 42.2% of women, while the levels of these antibodies only differed between the UM and uninfected groups (*P* = .004; Figure 1E).

Plasma Cytokine Levels in Severe Malaria During Pregnancy

Pregnant women suffering from SM displayed distinct cytokine profiles when compared to the uninfected controls (Figure 2). In particular, the levels of IFN-γ (*P* = .020), IL-6 (*P* < .0001) and IL-10 (*P* = .0002) were significantly elevated. Compared to women with UM, women with SM had significantly higher levels of TNF (*P* = .001), IL-6 levels (*P* = .005) and IL-8 (*P* = .001). No detectable levels of IL-12p70 were observed in any of the plasma samples and therefore excluded from subsequent analyses.

Association Between Antibodies to Malaria Antigens and Cytokines Categorized by Severe Malaria and Uncomplicated Malaria Groups

A correlation analysis was performed to determine the relationship between antibody and cytokine levels among pregnant women with SM and UM (Table 3A and 3B respectively). Antibodies to VAR2CSA-DBL5 were negatively correlated with IL-1β (*P* = .001), IL-6 (*P* = .004) and IL-8 (*P* = .020) among women with SM (Table 3A). No associations were observed between antibodies and cytokines among pregnant women with UM (Table 3B).

Association of Malaria Antibodies and Cytokines With Maternal Haemoglobin Levels and Parasite Density in Malaria Infected Women

Maternal haemoglobin levels were positively associated with opsonising antibodies to pregnancy-specific VSA (*P* = .028) and negatively associated with levels of TNF (*P* = .001), IL-1β (*P* = .0004), IL-6 (*P* = .003) and IL-8 (*P* = .001) (Table 4). Women with SA were excluded from this analysis. To examine the relationships between antibodies, cytokines and parasite density, we combined women with UM and SM, since the parasite density is similar between the two groups. Correlation

Table 1. Demographic and Clinical Characteristics of Study Participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Uninfected (n = 41)</th>
<th>UM (n = 41)</th>
<th>SM (n = 39)</th>
<th>All Participants (n = 121)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>29 (26,32)</td>
<td>27 (22,32)</td>
<td>28 (25,32)</td>
<td>28 (24,32)</td>
</tr>
<tr>
<td>Gravidity, number of women</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primigravida</td>
<td>14</td>
<td>16</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Secundigravida</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>Multigravida</td>
<td>18</td>
<td>18</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>29 (21,32)</td>
<td>29 (23,34)</td>
<td>30 (24,36)</td>
<td>29 (22,34)</td>
</tr>
<tr>
<td>Physical findings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever, % of women</td>
<td>4.88</td>
<td>97.6</td>
<td>100</td>
<td>71.1</td>
</tr>
<tr>
<td>Duration, median (IQR) days</td>
<td>0 (0,0)</td>
<td>2 (2,3)</td>
<td>2 (2,3)</td>
<td>2 (0,3)</td>
</tr>
<tr>
<td>Laboratory findings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin concentration, g/dL</td>
<td>10.6 (10.1, 10.9)</td>
<td>10.4 (9.8, 10.8)</td>
<td>8.8 (6.9, 10)</td>
<td>10.2 (8.9, 10.8)</td>
</tr>
<tr>
<td>Parasitaemia, Parasites/µL</td>
<td>0</td>
<td>9200 (4800, 17 100)</td>
<td>8840 (6500, 24 000)</td>
<td>9100 (5800, 20 000)</td>
</tr>
<tr>
<td>White blood cell count, cells/mL</td>
<td>. . .</td>
<td>5350 (5200, 5500)</td>
<td>5600 (4000, 6400)</td>
<td>5500 (4400, 6400)</td>
</tr>
<tr>
<td>Creatinine levels, mg/dL</td>
<td>. . .</td>
<td>0.9 (0.9, 0.9)</td>
<td>1 (0.9,1)</td>
<td>1 (0.9, 1)</td>
</tr>
<tr>
<td>Bilirubin, mg/dL</td>
<td>. . .</td>
<td>1.2 (1, 2)</td>
<td>2 (1, 2.5)</td>
<td>2 (1, 2.45)</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>. . .</td>
<td>101 (80, 110)</td>
<td>80 (39, 100)</td>
<td>95 (60, 108)</td>
</tr>
</tbody>
</table>

Data presented as Median and IQR (interquartile range) unless otherwise stated. Abbreviation: UM, uncomplicated malaria; SM, severe malaria.
analysis revealed a negative association between both total and opsonic antibodies to non-pregnancy-specific VSA and increasing parasite density (\(P = .05\) and \(P = .014\) respectively), and positive associations between parasite density and inflammatory cytokine levels, IL-6 (\(P = .020\)) and IL-8 (\(P = .005\)) (Table 4).

**DISCUSSION**

Pregnant women are highly susceptible to *P. falciparum* infection, and in areas with low or unstable malaria transmission they may be at increased risk for SM [19]. Antibodies that inhibit adhesion of pRBC to CSA, or that opsonise them for phagocytic clearance, have been associated with better pregnancy outcomes [10, 12, 13, 20, 21]. We measured humoral immunity to pregnancy-specific and non-pregnancy-specific *P. falciparum* VSA in pregnant Sudanese women with SM matched to uninfected pregnant women and those with UM to understand the relationship between antibodies to VSA, cytokines and development of SM in pregnancy.

Interestingly, the pregnant women with SM had lower levels of IgG to pregnancy-specific VSA than uninfected women, and the levels of pregnancy-specific VSA opsonising antibodies were low in all groups, consistent with limited previous exposure to malaria during pregnancy. In comparison, IgG and opsonising antibodies to non-pregnancy-specific VSA were frequently detected, but were not significantly different between the groups. The overall low antibody prevalence observed can be largely explained by the low malaria transmission pattern in the study area. Variation in malaria transmission may influence the acquisition and maintenance of antibodies to malaria during pregnancy. For instance, in the current cohort pregnancy-specific malaria antibody levels were not associated with gravidity or maternal age, agreeing with a previous study conducted in the same area reporting that susceptibility to SM is not age or parity-dependent [22]. In contrast, pregnant women residing in more highly-endemic regions demonstrate gravidity [23] and age dependent [3] changes, with primigravid young mothers (≤20 years) having lower pregnancy-specific antibody levels than older, multigravid mothers.

Antibodies to whole parasites (IgG or opsonising antibodies to CS2 parasite VSA) tended to be of lower levels and less prevalent in women with SM than in uninfected women, whereas antibodies to the DBL5ε domain of VAR2CSA, which have been associated with reduced risk of placental adhesion [24–26] and improved birth weight [10], were significantly more common in UM than uninfected women, and were more prevalent in infected than uninfected women (Table 2). These differences in prevalence and magnitude of pregnancy-specific antibody responses measured with different assays could have several causes. First, the low prevalence of malaria in the community suggests that many women were experiencing their first episode of malaria in pregnancy, and there may be variations in development of initial antibody response to different targets. Second, opsonising antibodies to VSA are primarily IgG1 and IgG3 [27], whereas the VSA assay measures total IgG which may have other functions in addition to opsonisation such as cytoadhesion inhibition that may be important for protecting uninfected women from SM, explaining the association between SM and low levels of these antibodies. Third, antibodies to VAR2CSA-DBL5 target only a single domain of a large complex protein, and antibodies to the full length VAR2CSA have been associated with a reduced risk of placental malaria compared to responses to individual domains (such as DBL5ε) [28]. Moreover, antibody responses to VAR2CSA-DBL5 are not confined to pregnant women [29], and thus may not solely reflect exposure to malaria in pregnancy. The observation of higher levels of antibodies to VAR2CSA-DBL5 in UM, and higher prevalence of these antibodies in UM and SM compared to uninfected women, may reflect broader differences in malaria exposure.

A primary mechanism by which the adaptive and innate immune systems combine to protect the host is through the secretion of cytokines. During normal pregnancy, Th2 T cells mediate dominant anti-inflammatory cytokine responses via secretion of IL-4, IL-5, IL-10, IL-13 and TGFβ, preventing the

**Table 2. Percentage of Women Seropositive to Malaria Antigens**

<table>
<thead>
<tr>
<th></th>
<th>Uninfected ((n = 41))</th>
<th>UM ((n = 41))</th>
<th>SM ((n = 39))</th>
<th>All Participants ((n = 121))</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG to pregnancy-specific VSA</td>
<td>26.80</td>
<td>17.00</td>
<td>12.80</td>
<td>19.00</td>
<td>.260</td>
</tr>
<tr>
<td>Total IgG to non-pregnancy-specific VSA</td>
<td>48.80</td>
<td>58.50</td>
<td>71.80</td>
<td>59.50</td>
<td>.110</td>
</tr>
<tr>
<td>Antibodies to VAR2CSA-DBL5</td>
<td>26.80</td>
<td>53.70</td>
<td>46.20</td>
<td>42.20</td>
<td>.040*</td>
</tr>
<tr>
<td>Opsonising antibodies to pregnancy-specific VSA</td>
<td>31.70</td>
<td>26.80</td>
<td>10.30</td>
<td>23.00</td>
<td>.100</td>
</tr>
<tr>
<td>Opsonising antibodies to non-pregnancy-specific VSA</td>
<td>39.00</td>
<td>41.50</td>
<td>23.10</td>
<td>34.70</td>
<td>.180</td>
</tr>
</tbody>
</table>

Data presented as percentage of women seropositive to each type of antigen for each group. Data analysed performing Chi2 test and statistically significant differences between the 3 groups reported (\(P\) value).

\(\cdot\) Denotes the significant difference in seropositivity to VAR2CSA-DBL5 between the 3 groups. The proportion of women seropositive to VAR2CSA-DBL5 was significantly higher in pregnant women with UM compared to uninfected (further analysis of seropositivity to VAR2CSA-DBL5 between uninfected and UM, Mann Whitney test, \(P = .013\)).
Figure 1. Total and functional antibodies to pRBCs expressing pregnancy-specific or non-pregnancy-specific VSA presented by disease severity. Graphs (A) and (B) represent the levels of total immunoglobulin G (IgG) against pregnancy-specific VSA (CS2 parasite line) and non-pregnancy-specific VSA (E8B parasite line), respectively. Y axis represents the geometric Mean Fluorescence Intensity (MFI) (adjusted for intra and inter-plate variations). Women diagnosed with SM had significantly lower IgG levels against pregnancy-specific VSA than uninfected, Mann Whitney test *P = .020. Graphs (C) and (D) represent opsonizing antibodies to pregnancy-specific and non-pregnancy-specific VSA, respectively. Each symbol represents an individual woman with percentage phagocytosis (calculated using positive and negative controls). Graph (E) represents the levels of antibodies to VAR2CSA-DBL5. Data presented as optical density at 410 nm wavelength. Women suffering from UM had significantly higher antibody levels to VAR2CSA-DBL5 compared to uninfected, Mann Whitney test, *P = .004. Horizontal lines in each graph represent the median. The dotted bottom line (-) represents the reference level for seropositivity and the top line (---) represents the reference level for the positive controls.
Figure 2. Analysis of plasma cytokine levels (A) IFNγ, (B) TNF, (C) IL1β, (D) IL-6, (E) IL-8, (F) IL-10 between uninfected, UM and SM groups. Each symbol represents an individual woman with their measured plasma cytokine levels in pg/mL. Cytokine levels measured in pg/ml for graphs (B–F) are presented on a log 10 scale with 0.01 indicating 0.00 pg/mL while IFNγ levels were presented on a linear scale (A). Horizontal bar denotes the median. Non-parametric Mann-Whitney and Kruskal-Wallis statistical tests were performed to determine the difference in cytokine levels between infected groups and the uninfected controls. N = 41 uninfected, n = 41 UM and n = 39 SM. The symbol ‘*’ denotes statistical significant differences in cytokine levels compared to those in uninfected group, ‘Ψ’ denotes statistical significant differences compared to UM group. Significant differences presented as ***/ΨΨΨ P < .0005, **/ΨΨ Ψ ≤ .005 and */ Ψ ≤ .05.
IL-10−IL-8 (B) Uncomplicated malaria

-IL-6

TNF

IFN

Opsonising antibodies to pregnancy-specific VSA

-0.10 (0.536)−0.15 (0.357)−0.30 (0.061)−0.14 (0.391)−0.14 (0.408)0.02 (0.910)

Opsonising antibodies to non-pregnancy-specific VSA

-0.03 (0.835)−0.21 (0.194)−0.22 (0.176)−0.21 (0.196)−0.29 (0.073)0.02 (0.910)

(B) Uncomplicated malaria

Total IgG to pregnancy-specific VSA

-0.03 (0.868)−0.26 (0.108)−0.20 (0.211)0.08 (0.636)−0.13 (0.425)0.08 (0.635)

Total IgG to non-pregnancy-specific VSA

0.14 (0.401)−0.07 (0.657)−0.11 (0.507)−0.16 (0.306)−0.14 (0.369)−0.02 (0.941)

Antibodies to VAR2CSA-DBL5

0.25 (0.112)−0.13 (0.420)−0.30 (0.054)0.22 (0.173)−0.02 (0.892)0.29 (0.070)

Opsonising antibodies to pregnancy-specific VSA

-0.02 (0.536)−0.05 (0.741)−0.20 (0.206)−0.15 (0.343)−0.01 (0.965)−0.20 (0.212)

Opsonising antibodies to non-pregnancy-specific VSA

0.04 (0.814)−0.19 (0.227)−0.03 (0.849)−0.02 (0.920)−0.14 (0.384)0.07 (0.666)

Data presented as Spearman rank correlation coefficient ρ (P-value) for comparisons between antibody and cytokine levels in pregnant women with SM and UM. N = 39 women (SM) and 41 women (UM).

** Represents statistical significance.

Table 3. Correlation Between Antibody Levels and Cytokines in Women

<table>
<thead>
<tr>
<th></th>
<th>IFNγ</th>
<th>TNF</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Severe malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgG to pregnancy-specific VSA</td>
<td>0.11 (0.523)</td>
<td>0.01 (0.940)</td>
<td>0.13 (0.436)</td>
<td>−0.14 (0.399)</td>
<td>0.25 (0.125)</td>
<td>−0.18 (0.270)</td>
</tr>
<tr>
<td>Total IgG to non-pregnancy-specific VSA</td>
<td>−0.15 (0.360)</td>
<td>−0.22 (0.173)</td>
<td>−0.14 (0.388)</td>
<td>−0.28 (0.082)</td>
<td>−0.14 (0.400)</td>
<td>−0.12 (0.475)</td>
</tr>
<tr>
<td>Antibodies to VAR2CSA-DBL5</td>
<td>0.16 (0.319)</td>
<td>−0.27 (0.097)</td>
<td>−0.50 (0.001*)</td>
<td>−0.45 (0.004*)</td>
<td>−0.37 (0.020*)</td>
<td>0.29 (0.07)</td>
</tr>
<tr>
<td>Opsonising antibodies to pregnancy-specific VSA</td>
<td>−0.10 (0.536)</td>
<td>−0.15 (0.357)</td>
<td>−0.30 (0.061)</td>
<td>−0.14 (0.391)</td>
<td>−0.14 (0.408)</td>
<td>0.02 (0.910)</td>
</tr>
<tr>
<td>Opsonising antibodies to non-pregnancy-specific VSA</td>
<td>−0.03 (0.835)</td>
<td>−0.21 (0.194)</td>
<td>−0.22 (0.176)</td>
<td>−0.21 (0.196)</td>
<td>−0.29 (0.073)</td>
<td>0.02 (0.910)</td>
</tr>
<tr>
<td>(B) Uncomplicated malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgG to pregnancy-specific VSA</td>
<td>−0.03 (0.868)</td>
<td>−0.26 (0.108)</td>
<td>−0.20 (0.211)</td>
<td>0.08 (0.636)</td>
<td>−0.13 (0.425)</td>
<td>0.08 (0.635)</td>
</tr>
<tr>
<td>Total IgG to non-pregnancy-specific VSA</td>
<td>0.14 (0.401)</td>
<td>−0.07 (0.657)</td>
<td>−0.11 (0.507)</td>
<td>−0.16 (0.306)</td>
<td>−0.14 (0.369)</td>
<td>−0.02 (0.941)</td>
</tr>
<tr>
<td>Antibodies to VAR2CSA-DBL5</td>
<td>0.25 (0.112)</td>
<td>−0.13 (0.420)</td>
<td>−0.30 (0.054)</td>
<td>0.22 (0.173)</td>
<td>−0.02 (0.892)</td>
<td>0.29 (0.070)</td>
</tr>
<tr>
<td>Opsonising antibodies to pregnancy-specific VSA</td>
<td>−0.02 (0.536)</td>
<td>−0.05 (0.741)</td>
<td>−0.20 (0.206)</td>
<td>−0.15 (0.343)</td>
<td>−0.01 (0.965)</td>
<td>−0.20 (0.212)</td>
</tr>
<tr>
<td>Opsonising antibodies to non-pregnancy-specific VSA</td>
<td>0.04 (0.814)</td>
<td>−0.19 (0.227)</td>
<td>−0.03 (0.849)</td>
<td>−0.02 (0.920)</td>
<td>−0.14 (0.384)</td>
<td>0.07 (0.666)</td>
</tr>
</tbody>
</table>

Data presented as Spearman rank correlation coefficient ρ (P-value) for comparisons between antibody and cytokine levels in pregnant women with SM and UM. N = 39 women (SM) and 41 women (UM).

** Represents statistical significance.

Table 4. Association Between Haemoglobin Levels and Parasite Density With Antibody and Cytokine Levels Among Pregnant Women With Malaria

<table>
<thead>
<tr>
<th></th>
<th>Maternal Haemoglobin Concentration (n = 70)</th>
<th>Parasite Density (n = 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG to pregnancy-specific VSA</td>
<td>−0.11 (0.376)</td>
<td>−0.08 (0.50)</td>
</tr>
<tr>
<td>Total IgG to non-pregnancy-specific VSA</td>
<td>−0.12 (0.309)</td>
<td>−0.22 (0.05*)</td>
</tr>
<tr>
<td>Antibodies to VAR2CSA-DBL5</td>
<td>0.22 (0.069)</td>
<td>0.07 (0.520)</td>
</tr>
<tr>
<td>Opsonising antibodies to pregnancy-specific VSA</td>
<td>0.26 (0.028*)</td>
<td>−0.06 (0.600)</td>
</tr>
<tr>
<td>Opsonising antibodies to non-pregnancy-specific VSA</td>
<td>−0.002 (0.989)</td>
<td>−0.28 (0.014*)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.01 (0.957)</td>
<td>0.04 (0.760)</td>
</tr>
<tr>
<td>TNF</td>
<td>−0.40 (0.001*)</td>
<td>0.16 (0.150)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>−0.41 (0.0004*)</td>
<td>0.18 (0.110)</td>
</tr>
<tr>
<td>IL-6</td>
<td>−0.35 (0.003*)</td>
<td>0.26 (0.020*)</td>
</tr>
<tr>
<td>IL-8</td>
<td>−0.41 (0.001*)</td>
<td>0.31 (0.005*)</td>
</tr>
<tr>
<td>IL-10</td>
<td>−0.09 (0.440)</td>
<td>0.02 (0.840)</td>
</tr>
</tbody>
</table>

Spearman rank correlation performed between antibody and cytokine levels with maternal haemoglobin levels and parasite density in pregnant women with SM and UM. Women with severe anaemia (n = 10) were excluded from the analysis of associations with haemoglobin levels. Parasite density between SM and UM groups were similar and not statistically different (data not shown) therefore both groups of women were included in the analysis.

Data presented as Spearman rank correlation coefficient ρ (P-value).

** Represents statistical significance.

In malaria, Th1 mediated cytokine responses dominate, with the transcription and secretion of pro-inflammatory cytokines TNF, IFNγ, IL-1β, IL-6, IL-8, IL-12 (reviewed in [14]) to facilitate clearance of the parasites. Our results demonstrated that some pro-inflammatory cytokines such as IFNγ and IL-6 are significantly elevated in women suffering from SM with concurrent increase in IL-10. Similar findings were reported from studies investigating cytokine profiles in SM in adults [31] and children [32, 33]. Although IL-10 is considered an anti-inflammatory cytokine [34], in the context of malaria in pregnancy it is positively associated with inflammation in the placenta [35]. Excessive IL-10 production may interfere with parasite clearance due to its ability to suppress cytokine secretion by monocyte/macrophages [34]. Elevated levels of pro-inflammatory cytokines, TNF, IL-6 and IL-8 in women with SM compared to women with UM suggest the possible involvement of these cytokines in SM pathology in pregnancy. It is noteworthy that elevated levels of inflammatory cytokines were also negatively associated with protective antibodies to VAR2CSA-DBL5 in pregnant women with SM. This raises the possibility that women lacking specific immunity to pregnancy-associated malaria may develop exaggerated inflammatory responses following infection, which may predispose to development of SM.

We found evidence that antibody and cytokine responses correlated with maternal haemoglobin concentration or with parasite density in infected women. Total IgG antibodies to pregnancy-specific VSA have been associated with protection against low birth weight and maternal anaemia in a number of rejection of the fetus (reviewed in [14, 30]).
studies [12, 13, 21, 36]. Opsonising antibodies to pregnancy-specific VSA were positively correlated with haemoglobin levels (Table 4), in keeping with other studies from our group [12, 21]. This finding adds further weight to our hypothesis that antibodies that mediate enhanced phagocytic clearance of pRBCs may protect women against malaria-induced anaemia during pregnancy. In addition, maternal haemoglobin levels were lower in women with elevated levels of the pro-inflammatory cytokines TNF, IL-1β, IL-6 and IL-8, while IL-6 and IL-8 correlated positively with parasite density, suggesting that parasite-induced secretion of these cytokines possibly leads to reduced maternal haemoglobin levels. However further studies are required to confirm these associations and clearly define the role of cytokines in SM in pregnancy.

Over all we conclude that low immunity to pregnancy-specific VSA and heightened pro-inflammatory cytokine responses are associated with SM in pregnancy. In the presence of infection most antibody responses are associated with controlling parasitaemia, preventing malaria induced anaemia and suppressing pro-inflammatory cytokine secretion. However the overall low prevalence and magnitude of antibody responses observed in the current cohort rendered it difficult to clearly understand their role in malaria infection and the small sample size impacted on the power of statistical comparisons. Further studies may provide greater insight into the role of deficient antibody immunity and exaggerated cytokine responses in SM in pregnancy in areas with unstable malaria transmission such as Sudan.

**Supplementary Data**

**Supplementary materials** are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**


