

PLACENTAL MALARIA IN WOMEN WITH SOUTH-EAST ASIAN OVALOCYTOSIS

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Abstract. Malaria during pregnancy, which is characterized by the accumulation of infected erythrocytes in the placenta, often has severe consequences for the mother and newborn. We assessed the effect of the genetic trait South-East Asian ovalocytosis (SAO) on placental malaria in women from Papua New Guinea. In children, this trait confers protection against cerebral malaria, but not against mild malaria disease, malaria parasitemia, or severe malaria anemia. Using a case-control approach, we found that SAO women suffer from placental malaria, and SAO-infected erythrocytes can sequester in the placenta, but heavy placental infections tended to be less common in SAO than in control pregnant women. Reduced prevalence and severity of placental infection associated with SAO were observed only for primigravid women, who are the group at highest risk of suffering from severe manifestations of placental malaria. Furthermore, we found that the prevalence of the SAO trait was lower among pregnant women than among non-pregnant controls.

INTRODUCTION

Individuals living in areas of high malaria transmission develop non-sterile immunity against this disease after repeated exposure, which confers relative protection against high-density parasitemia, clinical manifestations, and especially severe complications of the disease. Pregnant women are the exception among exposed adults, as they are highly susceptible to severe *Plasmodium falciparum* malaria infection.¹ Placental malaria, which is characterized by the accumulation of *P. falciparum*-infected erythrocytes in the intervillous spaces of the placenta,² is responsible for an increased risk of severe maternal anemia and infant low birth weight (LBW), because of either intrauterine growth retardation or preterm delivery.^{1,3,4} LBW increases the susceptibility of the infant to many diseases and consequently the risk of death early in life,⁵ whereas severe maternal anemia increases the risk of maternal death. Furthermore, in areas of low malaria endemicity, maternal deaths from cerebral malaria and fetal miscarriages and stillbirths often occur as a consequence of placental malaria.⁴ Thus, there is high morbidity and mortality for both the mother and the infant either directly or indirectly associated with placental malaria.

In areas of high malaria transmission, the risk of developing placental malaria and its severe complications is higher in primigravid women and decreases with parity,^{1,3,6,7} because women develop effective antibody-mediated immune responses against variant surface antigens from parasites causing placental malaria in a parity-dependent manner.^{8–11} These antibodies are associated with improved pregnancy outcomes.^{12,13} Variant surface antigens from parasites that produce placental malaria around the globe exhibit a remarkable degree of antigenic similarity,^{8,9,14–17} but are distinct from variant surface antigens from non-placental isolates.¹⁸ The molecular basis for this observation is that parasites that sequester in the placenta predominantly express relatively conserved forms of the cytoadherence ligand PfEMP1 that principally bind to the glycosaminoglycan chondroitin sulfate

(CSA), which is abundantly expressed at the surface of syncytiotrophoblasts in the placenta where infected erythrocytes accumulate, but not to the endothelial receptor CD36.^{14,16,19–22} In contrast, parasites infecting children and non-pregnant adults principally express highly polymorphic forms of PfEMP1 with opposite binding specificity.^{18,22}

Here we studied the development of placental malaria in women with the genetic trait South-East Asian ovalocytosis (SAO). This mutation, largely asymptomatic in heterozygotes but incompatible with life in homozygotes,^{23,24} is determined by a 27 base pairs deletion in the gene encoding band 3, the major erythrocyte transmembrane protein.²⁵ The SAO trait, which results in erythrocytes with decreased anion transport activity²⁶ and dramatically altered mechanical properties,²⁷ reaches a high prevalence of more than 20% in some areas of the Western Pacific, and its geographical distribution parallels that of malaria endemicity in Papua New Guinea (PNG).²⁴ Previous studies have demonstrated that the SAO trait confers protection against cerebral malaria. Remarkably, no single SAO individual was found to suffer from cerebral malaria in 2 independent studies.^{28,29} In contrast, SAO individuals are fully susceptible to malaria infection and disease, and also to some complications of the disease like severe malaria anemia.²⁹ However, none of the previous studies assessed whether this trait confers protection against placental malaria, which like cerebral malaria, is closely associated with sequestration of infected erythrocytes. In this study, we used a case-control approach to compare the level of placental infections between SAO and control women.

MATERIALS AND METHODS

Study area. Blood and placental samples were collected between June 2002 and December 2003 from women living in the North Coast of the Madang Province of PNG, an area of year-round high malaria transmission.³⁰ The epidemiology of malaria during pregnancy in this area and a nearby area has been described.^{31–33} The study area covers a strip parallel to the coast of about 27 km long and 8 km wide that includes a population of approximately 30,000 people living in 15 main village areas. The Adelbert Ranges provide a natural geographic division of coastal and mountainous villages, which determines differences in malaria transmission.³⁴ For the geo-

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graphic matching of samples, the study area was divided in 8 sub-regions. Within each of the sub-regions, the main geographic factors affecting malaria transmission (altitude and distance to the sea) were similar and the vast majority of the population belonged to the same ethnic (and language) group.

Sample collection. This study was approved by the PNG Medical Research Advisory Committee. After obtaining informed consent, all women enrolled were asked to answer a questionnaire for demographic, medical, and obstetric information.

Four hundred and four women at delivery and 281 pregnant women attending the antenatal clinic were enrolled for this study. Blood smears were missing for 2 of the women enrolled at delivery, so only 402 were included in the parasitological analysis. Blood was also collected in cross-sectional village surveys from 196 non-pregnant women matched by age and geography with the women enrolled at delivery and from 49 non-pregnant women individually age-matched and geography-matched with 49 of the women attending the antenatal clinic.

Pregnant women were recruited for this study at Alexishafen and Mugil health centers. All women participating in this study were prescribed weekly chloroquine prophylaxis in accordance with PNG Standard Treatment Guidelines, but compliance was not monitored. Previous studies in the area found that the level of compliance was high, but the efficacy of this prophylaxis was low³¹ and did not reduce the risk of infection at delivery.³³ From women attending the antenatal clinic, finger prick blood was taken for malaria diagnosis, genetic analysis, and hemoglobin (Hb) measurement (using a portable Hemocue Hb meter, HemoCue AB, Ängelholm, Sweden). From women attending for delivery, a sample of peripheral venous blood in EDTA; a sample of serum; thick and thin smear films from both venous and placental blood; and a section of the placenta were collected within 12 hours after delivery. Hb concentration, temperature, and birth weight were recorded. A biopsy specimen of placental tissue was obtained from the maternal surface near the center of the placenta,³⁵ placed in 10% neutral buffered formalin and stored at room temperature for up to 3 months until it was transported to Adelaide for histologic examination. Smears were prepared from a droplet of the blood welling into an incision in the paracentral area of the placenta.

The number of infected erythrocytes per 200 leukocytes was determined from Giemsa-stained smears. Parasite densities were determined by assuming a leukocyte count of 8,000 parasites/ μ L.

Genetic analysis. The SAO status of all patients was determined by PCR as previously described, using the primers P198 and P199.²⁵ DNA was prepared using the rapid boiling method.³⁶ In a small number of cases the PCR did not yield any band and nested PCR was used to determine the SAO status, using the primers Band3-5'ext. (5'-GGCGCTATCAGTCCAGCCC-3') and Band3-3'ext. (5'-AGTGGAGATCAGCAGCTCCG-3') for the primary reaction and the previously described primers²⁵ for the nested.

Placental examination. The placental biopsies were processed and embedded in paraffin wax by standard techniques. Four micron sections were stained with hematoxylin and eosin (H&E) and with Giemsa's stain. Slides were examined blind to flow cytometry results and to maternal SAO, ma-

larial, or parity status. H&E slides were examined with standard microscopy and also with polarization microscopy, which increases the sensitivity of detection of placental malaria.³⁷ To rule out the formation of formalin pigment that could be mistaken by malaria pigment, we tested a subset of slides by immersing in picric acid and we did not observe any reduction in the pigment, indicating that it was not formalin artifact that is removed by the picric acid. This is in good agreement with previous studies that found that the use of neutral buffered formalin instead of acid formalin prevents the formation of formalin pigment.^{7,35,37,38} All cases were classified by one observer (TYK) as *no infection* (no evidence of pigment or parasites); *active infection* (parasites or pigment in maternal erythrocytes and monocytes in the intervillous space); *past-chronic infection* (parasites not present but pigment confined to fibrin or cells within fibrin); or *active-chronic infection* (both active and chronic infection), as previously described in detail.³⁵ These categories correspond to no infection, acute infection, past infection, and chronic infection according to the criteria of Ismail and colleagues.⁷ The amount of malaria pigment deposits seen in chronic infections was also semi-quantitatively assessed as: *mild* (focally present), *moderate* (small dots diffusely distributed with focal coarse deposits), and *severe* (large amounts of malarial pigment).⁷ Active infections were also semi-quantitatively assessed as *mild*, *moderate*, or *severe* according to the proportion of infected erythrocytes. A sample of 46 randomly selected slides was examined independently by another observer (SJR). Concordance was reached in all but one case, which was then deliberated and agreed upon.

Flow cytometry. A randomly selected subset of serum samples were evaluated for antibodies to variant surface antigens expressed on erythrocytes infected with trophozoite stages of 2 laboratory lines, CS2 and E8B, as previously described.³⁹ Assays were performed blind to SAO and malarial status. Samples were analyzed on a Becton Dickinson FAC-Scalibur flow cytometer and mean fluorescence intensity (MFI) of infected erythrocytes was measured. Sera from 5 malaria-naïve donors were used to define a cut-off for positivity (MFI >mean + 2 SD of negative controls) and a pool of reactive sera from malaria-exposed pregnant women provided a positive control. Amounts of antibody in test samples were expressed relative to the positive control according to the formula: $MFI_{relative} = [(MFI_{sample} - MFI_{negative\ control}) / (MFI_{positive\ control} - MFI_{negative\ control})] \times 100$.

Data analysis. For some of the analysis, samples collected at delivery were individually matched for gravidity and geography. For each sample determined to carry the SAO trait, 3 controls were selected among the collected samples from non-SAO women of the same gravidity and living in the same sub-region. When more than 3 women matched these criteria, the 3 specimens collected on the nearest dates were selected. Gravidity and place of residence were considered the main factors affecting the risk of developing placental malaria, whereas seasonality is not presumed to have an effect in an area of year-round transmission. We did not observe differences in the prevalence of malaria infection between women delivering in different seasons (data not shown). Mean age was very similar between the SAO and control groups (mean age 27.5 years, range 19–40, and 27.6 years, range 16–40, in SAO and matched control women, respectively).

Odds ratios for the prevalence of infection or high-density

infection between individually matched SAO and control women were determined using conditional logistic regression. Other statistical tests were used as described in the text. Statistical analysis was performed using STATA software (version 6.0).

RESULTS

Epidemiology of placental malaria in the Madang North Coast. Giemsa-stained smears from both placental and venous blood were prepared from 402 women at delivery; 15.7% of the women were positive for *P. falciparum* in the placental smear, and both the percentage of positives and the mean parasite density tended to decrease with gravidity, with the exceptions of mean density in women of gravidity 2 that was higher than in women of gravidity 1, and prevalence in women of gravidity 3 that was higher than in women of gravidity 2 (Table 1). Presence and density of parasites in the venous smear (see Table 1) correlated well with the results of the placental smear. Fifty-five of the women (13.7%) were positive on both placental and venous smear, whereas in 8 cases (2.0%) only the placental smear was positive and in 4 cases (1.0%) only the venous smear was positive. Four of the women had a low density *P. vivax* infection according to the venous smear, 3 of which were also *P. vivax* positive on the placental smear, but none of the women in the study was positive for *P. malariae* or *P. ovale*. Histology of placental sections, which is the gold standard for the detection of placental malaria,^{7,40} was performed on 192 of the placentas (48 placentas from SAO women and 3 matched controls for each of them); 3.1% of them had an active infection, 12% had an active-chronic infection, and 27.1% had a past-chronic infection (see Table 1). Again, prevalence decreased with gravidity (see Table 1). There was good agreement between histology results for active infection (active only or active-chronic) and microscopy results: 21 women (10.9%) had a positive placental smear and had an active infection as determined by histology, whereas 5 (2.6%) had a positive placental smear only and 8 (4.2%) had an active infection by histology only.

The mean birth weight (BW) was 2.89 kg (see Table 1), with LBW (BW < 2.5 kg) occurring in 15.9% of the newborns. Mean BW increased significantly with gravidity ($P < 0.001$ using one-way analysis of variance), as previously observed in other settings.³ Mean Hb value at delivery was 9.5 g/dL (see Table 1). Maternal severe anemia (Hb below 7 g/dL) occurred in 12.2% of the women. Mean Hb decreased signifi-

cantly with gravidity ($P = 0.012$ using one-way analysis of variance), suggesting that factors other than malaria play a prominent role in determining Hb levels in this population. Blood loss from repeated pregnancies and/or transfer of iron to the fetus may account for the lower Hb levels in multi-gravidae.

Women with a *P. falciparum* infection (detected by microscopy) tended to have lower Hb values (average 0.25 g/dL lower) and babies with lower BW (average 62 g lower), but these tendencies were not statistically significant. The prevalences of infant LBW and of maternal severe anemia were not significantly different between women with or without an infection.

Prevalence of the South-East Asian ovalocytosis trait. The SAO status was determined by PCR for all women participating in the study. Forty-eight of 404 women (11.9%) from whom blood was collected at delivery carried the SAO trait. The prevalence of the trait among pregnant women attending the antenatal clinic was similar (13.2% in 281 women). In contrast, the prevalence of the SAO trait in a group of 196 non-pregnant women within the same age group and with the same geographic sub-region distribution as in women enrolled at delivery was 17.3%, and the prevalence for 49 non-pregnant women individually matched with 49 of the women attending the antenatal clinic was 22.4%. Overall, the SAO trait was present in 18.4% of 245 non-pregnant women but only in 12.4% of 685 pregnant women. The difference in the prevalence of the SAO trait between the 404 women at delivery and the 196 matched non-pregnant women approached significance ($P = 0.076$ using two-tailed Fischer's test), which together with the observation that this tendency was consistent for other groups of pregnant and non-pregnant women suggests that the SAO trait may be less common among pregnant women.

Placental infection in women with South-East Asian ovalocytosis. Sequestration of infected red blood cells in the placenta does occur in SAO women (Figure 1); 10.4% of the SAO women had an active infection (either active only or active-chronic), and 31.2% had a past-chronic infection, whereas active and past-chronic infection occurred in 16.7% and 25.7% of the control women, respectively (Table 2). These differences were not significant. However, high-density active infections (moderate or severe) were less common in SAO women than in control women (see Table 2), although this difference was not statistically significant (OR 0.19, 95% CI 0.02–1.55, $P = 0.121$). The same tendency was observed

TABLE 1
Parasitological and clinical parameters at delivery

	Microscopy*		Histology (%)†				Hb‡	BW§¶
	Placental	Venous	Neg.	Active	Active-chronic	Past-chronic		
Total (n = 402)	15.7 (758, 0-108440)	14.7 (583, 0-104760)	57.8	3.1	12.0	27.1	9.5 ± 2.2	2.89 ± 0.48
Gravidity 1 (n = 100)	24.0 (1123, 0-108440)	26.3 (613, 0-104760)	37.5	0.0	20.0	42.5	10.1 ± 2.1	2.69 ± 0.44
Gravidity 2 (n = 80)	12.7 (2494, 0-23040)	11.2 (1233, 0-38680)	50.0	2.8	16.7	30.6	9.6 ± 2.1	2.87 ± 0.43
Gravidity 3 (n = 67)	19.4 (737, 0-107640)	14.9 (820, 0-24440)	62.5	8.3	8.3	20.8	9.4 ± 2.3	2.92 ± 0.47
Gravidity > 3 (n = 155)	10.3 (205, 0-21640)	9.1 (256, 0-12640)	68.5	3.3	7.6	20.7	9.2 ± 2.2	3.02 ± 0.49

* Values are % *P. falciparum* positive, in brackets geometric mean and range of density of parasites/μl of blood.

† Histology data only for 192 placenta, of which 40 were of gravidity 1, 36 of gravidity 2, 24 of gravidity 3 and 92 of gravidity > 3.

‡ Hemoglobin. Values are mean ± std. dev., in g/dL.

§ Birth weight. Values are mean ± std. dev., in kg.

¶ Twins were excluded from the analysis.

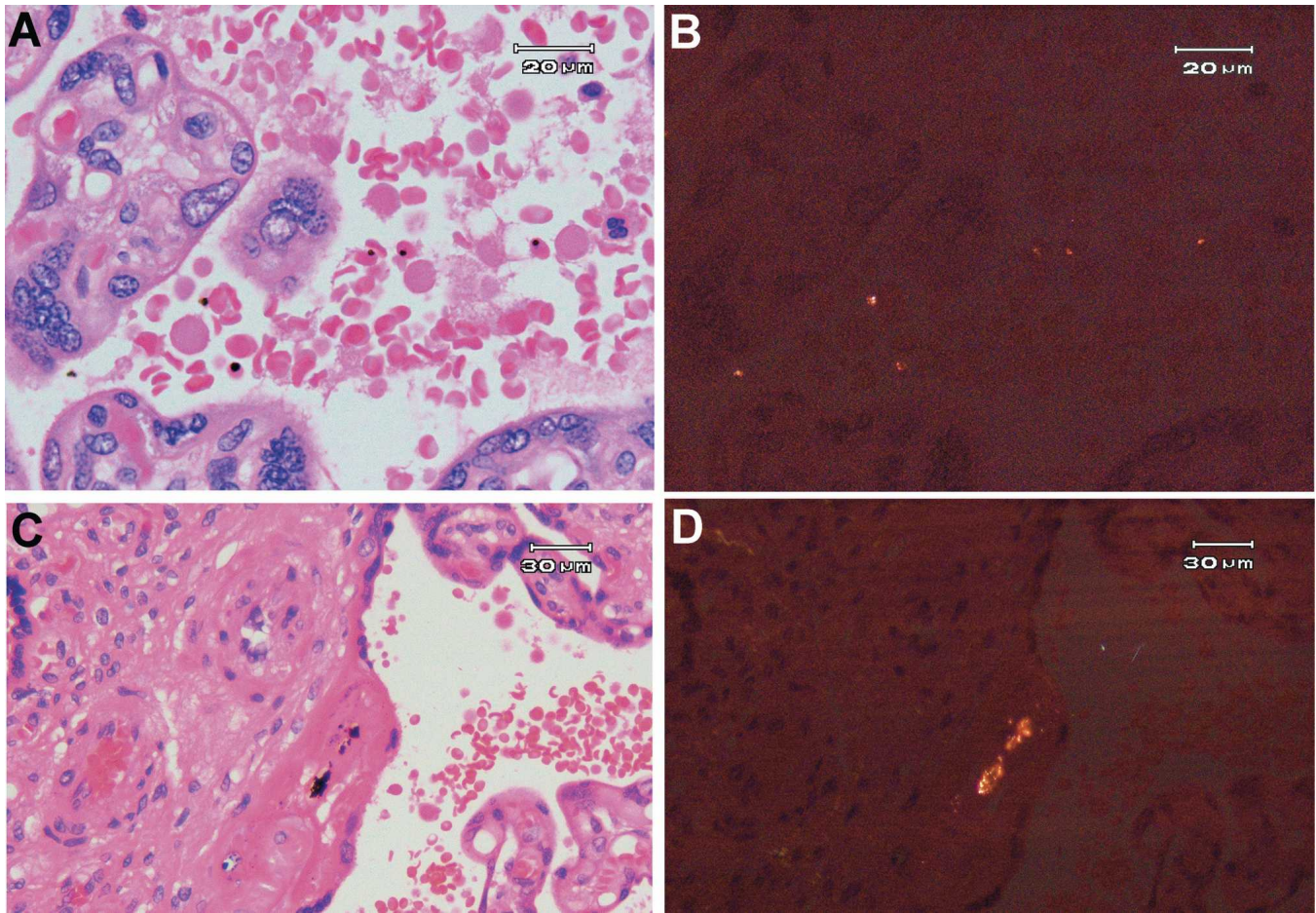


FIGURE 1. Histology of malaria-infected placentas from South-East ovalocytosis (SAO) women. Placentas are observed under normal (**A and C**) or polarized (**B and D**) light. **A and B**, SAO placenta showing an active infection (infected erythrocytes). **C and D**, SAO placenta showing a past-chronic infection (coarse malaria pigment within fibrin). This figure appears in color at www.ajtmh.org.

for past-chronic infections with high-density pigment deposition (moderate or severe) (OR 0.31, 95% CI 0.06–1.51, $P = 0.148$). Overall, the probability of having an infection scored as moderate or severe (either active or chronic) for SAO women was significantly lower than for control women (OR 0.22, 95% CI 0.06–0.81, $P = 0.022$).

Only 3 of 10 SAO primigravidae had an infection (either chronic or active), whereas 22 of the 30 control primigravidae had an infection (see Table 2). This difference was statistically

significant (OR 0.16, 95% CI 0.03–0.80, $P = 0.026$). In contrast, the prevalence of infection among multigravidae was similar between SAO and control women (OR 1.62, 95% CI 0.74–3.54, $P = 0.224$). Surprisingly, we did not observe gravidity-dependent changes in susceptibility to placental malaria among women with SAO. The prevalence of infection (either chronic or active) was similar between SAO primigravidae and SAO multigravidae (OR 1.89, 95% CI 0.42–8.43, $P = 0.405$), in contrast to the much higher prevalence in

TABLE 2
Infection by placental histology in matched South-East Asian ovalocytosis and control women in relation to gravidity

	% Neg.	Past-chronic infection*			Active infection*†		
		% Mild	% Mod.	% Sev.	% Mild	% Mod.	% Sev.
Control							
Total (n = 144)	57.6	14.6	10.4	0.7	7.6	6.3	2.8
Gravidity 1 (n = 30)	26.7	16.7	30.0	0.0	6.7	13.3	6.7
Gravidity >1 (n = 114)	65.8	14.0	5.3	0.9	7.9	4.4	1.8
SAO							
Total (n = 48)	58.3	27.1	2.1	2.1	8.3	2.1	0.0
Gravidity 1 (n = 10)	70.0	30.0	0.0	0.0	0.0	0.0	0.0
Gravidity >1 (n = 38)	55.3	26.3	2.6	2.6	10.5	2.6	0.0

* Infections were classified as mild, moderate, or severe as defined in the Materials and Methods section.

† Either active only or active-chronic.

TABLE 3
Infection by microscopy in matched South-East Asian ovalocytosis and control women in relation to gravidity

	Placental smear*				Venous smear*			
	% Neg.	% Low	% Med.	% High	% Neg.	% Low	% Med.	% High
Control								
Total (n = 144)	86.1	4.9	5.6	3.5	86.0	7.0	4.2	2.8
Grav. 1 (n = 30)	73.3	3.3	13.3	10.0	69.0	17.2	6.9	6.9
Grav. >1 (n = 114)	89.5	5.3	3.5	1.8	90.3	4.4	3.5	1.8
SAO								
Total (n = 48)	87.5	10.4	2.1	0.0	89.6	8.3	2.1	0.0
Grav. 1 (n = 10)	100.0	0.0	0.0	0.0	90.0	10.0	0.0	0.0
Grav. >1 (n = 38)	84.2	13.2	2.6	0.0	89.5	7.9	2.6	0.0

* Low corresponds to a *P. falciparum* density 1–500 parasites/ μ L of blood, med. 501–3000 parasites/ μ L, and high >3000 parasites/ μ L.

control primigravidae compared with control multigravidae (OR 0.19, 95% CI 0.08–0.46, $P = 0.000$) (see Table 2).

Parallel analysis using blood film data instead of histology data showed similar results (Table 3). SAO women had a lower probability of having a positive smear with a density above 500 parasites/ μ L, but the difference was not significant (for placental smears, OR 0.20, 95% CI 0.02–1.61, $P = 0.130$, for venous smears, OR 0.28, 95% CI 0.04–2.29, $P = 0.237$). Again, the difference between SAO and control women was larger among primigravidae (see Table 3).

Mean Hb and BW were very similar between SAO and control women, as were the prevalence of low BW and severe anemia (data not shown).

Antibodies against placental-binding-like isolates in women with South-East Asian ovalocytosis. As determined by flow cytometry, sera from SAO women at delivery contained antibodies against the parasite line CS2, which adheres to CSA and expresses variant surface antigens resembling those from placental-binding isolates (Table 4).³⁹ These antibodies occurred at similar levels between SAO and control women ($P = 0.46$ using Mann-Whitney test), and this was true for both gravidity groups ($P = 0.947$ for primigravidae and $P = 0.639$ for multigravidae) (see Table 4). The level of antibodies against CS2 surface antigens provides an indication of exposure to placental-binding parasites. As expected, antibodies against CS2 were more abundant in multigravidae than in primigravidae ($P = 0.002$ using Mann-Whitney test), both among SAO ($P = 0.028$) and among control ($P = 0.027$) women (see Table 4).

In contrast, gravidity did not affect the level of antibodies against the E8B parasite line ($P = 0.772$) (see Table 4), which adheres to CD36 and ICAM-1 and expresses variant surface antigens similar to isolates that commonly infect children.³⁹ SAO and control women had similar levels of antibodies against E8B parasites ($P = 0.616$) (see Table 4).

Pregnancy-related clinical malaria in women with South-East Asian ovalocytosis. Blood was collected from 197 pregnant women attending the antenatal clinic and experiencing either fever, history of fever in the past week, or headache. Fifty-one of them had a *P. falciparum* positive slide, of whom 28 had a parasite density higher than 1000 parasites/ μ L. We consider that, in these women, fever was most likely attributable to placental malaria, because this level of parasitemia is rarely seen among non-pregnant adult women. For instance, only 1 of 196 women of similar age living in the same area had a parasitemia above this threshold in a cross-sectional survey conducted on non-pregnant women. Furthermore, others

have shown that parasites in peripheral blood from pregnant women living in endemic areas usually originate from an ongoing placental infection.⁴¹

Four of the 28 women (14%) presumptively suffering from clinical malaria in pregnancy carried the SAO trait, suggesting that this trait does not provide protection, or provides incomplete protection, against clinical malaria in pregnancy.

DISCUSSION

The SAO trait, which reaches a high prevalence in some malarious areas of the Western Pacific, has been shown to confer specific protection against cerebral malaria.^{28,29} Sequestration of infected erythrocytes in the microvasculature of the brain is a major factor associated with the development of this pathology.⁴² Cerebral malaria is a relatively rare syndrome in Melanesian populations,⁴³ which led us to speculate that, in addition to conferring protection against cerebral malaria, the SAO trait might confer a selective advantage against another pathology closely associated with sequestration of infected erythrocytes, placental malaria. This would explain the high prevalence of the trait in some Melanesian populations because it would increase the chance of survival for both mothers carrying the trait and their infants (who are more likely to carry and perpetuate the trait than descendants of non-SAO mothers).

The main finding of this study was that SAO women do suffer from placental malaria, and SAO-infected erythrocytes can sequester in the placenta. Using a case-control approach, we found that the prevalence of placental infection

TABLE 4
Antibodies against CS2 and E8B parasites in South-East Asian ovalocytosis and control women at delivery, determined by flow-cytometry

	CS2		E8B	
	% Positive	Median MFI*	% Positive	Median MFI*
Control				
Gravidity 1 (n = 18)	83.3	24.7	72.2	12.6
Gravidity >1 (n = 35)	97.1	49.2	62.9	8.3
SAO				
Gravidity 1 (n = 6)	100.0	23.5	66.7	10.8
Gravidity >1 (n = 18)	88.9	62.7	83.3	12.1

These experiments were performed only with sera from a subset of the women. (The numbers of sera are indicated in the first column.)

* MFI, mean fluorescence intensity, calculated relative to the positive control according to the formula detailed in the Materials and Methods.

was not significantly different between control and SAO women when all gravidity groups were analyzed together. However, the prevalence of moderate or severe placental infections (either active or chronic) was lower in SAO than in control women. High-density active infections clearly indicate accumulation of large numbers of parasites in the placenta, but the density of pigment deposition in past-chronic infections can be indicative of either the time elapsed from a past infection or its severity. However, we believe that the density of pigment deposition relates to the severity of the past infection, because in non-SAO women the percentage of moderate and severe past infections decreased with gravidity, as expected for a true marker of severity. When data was stratified by gravidity, it became apparent that differences between SAO and control women in the prevalence and severity of placental infection only occurred in primigravidae. SAO primigravidae had significantly lower prevalence of placental infection than normal (non-SAO) primigravidae, who are at the highest risk of suffering placental malaria and its associated complications.^{1,3,6,7} On the other hand, the prevalence and severity of placental infections in multigravidae was similar between SAO and controls, presumably because with successive pregnancies normal women develop immune responses that reduce their risk of suffering from placental malaria to the levels observed in SAO women from the first pregnancy. Because histologic determination of the severity of placental infections is only semi-quantitative and the number of SAO primigravidae in our study was low, this intriguing observation must be taken with caution, but it clearly opens the way for larger studies specifically designed to address this issue.

The observation that reduced risk of placental infection in SAO women occurred at first pregnancy suggests that either immunologic mechanisms are not involved in the protection observed or SAO women mount more effective immune responses against placental parasites during the first pregnancy. However, our flow cytometry experiments did not reveal any major difference between SAO and control women in the acquisition of antibodies against variant surface antigens from placental-binding-like parasites. Although we only determined these antibody titers in a small subset of sera and we can not draw conclusions about their prevalence in this population, these results clearly indicate that SAO women are exposed to placental binding parasites and that they do not develop drastically higher titers of antibodies against their surface antigens. Thus, although we can not completely rule out the possibility that antibodies to placental-binding parasites in SAO women have different specificity that confers them a higher protective effect, it is likely that the protection against high-density placental infection observed in SAO primigravidae operates via non-immune mechanisms.

The observation in children that the SAO trait confers protection against cerebral malaria but not against malaria parasitemia or severe malaria anemia²⁹ suggests that protection acts via post-invasion mechanisms. This has led several authors to hypothesize that the SAO trait might prevent sequestration of infected erythrocytes.^{24,28,29} Our *in vitro* studies support the view that differences in the invasion of SAO erythrocytes alone are not likely to explain the protection conferred,⁴⁴ but we found that SAO infected erythrocytes cytoadhere *in vitro* to the principal receptors for sequestration, including CSA, suggesting that sequestration of SAO

infected erythrocytes does occur.⁴⁵ Here we show by histologic observation of placental sections that infected erythrocytes from SAO women accumulate in the placenta. This result confirms the predictions from our *in vitro* experiments, and provides the first *in vivo* evidence that SAO erythrocytes do sequester, using the only model amenable for the observation of sequestered infected erythrocytes *in vivo* in a large number of samples.

Our *in vitro* studies also showed that SAO-infected erythrocytes exhibit an altered adhesive behavior,⁴⁵ but the alteration was in the opposite direction to that previously predicted. Under conditions of flow, SAO erythrocytes infected with the CD36-binding line 3D7 bind more efficiently than normal infected erythrocytes to the principal endothelial receptor for sequestration, CD36.⁴⁵ Thus, the SAO trait may affect the tissue distribution of sequestered infected erythrocytes for parasites with dual binding to CD36 and other receptors.⁴⁵ Parasites that sequester in the placenta do not bind to CD36 because since binding to CD36 and CSA are mutually exclusive.²² Therefore, the increase in binding to CD36 associated with SAO would not affect the distribution of placental binding parasites. Thus, the observation that SAO-infected erythrocytes do sequester in the placenta is not at odds with our previous findings.

An intriguing observation of this study was the lower prevalence of the SAO trait among pregnant women. It is unlikely that low fertility is associated with this trait, because some SAO women had many descendants (up to 8 for the SAO women enrolled in this study), and the average number of descendants was similar between SAO and control pregnant women. It is also unlikely that the difference is explained by miscarriages of SAO homozygous fetus, because those could only account for 25% of the conceptions when the father is also SAO and 0% when the father is not SAO. One possible explanation would be that the combination of SAO with some other trait produces sterility, whereas women who do not carry this hypothetical other trait would have normal fertility. The reduced prevalence of the SAO trait among pregnant women will have to be taken into consideration when designing any further study that assesses the effect of the SAO trait on placental malaria. The power of our study to detect a protective effect of this trait was reduced because our calculations were based on previous estimates of a 35% prevalence of the SAO trait in this area,²⁴ whereas the actual prevalence that we found in pregnant women was only 12.4%. Furthermore, we believe that 18% might provide an accurate estimation of the prevalence of the SAO trait in non-pregnant individuals from the Madang North Coast area. We collected samples from many different villages along the full area and avoided collecting samples from multiple individuals of the same family, and did not observe as high a prevalence of the SAO trait as previously reported.²⁴ Another recent study conducted in Liksul, at the northern end of our study area, found a prevalence of the SAO trait of less than 15%.⁴⁶

The enormous burden of malaria has shaped the human genome in numerous ways to increase the chances of survival from this disease.⁴⁷ Here we describe that the SAO trait, well known to confer specific protection against cerebral malaria, was associated in our study with some level of protection against placental malaria in primigravidae. Although this observation will have to await confirmation from larger studies, a common fundamental step for both pathologies is the se-

questration of infected erythrocytes, which makes it likely that the altered adherent behavior caused by the deletion in band 3 lies behind the mechanism of protection against both pathologies. A selective advantage against placental malaria would implicate that the mutation is selected and perpetuated not only by conferring a selective advantage against malaria to its carriers but also to the descendants of female carriers, but this could not be completely confirmed because we did not observe an improved birth weight outcome in newborns from SAO women. Because of the intrinsic variability in BW and the relatively low prevalence of LBW, this would require a study with an enormous sample size.

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