Brief Definitive Report

Evidence for the Involvement of VAR2CSA in Pregnancy-associated Malaria

Ali Salanti,[1,2] Madeleine Dahlbäck,[1,2] Louise Turner,[1,2] Morten A. Nielsen,[1,2] Lea Barfod,[1,2] Pamela Magistrado,[1,2] Anja T.R. Jensen,[1,2] Thomas Lavstsen,[1,2] Michael F. Ofori,[2,3] Kevin Marsh,[4] Lars Hviid,[1,2] and Thor G. Theander[1,2]

[1]*Centre for Medical Parasitology, Department of Medical Microbiology and Immunology, University of Copenhagen and* [2]*Department of Infectious Diseases, Copenhagen University Hospital, 2200 Copenhagen, Denmark*
[3]*Noguchi Memorial Institute for Medical Research, University of Ghana, 80108 Legon, Ghana*
[4]*Kenya Medical Research Institute, Centre for Geographic Medicine Research, Coast Kilifi, Kenya*

Abstract

In *Plasmodium falciparum*–endemic areas, pregnancy-associated malaria (PAM) is an important health problem. The condition is precipitated by accumulation of parasite-infected erythrocytes (IEs) in the placenta, and this process is mediated by parasite-encoded variant surface antigens (VSA) binding to chondroitin sulfate A (CSA). Parasites causing PAM express unique VSA types, VSA_{PAM}, which can be serologically classified as sex specific and parity dependent. It is sex specific because men from malaria-endemic areas do not develop VSA_{PAM} antibodies; it is parity dependent because women acquire anti-VSA_{PAM} immunoglobulin (Ig) G as a function of parity. Previously, it was shown that transcription of *var2csa* is up-regulated in placental parasites and parasites selected for CSA binding. Here, we show the following: (a) that VAR2CSA is expressed on the surface of CSA-selected IEs; (b) that VAR2CSA is recognized by endemic plasma in a sex-specific and parity-dependent manner; (c) that high anti-VAR2CSA IgG levels can be found in pregnant women from both West and East Africa; and (d) that women with high plasma levels of anti-VAR2CSA IgG give birth to markedly heavier babies and have a much lower risk of delivering low birth weight children than women with low levels.

Key words: *var* gene • *var2csa* • *Plasmodium falciparum* • PfEMP1 • vaccine

Introduction

Individuals living in areas of intense *Plasmodium falciparum* transmission have acquired protective immunity to malaria by the time they reach sexual maturity. Pregnant women constitute an important exception to this rule, and pregnancy-associated malaria (PAM) is an important cause of maternal and perinatal morbidity and mortality in such areas (1). PAM is characterized by placental accumulation of parasite-infected erythrocytes (IEs), which are unusual in being able to bind to chondroitin sulfate A (CSA) in vitro (2) and in not being recognized by IgG in the plasma of malaria-exposed men who have IgG with specificity for IEs infecting nonpregnant individuals (3, 4).

These data suggest that the variant surface antigens (VSAs) responsible for placental adhesion to CSA are not only functionally and antigenically distinct from other molecules present at the iRBC surface, but also that they share relatively conserved antigenic determinants (2, 3, 5). Specific acquired immunity to PAM increases with increasing parity (3–5) and is mediated by IgG with specificity for PAM-type VSA (VSA_{PAM}; 6, 7). Parasites that express VSA_{PAM}-type antigens on the IE surface selectively transcribe an unusually structured *var* gene (*var2csa*) at high levels (8), but the presence of a short open reading frame upstream of the *var2csa* initiation codon (9, 10) has raised concern that *var2csa* mRNA may not be translated into protein despite high transcription rates (11). Here, we present evidence that VAR2CSA, which is a relatively conserved (8) member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family (12) is expressed on the surface of CSA-selected IEs and that plasma levels of VAR2CSA-specific IgG correlate with protection from PAM.

Address correspondence to Ali Salanti, Centre for Medical Parasitology, Dept. of Medical Microbiology and Immunology, Panum Institute 24-2, Blegdamsvej 3, 2200 Copenhagen, Denmark. Phone: 45-35-32-76-76; Fax: 45-35-32-78-51; email: salanti@cmp.dk

Materials and Methods

Recombinant Proteins. A synthetic *var2csa* gene based on the *P. falciparum* clone 3D7 sequence (PlasmoDB accession no.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/11/1197/7 $8.00
Volume 200, Number 9, November 1, 2004 1197–1203
http://www.jem.org/cgi/doi/10.1084/jem.20041579

PFL0030c) was generated and optimized for codon usage by *Trichoplusia ni* insect cell lines. Repeats, poly-A sites, and instability elements were removed without altering the protein sequence (Geneart). Domains of the synthetic *var2csa* gene were cloned into the pBAD-TOPO vector (Invitrogen) by PCR using the following primers: DBL1-X, Fw: 5′-CGGAATTCATGGACA-AGTCCTCCATC-3′, DBL1-X, Rv: 5′-GATGCAGGTCTT-GTTGCT-3′; and DBL5-ε, Fw: 5′-CGGAATTCCTGGACA-GATGCTTCGAC-3′, and DBL5-ε, Rv: 5′-CTTGTTGCAG-ATGTAGTC-3′. An exon 2 construct and the DBL5-ε domain of *var4* gene were cloned (13). Recombinant proteins based on these constructs were produced in *Baculovirus*-infected Sf9 cells and purified (13).

Animal Antisera. Antisera were raised by injection of 20 μg of recombinant proteins (s.c.) into rabbits in Freund's complete adjuvant, followed by four booster injections of 20 μg of protein in Freund's incomplete adjuvant at 3-wk intervals. Antisera were collected 15 d after the final booster injection. The total amount of IgG in the rabbit sera was measured using a rabbit serum IgG ELISA kit.

Human Plasma Samples. Three panels of plasma samples from Ghanaian individuals exposed to hyperendemic and seasonal *P. falciparum* transmission were used. Ethical clearance was granted by the Ministry of Health, Ghana. The first panel was collected from 39 men and 46 women, the second was from 30 men and 27 term-pregnant women, and the third was obtained at the time of delivery from 62 pregnant women of different parities. To analyze the relationship between IgG reactivity and recombinant proteins and birth weight of offspring, we used a plasma sample set obtained at the time of delivery from 110 Kenyan women with histological evidence of active-chronic placental *P. falciparum* infection (14). Ethical clearance was granted by the Kenyan Medical Research Institute National Ethical Review Committee. Plasma samples from six adults without *P. falciparum* exposure were used as negative controls.

Malaria Parasites and In Vitro Selection Procedures. All *P. falciparum* lines used were maintained in culture (15). Selection for expression of VSA_{PAM}-type antigens on the IE surface was achieved by repeated panning of IEs on CSA in vitro (3, 4). Selection for expression VAR2CSA was achieved by repeated panning of IEs using IgG-reactive DynaBeads VAR2CSA DBL5-ε–specific antisera (15).

Measurement of var2csa-specific Antibody Levels in Plasma. Plasma levels of *P. falciparum* antigen-specific IgG were measured in standard ELISA assays (16). Cut-off values were set to mean plus two standard deviations of readings obtained with the negative control plasma samples.

Measurement of var2csa-specific, IE Surface-reactive Antibodies. IgG reactivity to antigens expressed on the surface of intact *P. falciparum*–infected erythrocytes were evaluated by flow cytometry and confocal microscopy. Analysis by flow cytometry was performed as described (13), except for the sequential usage of 4 μl of goat anti–rabbit IgG (401311; Calbiochem), 4 μl of biotinylated rabbit anti–goat Ig (DakoCytomation), and 0.5 μl of FITC-conjugated streptavidin (BD Biosciences) for detection of rabbit IgG on the IE surface. Confocal microscopy was done on an LSM5 scanning microscope (Carl Zeiss MicroImaging, Inc.) using wet-mounted antibody-labeled IEs.

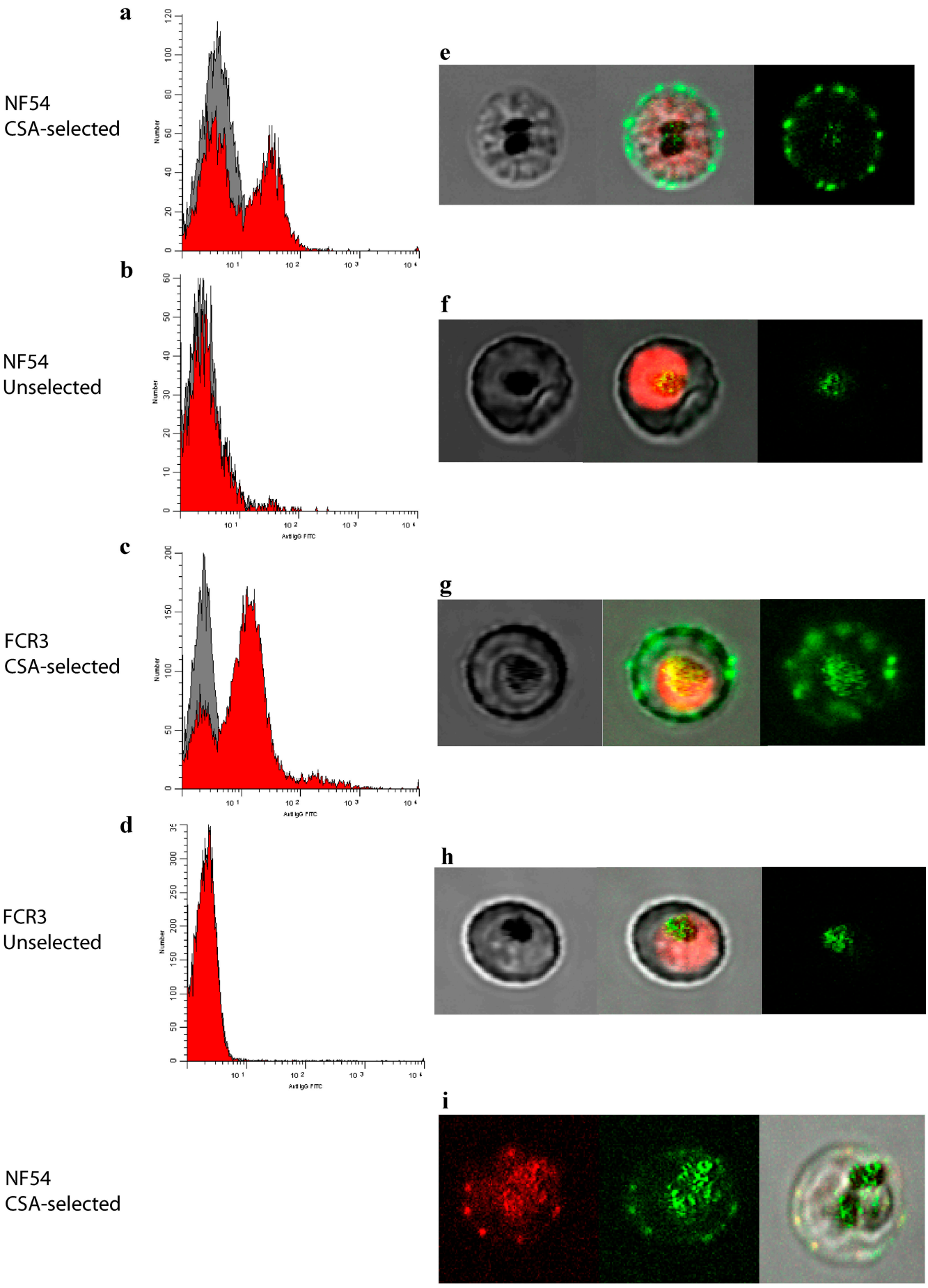
Statistical Analysis. Sex specificity of IgG levels was evaluated by Mann-Whitney rank sum test (T), whereas parity dependency was evaluated by Spearman's rank correlation coefficient (rs). Stata v. 7 (Stata Corporation), SigmaStat v. 3.0 (SPSS), and CIA v. 2.0 software were used.

Results and Discussion

P. falciparum parasites implicated in PAM express a serologically defined VSA subset (VSA_{PAM}) on the IE surface. VSA_{PAM} are not recognized by IgG in the plasma of malaria-exposed men (sex specificity), and levels of VSA_{PAM}-specific IgG in the plasma of women depend on their number of pregnancies (parity dependency; references 3–5). To investigate whether *var2csa*-encoded proteins (VAR2CSA) are expressed on the surface of such IEs, we determined the IE surface reactivity of antisera raised against recombinant proteins corresponding to two *var2csa* domains.

We found that IgG in rabbit antisera raised against DBL5-ε domain of VAR2CSA specifically labeled the surface of erythrocytes infected by two VSA_{PAM}-expressing parasite lines (Fig. 1, a and c). Furthermore, examination of IEs by confocal microscopy showed a punctate surface labeling characteristic of PfEMP1 surface localization (Fig. 1, e, g, and i). VAR2CSA-specific antisera did not label erythrocytes infected by isogenic parasite lines not expressing VSA_{PAM} (Fig. 1, b, d, f, and h). Experiments using antisera raised against DBL1-X gave identical results (unpublished data). VSA_{PAM}-expressing IEs have previously been shown to have a generally increased reactivity with IgG and IgM (17, 18). Therefore, we tested control rabbit antisera containing the same amount of total IgG and raised against a surface-exposed domain (DBL5-ε) of a PfEMP1 associated with severe *P. falciparum* malaria in children (VAR4; references 13, 15). Neither VAR4-specific antiserum (Fig. 1, a–d) nor rabbit prebleed control serum (not depicted) reacted with the surface of intact erythrocytes infected by any of the aforementioned parasite lines. The double peak in the flow diagram of anti-VAR2CSA–labeled CSA-selected NF54 (Figs. 1 a and 2 a) indicated that approximately half of these parasites expressed VAR2CSA, suggesting that the remaining parasites expressed other VSA_{PAM} than VAR2CSA or that some of the parasites had changed VSA expression since CSA selection. To investigate this, we selected the NF54CSA isolate on VAR2CSA antibodies using IgG-reactive DynaBeads and obtained a parasite line in which a markedly larger proportion of the IEs reacted with VAR2CSA IgG (Fig. 2, a and b). After IgG selection, the parasites were also better recognized by plasma from term-pregnant women and mostly unrecognized by plasma from sympatric men (Fig. 2, c and d).

Figure 1. Analysis by flow cytometry (a–d) and confocal microscopy (e–h) of intact erythrocytes infected by *P. falciparum* line NF54 (a, b, e, f, and i) or FCR3 (c, d, g, and h) and labeled by IgG in rabbit antisera raised against VAR2CSA DBL5-ε (a–d, solid red, and e–h) or VAR4 DBL5-δ (a–d, solid gray) as a control serum. Infected erythrocytes subjected to several rounds of in vitro selection for adhesion to CSA (a, c, e, g, and i) or unselected (b, d, f, and h) are shown. Confocal microscopy images (e–h) were unstained (left), stained with ethidium bromide and labeled by anti-VAR2CSA IgG (middle), or stained by anti-VAR2CSA IgG only. Confocal panel i shows the double stained parasites. The left image (red) is IgG staining with pooled plasma from pregnant women; the middle image (green) is stained with anti-VAR2CSA IgG; and the right image is an overlay of the two first images. The staining of the central spot in the confocal images without EtBr is caused by autofluorescence of hemozoin.


a
b
c
d
e
f
g
h
i
NF54
CSA-selected
NF54
Unselected
FCR3
CSA-selected
FCR3
Unselected
NF54
CSA-selected
Number
Anti IgG FITC

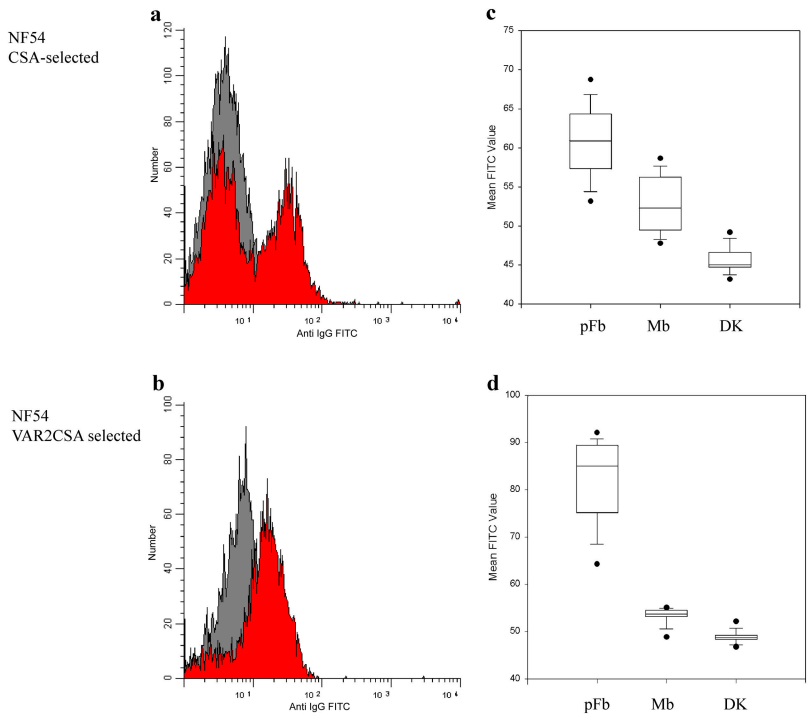


Figure 2. Flow cytometry analyses of intact infected erythrocytes (a and b) labeled by IgG against VAR2CSA DBL5-ε (solid red) and VAR4 DBL5-δ (solid gray). Erythrocytes were infected with CSA-selected NF54 (a) or a subline of this parasite, which had been subjected to additional in vitro selection for binding to VAR2CSA DBL5-ε–specific IgG (b). (c and d) The recognition of the two parasite lines by plasma IgG from 10 Ghanaian term-pregnant women (pFb), 10 sympatric Ghanaian men (Mb), and 10 Danish controls (DK). For each group, the median (central line), the central 50% (box), the central 80% (bars), and the central 90% (•) of data points are shown.

To investigate the relationship between VAR2CSA and VSA_{PAM} further, we used flow cytometry to analyze IEs double labeled with an antiserum to VAR2CSA DBL5-ε and plasma pools from *P. falciparum*–exposed men and pregnant women, respectively. VSA_{PAM}-expressing, CSA-selected IEs appeared as a double positive population (Fig. 3 a) when using the pool from pregnant women, but as a single (VAR2CSA DBL5-ε) positive population when using the male pool (Fig. 3 b). In contrast, unselected IEs expressing non-PAM–type VSA were not labeled by the DBL5-ε antiserum and were recognized to a similar degree by the plasma pools from men and pregnant women (Fig. 3, c and d). Examination of IEs by confocal microscopy showed that the targets of IgG in the DBL5-ε antiserum and the pooled plasma from pregnant women colocalized on the IEs surface (Fig. 1 i). Together, these results suggest that VAR2CSA is selectively expressed on the surface of IEs that have been selected for adhesion to CSA in vitro and that have a sex-specific and parity-dependent VSA_{PAM}-type IgG recognition profile. The data also indicate that the targets of the VAR2CSA-specific and VSA_{PAM}-specific IgG are identical or closely associated.

To examine whether human IgG recognition of VAR2CSA depended on plasma donor sex and parity, we used ELISA to measure levels of VAR2CSA-specific IgG in panels of individual plasma samples from *P. falciparum*–exposed men and women. Median levels of IgG specific for recombinant proteins corresponding to the DBL1-X and DBL5-ε domains of VAR2CSA were significantly different (DBL1-X: $P < 0.001$ [T], median difference [95% CI] = 0.10 [0.054–0.20]; DBL5-ε: $P < 0.001$ [T], median difference [95% CI] = 0.14 [0.081–0.27]) in plasma from women than in plasma from men (Fig. 4 a). This pattern was even more pronounced when examining samples from a separate cohort of sympatric men and term-pregnant women (DBL1-X: $P < 0.001$ [T], median difference [95% CI] = 2.13 [1.54–2.45]; DBL5-ε: $P < 0.001$ [T], median difference [95% CI] = 2.34 [1.46–2.59]; Fig. 4 b). Negative control plasma from donors without *P. falciparum* exposure did not contain VAR2CSA-specific IgG (unpublished data).

To exclude that the differences in VAR2CSA-specific IgG reactivity were simply due to a generally increased *P. falciparum*–specific IgG reactivity among the women, we also measured levels of IgG specificity for another, non-PAM–type PfEMP1 (VAR4; reference 13) and the non-PfEMP1 antigen, GLURP (16). We did not observe any significant sex-specific differences in the IgG levels for either of these antigens (VAR4 DBL5-δ: $P = 0.096$ [T], median difference [95% CI] = −0.047 [−0.22–0.16] [Fig. 4 a]; $P = 0.78$ [T], median difference [95% CI] = 0.00 [−0.18–0.13]; Fig. 4 b) or GLURP ($P = 0.39$ [T], median difference [95% CI] = −0.11 [−0.46–0.16] and $P = 0.23$ [T], median difference [95% CI] = 0.27 [−0.14–0.91]; Fig. 4 c). In addition to the sex specificity of VAR2CSA-specific IgG levels, we observed that these levels also depended on donor parity in individual plasma samples from term-pregnant women with parities ranging from 0 to 10 ($rs = 0.38$, $P = 0.0027$; Fig. 4 d). This correlation remained significant in a linear regression model, including both parity and age as explanatory variables ($P = 0.03$). In contrast, GLURP-specific IgG levels did not depend significantly on parity ($P = 0.94$; unpublished data).

Plasma levels of VSA_{PAM}-specific IgG correlate with acquisition of protective immunity to PAM; in fact, there is strong recent evidence to suggest a causal relationship between

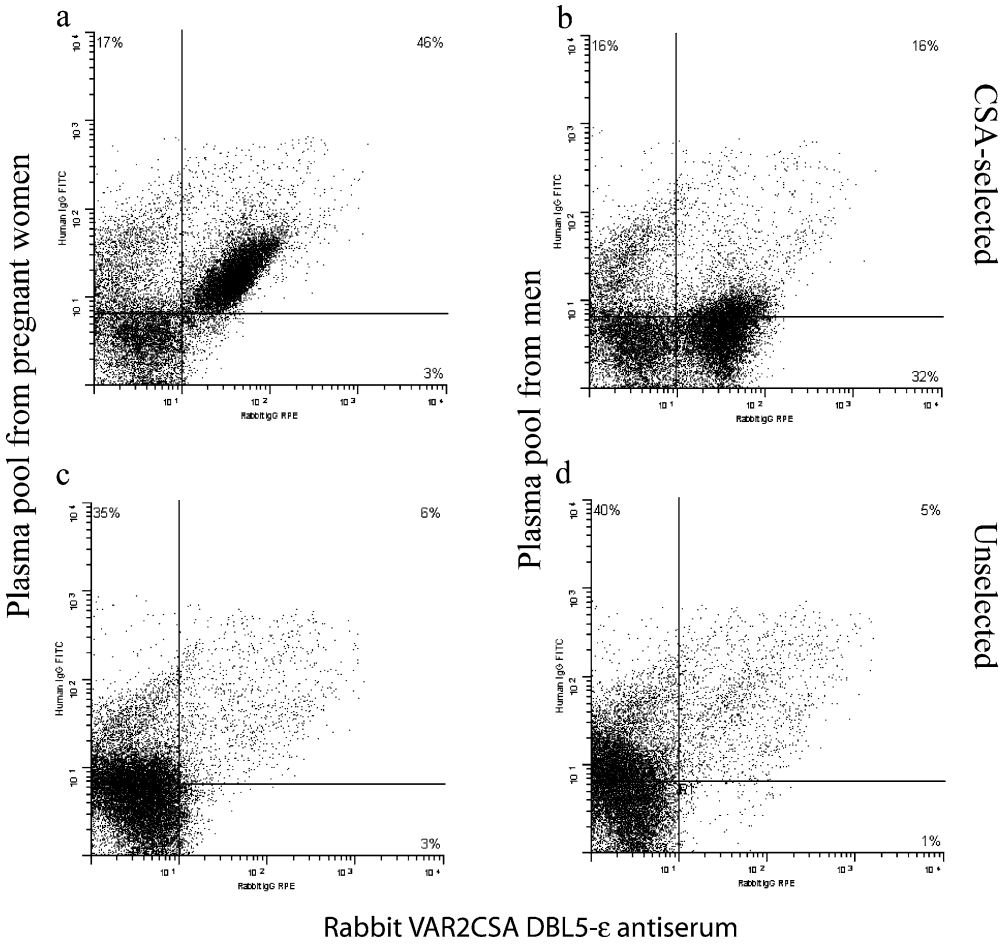


Figure 3. Colabeling of intact erythrocytes infected by *P. falciparum* line FCR3 by IgG in rabbit antisera raised against VAR2CSA DBL5-ε and by plasma pools from *P. falciparum*–exposed, term-pregnant women (a and c) or from sympatric men (b and d). Infected erythrocytes subjected to several rounds of in vitro selection for adhesion to CSA (a and b) or without pretreatment (c and d) are shown.

high levels of VSA_{PAM}-specific antibodies and protection from maternal anemia, low birth weight, and prematurity due to PAM (6, 7). Adverse PAM-related pregnancy outcomes are concentrated among women with an ongoing placental infection of sufficient duration to allow detection of both IEs and hemozoin-laden phagocytes in the placenta at the time of delivery (active-chronic–type PAM; reference 14), and it is also among women with active-chronic PAM that the protective effect of VSA_{PAM}-specific IgG is most readily detected (7).

To investigate whether a similar relationship exists between levels of IgG specific for VAR2CSA and birth weight of offspring, we studied the relationship between pregnancy outcome and VAR2CSA-specific plasma IgG levels at the time of delivery in a group of *P. falciparum*–exposed women participating in a study of the impact of PAM on pregnancy outcome. The samples studied here belonged to the random subset of the original cohort used to study the relationship between VSA_{PAM}-specific IgG levels and pregnancy outcome (7), and included all the 110 women in that subset who were found to suffer from active-chronic PAM. Logistic regression analysis showed that high (i.e., above median) plasma levels of VAR2CSA DBL5-ε–specific plasma IgG were associated with a fourfold lower risk of giving birth to a seriously underweight (<2.500 g) infant (Table I). Correspondingly, women with high (i.e., above median) plasma levels of VAR2CSA DBL5-ε–specific plasma IgG delivered babies weighing 2.864 g (95% CI: 2.700–3.029 g) compared with 2.436 g (95% CI: 2.264–2.608 g) for women with low (i.e., below median) VAR2CSA DBL5-ε–specific plasma IgG levels.

We have shown previously that the *var2csa* gene is remarkably conserved between parasite isolates (8). Here, we show that 3D7 VAR2CSA-specific antisera labeled different CSA-selected parasite lines (Fig. 1), and that 3D7 VAR2CSA-specific IgG could be detected in plasma from pregnant women from both West and East Africa (Figs. 2–4). Together, these findings indicate substantial serological cross-reactivity between VAR2CSA from different parasites. The fact that levels of VAR2CSA-specific IgG correlated with sex and parity in plasma from *P. falciparum*–exposed individuals (a characteristic shared with the VSA_{PAM}-specific IgG mediating protection against adverse pregnancy outcome due to placental *P. falciparum* infection; Fig. 4 and references 6, 7) is consistent with the paradigm that PAM develops in otherwise malaria-immune women when they are infected with parasites that rely on placental binding to CSA for survival and that express IE surface antigens to which the women do not have specific protective immunity.

Several groups have identified CSA-binding domains in PfEMP1 molecules (19-21). The most extensively studied of these genes (*var1csa*) does not show enhanced transcription in

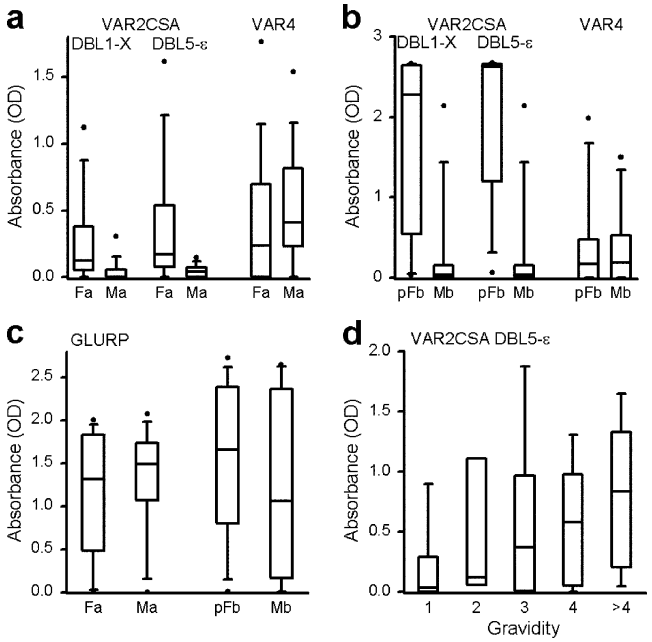


Figure 4. Plasma levels of IgG with specificity for the DBL1-X and DBL5-ε domains of VAR2CSA and the DBL5-δ domain of VAR4 in 46 women (Fa) and 39 sympatric men (Ma) from an area of hyperendemic seasonal malaria transmission (a) and in 27 term-pregnant women (pFb) and 30 sympatric 39 men (Mb) from an area of hyperendemic stable malaria transmission (b). (c) Levels of GLURP-specific IgG in the same groups of malaria-exposed individuals as in a and b. Plasma levels of VAR2CSA DBL5-ε–specific IgG at the time of delivery in 62 pregnant women (gravidities ranging from 1 to 10) from an area of hyperendemic seasonal malaria transmission (d). For each group, the median (central line), the central 50% (box), the central 80% (bars), and the central 90% (•) of data points are shown.

placental or CSA-selected parasites (22, 23) and is transcribed by parasites from nonpregnant females and children (24, 25). Although antibodies against VAR1CSA constructs have been reported to react selectively with the surface of placental and CSA-selected isolates (26, 27), the role of nonspecific IgG and IgM binding to placental and CSA-selected isolates (17, 18) and the impact of selection on *Saimiri* brain endothelial cells used in these experiments (28, 29) have only been partially resolved. Furthermore, sex-specific and parity-dependent plasma IgG recognition of IEs after *Saimiri* brain endothelial cell selection has not been reported, but domains from VAR1CSA do not show the expected VSA_{PAM}-type sex-specific and parity-dependent IgG recognition (30). Therefore, the presence of CSA-binding domains that can be inhibited by specific IgG or CSA in itself is not sufficient evidence for involvement in the pathogenesis of PAM (29).

In conclusion, we find that IEs with the VSA_{PAM} phenotype implicated in PAM selectively express VAR2CSA and that high VAR2CSA-specific IgG levels are related to favorable birth outcome. This raises hope that VAR2CSA-specific IgG induced by vaccination of women in malaria-endemic areas before their first pregnancy can protect against adverse consequences of malaria in pregnancy.

We are indebted to the individuals who donated parasite and plasma samples for the paper. We thank all the staff involved in the clinical studies. We thank T. Staalsoe for cultivation and in vitro selection of the parasite lines used. We thank A. Corfitz for excellent technical assistance.

The work received financial support from the Commission of European Union (grant nos. QLK2-CT-2001-01302, PAMVAC and QLK2-CT-2002-01197, EUROMALVAC2), the Danish Medical Research Council (grant nos. 22-02-0220 and 22-03-0333), and the Danish Research Council for Development Research (grant no. 104.Dan.8.L.306). A. Salanti and P. Magistrado are supported by Ph.D. studentships from the Gates Malaria Partnership. M. Dahlback and M. Ofori are supported by Ph.D. studentships from RUF. M. Nielsen and T. Lavstsen are supported by Ph.D. studentships from the Faculty of Health Sciences, University of Copenhagen. L. Barfod is also supported by a Ph.D. studentship.

The authors have no conflicting financial interests.

Submitted: 6 August 2004
Accepted: 7 September 2004

Table I. *Multiple Logistic Regression Analysis of Risk of Low Birth Weight (<2.500 g) in 110 Delivering Women with Histological Evidence of Active-Chronic Placental P. falciparum Infection*

	Odds ratio [95% CI]	P > z
VAR2CSA DBL5-ε IgG > median	0.25 [0.10–0.63]	0.003
Weight of mother	0.84 [0.75–0.94]	0.002
Middle arm circumference of mother	1.41 [1.02–1.93]	0.03

References

1. Brabin, B.J. 1983. An analysis of malaria in pregnancy in Africa. *Bull. World Health Organ.* 61:1005–1016.
2. Fried, M., and P.E. Duffy. 1996. Adherence of *Plasmodium falciparum* to chondroitin sulphate A in the human placenta. *Science.* 272:1502–1504.
3. Ricke, C.H., T. Staalsoe, K. Koram, B.D. Akanmori, E.M. Riley, T.G. Theander, and L. Hviid. 2000. Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulphate A. *J. Immunol.* 165:3309–3316.
4. Staalsoe, T., R. Megnekou, N. Fievet, C.H. Ricke, H.D. Zornig, R. Leke, D.W. Taylor, P. Deloron, and L. Hviid. 2001. Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes that protect against placental parasitemia. *J. Infect. Dis.* 184:618–626.
5. Fried, M., F. Nosten, A. Brockman, B.T. Brabin, and P.E. Duffy. 1998. Maternal antibodies block malaria. *Nature.* 395: 851–852.
6. Duffy, P.E., and M. Fried. 2003. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. *Infect. Immun.* 71:6620–6623.
7. Staalsoe, T., C.E. Shulman, J.N. Bulmer, K. Kawuondo, K. Marsh, and L. Hviid. 2004. Variant surface antigen-specific IgG and protection against clinical consequences of preg-

nancy-associated *Plasmodium falciparum* malaria. *Lancet*. 363: 283–289.
8. Salanti, A., T. Staalsoe, T. Lavstsen, A.T. Jensen, M.P. Sowa, D.E. Arnot, L. Hviid, and T.G. Theander. 2003. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol. Microbiol*. 49: 179–191.
9. Gardner, M.J., N. Hall, E. Fung, O. White, M. Berriman, R.W. Hyman, J.M. Carlton, A. Pain, K.E. Nelson, S. Bowman, et al. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*. 419:498–511.
10. Lavstsen, T., A. Salanti, A.T. Jensen, D.E. Arnot, and T.G. Theander. 2003. Sub-grouping of *Plasmodium falciparum* 3D7 *var* genes based on sequence analysis of coding and non-coding regions. *Malar. J*. 2:27.
11. Kozak, M. 2002. Pushing the limits of the scanning mechanism for initiation of translation. *Gene*. 299:1–34.
12. Su, X., V.M. Heatwole, S.P. Wertheimer, F. Guinet, J.A. Herrfeldt, D.S. Peterson, J.A. Ravetch, and T.E. Wellems. 1995. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell*. 82:89–100.
13. Jensen, A.T., P. Magistrado, S. Sharp, L. Joergensen, T. Lavstsen, A. Chiucchiuini, A. Salanti, L.S. Vestergaard, J.P. Lusingu, R. Hermsen, et al.. 2004. *Plasmodium falciparum* associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A *var* genes. *J. Exp. Med*. 199:1179–1190.
14. Shulman, C.E., T. Marshall, E.K. Dorman, J.N. Bulmer, F. Cutts, N. Peshu, and K. Marsh. 2001. Malaria in pregnancy: adverse effects on haemoglobin levels and birthweight in primigravidae and multigravidae. *Trop. Med. Int. Health*. 6:770–778.
15. Staalsoe, T., M.A. Nielsen, L.S. Vestergaard, A.T. Jensen, T.G. Theander, and L. Hviid. 2003. In vitro selection of *Plasmodium falciparum* 3D7 for expression of variant surface antigens associated with severe malaria in African children. *Parasite Immunol*. 25:421–427.
16. Dodoo, D., M. Theisen, J.A. Kurtzhals, B.D. Akanmori, K.A. Koram, S. Jepsen, F.K. Nkrumah, T.G. Theander, and L. Hviid. 2000. Naturally acquired antibodies to the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. *J. Infect. Dis*. 181:1202–1205.
17. Flick, K., C. Scholander, Q. Chen, V. Fernandez, B. Pouvelle, J. Gysin, and M. Wahlgren. 2001. Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science*. 293: 2098–2100.
18. Creasey, A.M., T. Staalsoe, A. Raza, D.E. Arnot, and J.A. Rowe. 2003. Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of *Plasmodium falciparum* isolates implicated in malaria during pregnancy. *Infect. Immun*. 71:4767–4771.
19. Buffet, P.A., B. Gamain, C. Scheidig, D. Baruch, J.D. Smith, R. Hernandez-Rivas, B. Pouvelle, S. Oishi, N. Fujii, T. Fusai, et al. 1999. *Plasmodium falciparum* domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc. Natl. Acad. Sci. USA*. 96:12743–12748.
20. Reeder, J.C., A.F. Cowman, K.M. Davern, J.G. Beeson, J.K. Thompson, S.J. Rogerson, and G.V. Brown. 1999. The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. *Proc. Natl. Acad. Sci. USA*. 96:5198–5202.
21. Degen, R., N. Weiss, and H.P. Beck. 2000. *Plasmodium falciparum*: cloned and expressed CIDR domains of PfEMP1 bind to chondroitin sulfate A. *Exp. Parasitol*. 95:113–121.
22. Salanti, A., A.T. Jensen, H.D. Zornig, T. Staalsoe, L. Joergensen, M.A. Nielsen, A. Khattab, D.E. Arnot, M.Q. Klinkert, L. Hviid, and T.G. Theander. 2002. A sub-family of common and highly conserved *Plasmodium falciparum* var genes. *Mol. Biochem. Parasitol*. 122:111–115.
23. Rowe, J.A., S. Kyes, S. Rogersen, H. Babiker, and A. Raza. Identification of a conserved *Plasmodium falciparum* var gene implicated in malaria in pregnancy. *J. Infect. Dis*. 185:1207–1211.
24. Fried, M., and P.E. Duffy. 2002. Two DBLgamma subtypes are commonly expressed by placental isolates of *Plasmodium falciparum*. *Mol. Biochem. Parasitol*. 122:201–210.
25. Winter, G., Q. Chen, K. Flick, P. Kremsner, V. Fernandez, and M. Wahlgren. 2003. The 3D7var5.2 (var COMMON) type var gene family is commonly expressed in non-placental *Plasmodium falciparum* malaria. *Mol. Biochem. Parasitol*. 127: 179–191.
26. Lekana Douki, J.B., B. Traore, F.T. Costa, T. Fusai, B. Pouvelle, Y. Sterkers, A. Scherf, and J. Gysin. 2002. Sequestration of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A, a receptor for maternal malaria: monoclonal antibodies against the native parasite ligand reveal pan-reactive epitopes in placental isolates. *Blood*. 100:1478–1483.
27. Costa, F.T., T. Fusai, D. Parzy, Y. Sterkers, M. Torrentino, J.B. Douki, B. Traore, S. Petres, A. Scherf, and J. Gysin. 2003. Immunization with recombinant duffy binding-like-gamma3 induces pan-reactive and adhesion-blocking antibodies against placental chondroitin sulfate A-binding *Plasmodium falciparum* parasites. *J. Infect. Dis*. 188:153–164.
28. Pouvelle, B., P. Meyer, C. Robert, L. Bardel, and J. Gysin. 1997. Chondroitin-4-sulphate impairs in vitro and in vivo cytoadherence of *Plasmodium falciparum* infected erythrocytes. *Mol. Med*. 3:508–518.
29. Gamain, B., J.D. Smith, M. Avril, D.I. Baruch, A. Scherf, J. Gysin, and L.H. Miller. 2004. Identification of a 67-amino-acid region of the *Plasmodium falciparum* variant surface antigen that binds chondroitin sulphate A and elicits antibodies reactive with the surface of placental isolates. *Mol. Microbiol*. 53:445–455.
30. Jensen, A.T., H.D. Zornig, C. Buhmann, A. Salanti, K.A. Koram, E.M. Riley, T.G. Theander, L. Hviid, and T. Staalsoe. 2003. Lack of gender-specific antibody recognition of products from domains of a var gene implicated in pregnancy-associated *Plasmodium falciparum* malaria. *Infect. Immun*. 71:4193–4196.