Antibodies from malaria-exposed pregnant women recognize trypsin resistant epitopes on the surface of Plasmodium falciparum-infected erythrocytes selected for adhesion to chondroitin sulphate A

Citation for published version:
Sharling, L, Sowa, KMP, Arnot, DE, Enevold, A & Staalsoe, T 2004, 'Antibodies from malaria-exposed pregnant women recognize trypsin resistant epitopes on the surface of Plasmodium falciparum-infected erythrocytes selected for adhesion to chondroitin sulphate A', Malaria Journal, vol. 3, no. 31. https://doi.org/10.1186/1475-2875-3-31

Digital Object Identifier (DOI):
10.1186/1475-2875-3-31

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Malaria Journal

Publisher Rights Statement:
This is an open-access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Antibodies from malaria-exposed pregnant women recognize trypsin resistant epitopes on the surface of *Plasmodium falciparum*-infected erythrocytes selected for adhesion to chondroitin sulphate A

Lisa Sharling*1, Anders Enevold2, Kordai MP Sowa1, Trine Staalsoe2 and David E Arnot1

Address: 1Institute of Cell, Animal and Population Biology, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh, EH9 3JT, Scotland, UK and 2Centre for Medical Parasitology, Department of Infectious Diseases M7641. Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark

Email: Lisa Sharling* - L.sharling@sms.ed.ac.uk; Anders Enevold - aecmp@rh.dk; Kordai MP Sowa - M.Sowa@ed.ac.uk; Trine Staalsoe - tscmp@rh.dk; David E Arnot - d.e.arnot@ed.ac.uk

* Corresponding author

Abstract

**Background:** The ability of *Plasmodium falciparum*-infected erythrocytes to adhere to the microvasculature endothelium is thought to play a causal role in malaria pathogenesis. Cytoadhesion to endothelial receptors is generally found to be highly sensitive to trypsinization of the infected erythrocyte surface. However, several studies have found that parasite adhesion to placental receptors can be markedly less sensitive to trypsin. This study investigates whether chondroitin sulphate A (CSA) binding parasites express trypsin-resistant variant surface antigens (VSA) that bind female-specific antibodies induced as a result of pregnancy associated malaria (PAM).

**Methods:** Fluorescence activated cell sorting (FACS) was used to measure the levels of adult Scottish and Ghanaian male, and Ghanaian pregnant female plasma immunoglobulin G (IgG) that bind to the surface of infected erythrocytes. *P. falciparum* clone FCR3 cultures were used to assay surface IgG binding before and after selection of the parasite for adhesion to CSA. The effect of proteolytic digestion of parasite erythrocyte surface antigens on surface IgG binding and adhesion to CSA and hyaluronic acid (HA) was also studied.

**Results:** *P. falciparum* infected erythrocytes selected for adhesion to CSA were found to express trypsin-resistant VSA that are the target of naturally acquired antibodies from pregnant women living in a malaria endemic region of Ghana. However in vitro adhesion to CSA and HA was relatively trypsin sensitive. An improved labelling technique for the detection of VSA expressed by CSA binding isolates has also been described.

**Conclusion:** The VSA expressed by CSA binding *P. falciparum* isolates are currently considered potential targets for a vaccine against PAM. This study identifies discordance between the trypsin sensitivity of CSA binding and surface recognition of CSA selected parasites by serum IgG from malaria exposed pregnant women. Thus, the complete molecular definition of an antigenic *P. falciparum* erythrocyte surface protein that can be used as a malaria in pregnancy vaccine has not yet been achieved.
Background

Rapid clearance of parasitaemia following transfusion of IgG from malaria immune adults to clinically ill recipients illustrates that naturally acquired antibodies have a parasite clearing role in human malaria infection [1-3]. Neither the nature of the protective immune response nor the target antigens and epitopes recognized by infection clearing antibodies are fully understood. Evidence is accumulating to suggest that the acquisition of antibodies binding the VSA on infected erythrocytes plays a major role in the development of age and exposure dependent immunity [4-8]. The evidence for protective anti-VSA responses is particularly strong for the PAM syndrome [9,10].

PAM is characterized by the sequestration of *Plasmodium falciparum* infected erythrocytes in the intervillous spaces of the placenta. Infected erythrocytes adhere to low-sulphated forms of CSA present on the extracellular proteoglycan matrix of syncytiotrophoblasts [11]. *In vitro* selection of infected erythrocytes for adhesion to CSA concomitantly selects for expression of VSA that share characteristics with postnatal placental isolates. Thus plasma antibodies from malaria exposed pregnant, or multigravid women, recognize the VSA of CSA binding parasites (here referred to as VSA\textsubscript{PAM}). These sera can also block adhesion of CSA-selected infected erythrocytes to CSA *in vitro* [12]. Interestingly, antibodies that bind CSA-selected parasites and block adhesion are not acquired by malaria-exposed males. There is a striking female-specific antibody response recognizing both *in vitro* CSA-selected parasites [12,13] and *P. falciparum* isolates taken from infected placentae at delivery [14-16]. Furthermore, the levels of CSA-adhesion blocking plasma IgG have been shown to increase with adult female parity. Recent immuno-epidemiological studies also show a strong positive correlation between the levels of antibodies that recognize the infected erythrocyte surface[15], the level of CSA-adhesion blocking antibody [17] and positive birth outcomes as measured by birth weight.

To date, the best characterized VSA is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a polymorphic, high molecular weight membrane protein (200–450 kDa) encoded by the var multi-gene family [18-20]. Members of the PfEMP-1 family function as adhesion molecules binding to various host endothelial receptors. They are situated in the knob-like protrusions associated with the parasitized erythrocyte surface.

Since var genes encode large extracellular domains rich in lysine and arginine residues, it is not surprising that PfEMP-1 molecules and adhesion to endothelial receptors have been reported to be highly sensitive to trypsin treatment [18,21-24]. Less expected was the finding that parasite adhesion to the placental receptor CSA, when immobilized [25-27] or when cell surface associated [28,29], can be relatively trypsin resistant. This study investigates the protease-sensitivity profile of the VSA\textsubscript{PAM} expressed by CSA-selected parasite clone FCR3 with regard to recognition by antibodies acquired during PAM and adhesion to placental receptors.

Methods

Parasite isolates

Parasites were maintained in group O erythrocytes under standard conditions [30], using RPMI 1640 medium containing 25 mM HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 25 µg/ml gentamycin and 10% pooled normal human serum. The pH was adjusted to between 7.2 and 7.4 with 1 M NaOH. Culture flasks at 5% haematocrit were gassed with 96% nitrogen, 3% carbon dioxide and 1% oxygen. The laboratory clone FCR3 originates from peripheral blood collected in the Gambia. FCR3CSA was obtained from the Malaria Research and Reference Reagent Resource Centre (ATCC) [31], and was confirmed, using genetic markers to be identical to the laboratory clone FCR3 kept in the original W.H.O. strain registry collection in Edinburgh (D. Walliker, pers. comm.). CSA binding was maintained by panning late stage infected erythrocytes fortnightly on bovine tracheal CSA (10 µg/ml) (Sigma) immobilized on polystyrene Petri dishes (Falcon), as previously described [26]. Prior to protease treatment and analysis by flow cytometry, cultures were synchronized by sorbitol treatment to obtain cultures enriched for late stage parasites.

Plasma donors

Serum samples from 20 men living in a malaria endemic region of Ghana were pooled to produce the male serum pool. Serum samples collected at the time of birth from the placentas of 15 women living in a malaria endemic region of Ghana were pooled to produce the pregnant female serum pool. This pool included five primigravidae, nine secundigravidae and one multigravid woman. Serum samples from six Scottish malaria naïve individuals were pooled and used as a control.

Protease treatment

Protease treatment of infected erythrocytes was carried out as previously described [26]. Briefly, samples containing 3 × 10^6 cells from sorbitol treated late stage cultures of 8–10
% parasitaemia were washed twice with phosphate-buffered saline (PBS) and then incubated with the appropriate concentration of trypsin-TPCK (Worthington Biochemicals) or pronase (Boehringer-Mannheim) in a final volume of 1.0 ml in PBS, for 10 minutes at 37°C. The reaction was terminated either by adding soybean trypsin inhibitor (Worthington Biochemicals) to a final concentration of 1 mg/ml or by adding 10% human serum. Cells were washed twice with PBS before further use.

**Analysis of VSA specific antibodies by flow cytometry**

Flow cytometry was used to measure the levels of plasma IgG binding to the VSA of late stage parasites essentially following the method previously described by Staalsoe et al [13,32]. 3 x 10^6 cells form late stage *P. falciparum* cultures of 8–10% parasitaemia were washed twice with PBS. Cells were incubated sequentially with plasma antibodies diluted 1:20 in PBS, goat anti-human IgG diluted 1:200 in PBS (Dako) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat (Dako) diluted 1:25 in PBS. All incubations were in a total volume of 100 µl for 30 minutes at room temperature and were followed by two washes with 1 ml of PBS. Samples were analysed immediately on a FACSCAN apparatus (Becton-Dickinson). FITC fluorescence due to cell surface antibody recognition was determined for 5000–10000 ethidium bromide gated infected erythrocytes.

**Modified labeling procedure for FACS analysis**

In order to circumvent the non-specific labeling of the VSA by the tertiary antibody, new reagents have been introduced. The procedure follows the method detailed above with the following modifications. A biotinylated rabbit anti-human IgG antibody (DAKO) was used diluted 1:25 to replace the secondary antibody. In the place of a tertiary antibody, FITC-conjugated streptavidin (DAKO) was used at a 1:2000 dilution. In these experiments the control sera was a pool of malaria naive Danish volunteer serum.

**Binding assays**

Human umbilical cord hyaluronic acid (Sigma) and bovine trachea CSA (Sigma) were used at a concentration of 10 µg/ml in PBS (pH 7.2). 20 µl of each receptor was spotted in triplicate onto 5 cm diameter petri dishes (Falcon). Receptors were adsorbed onto the plastic petri dishes overnight at 4°C. 10 µg/ml BSA in PBS was similarly adsorbed as a negative control. Plates were then blocked by removing the receptor solution and adding 20 µl of 2% BSA in PBS. Following the removal of this blocking solution late stage parasites, suspended in 2 ml of complete RPMI-HEPES medium (8–10% parasitaemia, 5% haematocrit), were added to the petri dish. Parasites were incubated with the immobilized receptor for 60 minutes at 37°C with occasional agitation. Unbound cells were removed by four gentle washes with incomplete RPMI-HEPES medium; bound cells were fixed with 0.5% (v/v) glutaraldehyde in PBS for 10 minutes and Giesma Stained. Bound cells were counted by light microscopy. Protease treatment of intact cells was carried out as described above.

**Statistical analysis**

Statistical analyses were performed using Analyses of Variance in Minitab 13.30 (Minitab Inc.), using protease, protease concentration and serum pool as explanatory variables. Statistical models were tested for homogeneity of variance and normality of error distributions. Where possible, maximal models with interactions between these variables were fitted first, after which models were minimized by removing nonsignificant (p > 0.05) terms.

**Results**

Concomitant selection of a trypsin-resistant VSA following parasite selection for CSA adhesion

It was first established that selection of clone FCR3 for adhesion to CSA resulted in the concomitant selection for VSA specifically recognized by plasma IgG from malaria exposed Ghanaian pregnant women (IgGpreg) (figure 1). However there was no increase in the binding of IgG from a pool of plasma from malaria exposed Ghanaian men (IgGmale). The unselected FCR3 clone expressed VSA that were equally well recognized by antibodies in the IgGmale and IgGpreg serum pools (figure 1). These interactions between serum antibody binding and selection for CSA adhesion were highly significant (F2,29 9.5, P = 0.001).

The trypsin sensitivity of this VSA/IgG binding interaction and of parasite adhesion to CSA was then measured. Parasitized erythrocyte surface trypsinization at a concentration of 0.1 mg/ml showed that the IgGpreg binding of FCR3CSA was significantly more trypsin-resistant than was binding of the same serum to the unselected clone (figure 2A &2B; F1,14 16.4, P = 0.015). Although the mean surface fluorescence due to the IgGpreg binding of FCR3CSA was slightly reduced by 0.1 mg/ml trypsin this reduction was not significant (figure 2A; F1,2 11.3, P = 0.078). The effect of 0.1 mg/ml trypsin on VSA recognition by IgGmale and IgGcontrol was comparable before and after CSA selection of the parasite (figure 2).

The effect of a 10-fold higher trypsin concentration and the effect of the non-specific protease, pronase, on IgG recognition of FCR3CSA was also determined. Trypsinization with 0.1 mg/ml did not significantly reduce the mean surface fluorescence due to IgGpreg binding to FCR3CSA (figure 3; F1,4 0.35, p = 0.587). However, treatment of the intact infected erythrocyte with 0.1 mg/ml pronase did significantly reduce IgGpreg recognition of FCR3CSA (figure 3). Pronase treatment also significantly reduced...
Surprisingly, IgGcontrol binding to the infected erythrocyte surface increased following CSA selection of the parasite (figure 1); however, this non-immune recognition was found to be significantly more trypsin sensitive than IgGpreg recognition (figure 3; $F_{4,183.11} = 0.041$). This indicates that the epitopes recognized by the IgGcontrol serum pool and the epitopes recognized by the IgGpreg serum pool are distinct entities. An increase in apparent non-immune immunoglobulin binding to the infected erythrocyte surface has been observed for a number of parasite clones after selection for adhesion to CSA (data not shown). The source of this background labelling of FCR3CSA by naïve sera was found to be due to non-specific binding by the FITC-labelled tertiary rabbit anti-goat antibody. By using the modified antibody labelling procedure, which employs a biotin-labelled secondary antibody and FITC-labelled streptavidin, binding of malaria
naive IgG to FCR3CSA (mean fluorescence index = 16) was comparable to the unselected parasite (mean fluorescence index = 17). Thus the recognition of VSA PAM by malaria naive IgG was abolished (figure 4).

**Discordance between the protease sensitivity of the CSA adhesion interaction and IgG binding**

Following the identification of trypsin-resistant epitopes that appear to be concomitantly selected with CSA adhesion, the trypsin sensitivity of CSA adhesion itself was determined. FCR3CSA binding to immobilised CSA was markedly more sensitive to trypsin than IgGpre recognition of the infected erythrocyte surface (figure 5). Parasite adhesion was reduced by 81% and 91% following treatment with 0.1 mg/ml trypsin or 0.1 mg/ml pronase respectively (figure 5). A trypsin concentration of 1 mg/ml reduced binding as efficiently as 0.1 mg/ml pronase, and although 0.1 mg/ml pronase significantly reduced cell surface fluorescence due to IgGpre antibody binding, 1 mg/ml trypsin had no significant effect on IgGpre antibody binding. There is, thus, significant discordance between the high trypsin sensitivity of CSA adhesion and the relatively trypsin-insensitive binding of IgGpre serum antibodies to the infected erythrocyte surface ($F_{1,8} 14.4$, $p = 0.005$).

**Figure 3**

**FCR3CSA expresses surface antigens exhibiting differential protease sensitivity.** Intact infected erythrocytes were treated with 1.0 mg/ml trypsin or 0.1 mg/ml pronase prior to FACS analysis. Serum pools are the same as those described in Figure 1. The bar chart shows mean and standard error of the means for three independent experiments.

**Figure 4**

**A modified antibody labelling procedure for FACS analysis of CSA selected parasites.** In order to circumvent the non-specific labelling of FCR3CSA VSA seen when using the FITC rabbit anti-goat tertiary antibody, a biotinylated rabbit anti-human antibody in combination with FITC-conjugated streptavidin was used. Panels A and B show FCR3CSA and FCR3 infected erythrocytes respectively. In these experiments the control serum was a pool of sera from malaria naive Danish volunteers, here shown as a solid grey histogram. The IgGmale serum pool is shown as a lightweight line and the IgGpreg serum pool as a heavyweight line.

**Figure 5**

**The effect of increasing concentrations of trypsin on parasite adhesion to immobilised CSA and HA.** Parasite adhesion to 10 µg/ml human umbilical cord HA and bovine trachea CSA, adsorbed onto the plastic petri dishes, was determined following protease treatment of the intact infected erythrocyte. Bound cells were Giemsa stained and counted by light microscopy. Panels A and B show receptor binding for FCR3 and FCR3CSA infected erythrocytes respectively. The bar chart shows mean and standard error of the means for three independent experiments.
Human umbilical cord hyaluronic acid (HA) was also included in these assays to investigate the binding capacity of the CSA selected clone with respect to this receptor. FCR3CSA was found to bind both HA and CSA, although binding to HA was significantly lower (figure 5B; $F_{3,19}$ 20.44, $p < 0.001$), at 71% that observed for CSA. Interestingly, as has previously been shown for other P. falciparum isolates [27], the trypsin-sensitivity of parasite adhesion to HA and CSA differed at low trypsin concentrations (0.01 mg/ml) (figure 5B; $F_{1,8}$ 7.7, $p = 0.024$). Parasite adhesion to hyaluronic acid was found to be more sensitive to trypsinization than adhesion to CSA.

**Discussion**

The acquisition of antibodies to the surface of placental isolates correlates with protection from malaria in pregnancy and the targets of these antibodies are potential vaccine candidates [13,15]. Two variants of the well characterized VSA, PfEMP1, have been shown to have distinct CSA-binding domains [29,33] and antibodies raised against these domains have been reported to recognize the infected erythrocyte surface [34] and in some cases block parasite adhesion [35,36]. However, in a recent study of var gene transcription in CSA-selected clones, a third potential CSA-binding PfEMP1 (var2csa) was identified. Var2csa is predicted to possess distinctly different DBL domains and appears to be the major var expressed by CSA-selected parasites that are recognized by parity-dependent antibodies [14]. Proteomic analysis of CSA-selected parasites has also identified four additional potential CSA binding PfEMP1 molecules [37]. The molecular identity of the surface antigens expressed at the infected erythrocyte surface remains unclear [38]. However, the differential protease sensitivity of the epitopes described here would allow treatment of the infected erythrocyte surface with trypsin thereby simplifying the surface complexity, thus, potentially making proteomic approaches more straightforward.

Although PfEMP1-mediated CSA adhesion appears to play a role in placental malaria the molecular interactions triggering this syndrome are more complex than initially thought. Several studies implicate additional receptors and binding phenotypes of placental parasites, such as non-immune IgM [39], hyaluronic acid [25,27,40] and non-immune IgG [41]. CSA-binding laboratory clones and placental CSA binding isolates also appear to express some parasite encoded surface antigens other than PfEMP1, such as ring surface proteins 1 and 2 (RSP 1 and 2) [42]. Interestingly, a gene ‘knock-out’ of the CSA binding var (FCR3varCSA) in parasite clone FCR3 abolishes CSA binding, but the ‘knock-out’ parasites still bind the syncytiotrophoblast of ex vivo placental cryosections [43]. Monoclonal antibodies raised against the CSA binding DBLγ domain also show this domain to be sensitive to surface proteolysis using relatively low trypsin concentrations (100 µg/ml) [34]. It is certainly possible that the trypsin-resistant VSA described here are not of the PfEMP1/CSA binding type.

Surface epitopes of the FCR3CSA parasite are both highly resistant to trypsin and are recognized by antibodies from malaria-exposed pregnant women. This agrees with a number of studies that have found parasite adhesion to placental receptors to be resistant to surprisingly high trypsin concentrations. However, binding assays with the parasite clone used in this study showed CSA and HA adhesion to be relatively trypsin-sensitive. This is also compatible with the results of Beeson and his colleagues who demonstrated trypsin-resistant CSA adhesion to be a clone dependent phenomenon [27]. Another recent study by the same group showed sera that is strongly reactive to the surface of CSA selected parasites is not always capable of inhibiting CSA adhesion [44]. Thus this study supports the view that erythrocyte surface epitopes distinct from those involved in CSA adhesion may be targets of the antibodies acquired during PAM and suggests that these two epitopes could be on different molecules. One further implication for vaccine development is that a candidate vaccine raising only CSA adhesion blocking antibodies may not mimic protective surface reactive gender-specific immune responses.

**Conclusion**

This study supports the view that major differences exist between VSA-PAM and previously characterized VSA. Apart from being recognized only by female sera in a parity-dependent manner, VSA-PAM show other distinct characteristics such as: i) VSA-PAM rarely form infected erythrocyte rosettes when compared to CD36 binding VSA [27,45], ii) with the exception of rosetting isolates, non-immune IgM binding is a phenomenon only seen with CSA-binding clones [39], iii) VSA-PAM do not generally mediate adhesion to CD36 [27,46], and iv) VSA-PAM mediated adhesion to the placenta and CSA can be resistant to concentrations of trypsin known to remove most PfEMP1 molecules from the infected cell surface. In combination with the findings of this study, these distinct properties of VSA-PAM suggest the involvement of either an unusually protease-resistant PfEMP1 structure, such has been shown to exist in the A4tres PfEMP1 molecule [47] or an alternative class of VSA in placental adhesion. The differential protease sensitivity exhibited by VSA-PAM can be exploited in comparative proteomic analysis to aid in the identification of the molecules whose phenotype is described here.

**List of abbreviations**

TPCK – L-(tosylamido-2-phenyl) ethyl chloromethyl ketone, CSA – chondroitin sulphate A, PfEMP1 – P. falciparum erythrocyte protein 1, PAM – Pregnancy associated
malaria, VSA – variant surface antigens, VSA_PAM – variant surface antigens expressed by placental or CSA binding parasites, IgG – immunoglobulin G, DBL-γ-Duffy like binding domain-gamma, FITC – fluorescein isothiocyanate.

Authors' contributions
LS conceived of the study, maintained P. falciparum culture, performed FACS analysis and binding assays, AE performed the modified labelling FACS experiments and participated in manuscript preparation, MS participated in the design of the study, TS helped develop some methodologies used in this study. DA helped conceive and fund the study and write the manuscript. All authors read and approved the final manuscript.

Declaration
None declared.

Acknowledgements
LS is sponsored by a Wellcome Trust Studentship. This work was also supported by EU PAMVAC contract QLK2-CT-2001-01302. We thank Andrew Sanderson for his help with performing the FACS analysis, David Walliker for his help in strain genotyping, Mike Ofori, Maja Lundquist, Edmund Nii-Laryea Browne and Victoria Bam for serum collection. Jaap de Sanderson for his help with performing the FACS analysis, David Edmund Nii-Laryea Browne and Victoria Bam for serum collection. Jaap de

References
1. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, Foucault C, Chongsuphajaisiddhi T, Druilhe P: Parasitologic and clinical human response to immunoglobulin-binding domain-gamma in Plasmodium falciparum. Am J Trop Med Hyg 1991, 45:297-308.
2. Cohen S, McGregor IA, Carrington S: Gamma-globulin and acquired immunity to human malaria. Nature 1961, 192:733-737.
3. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P: Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytess. J Exp Med 1990, 172:1633-1641.
4. Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K: Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat Med 1998, 4:358-360.
5. Marsh K, Otoo L, Hayes RJ, Carson DC, Greenwood BM: Antibodies to blood stage antigens of Plasmodium falciparum in rural Gambians and their relation to protection against infection. Trans R Soc Trop Med Hyg 1989, 83:293-303.
6. Dodoo D, Staalsoe T, Ghia H, Kivrak JA, Akanmori BD, Koram K, Dunyo S, Nkumah FK, Hviid L, Theander TG: Antibodies to variant antigens on the surfaces of infected erythrocytes are associated with protection from malaria in Ghanaian children. Infect Immun 2001, 69:3713-3718.
7. Nielsen MA, Staalsoe T, Kurtzhals JA, Goka BQ, Dodoo D, Allifarangis M, Theander TG, Akanmori BD, Hviid L: Plasmodium falciparum variant surface antigen expression varies between isolates causing severe and nonsevere malaria and is modified by acquired immunity. J Immunol 2002, 168:3444-3450.
8. Ofori MF, Dodoo D, Staalsoe T, Kurtzhals JA, Koram K, Theander TG, Akanmori BD, Hviid L: Malaria-induced acquisition of antibodies to Plasmodium falciparum variant surface antigens. Infect Immun 2002, 70:2982-2988.
9. Gilles HM, Lawson JB, Sibelas M, Voller A, Allan N: Malaria, anemia and pregnancy. Ann Trop Med Parasitol 1969, 63:245-263.
10. McGregor IA, Wilson ME, Billewicz WZ: Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birthweight and placental weight. Trans R Soc Trop Med Hyg 1983, 77:232-244.
11. Achur RN, Valiavsetti M, Aikkilä A, Ockenhouse CF, Gowda DC: Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillosus spaces that mediate the adherence of Plasmodium falciparum-infected erythrocytes to the placenta. J Biol Chem 2000, 275:40334-40356.
12. Staalsoe T, Megnekou R, Fivet N, Ricke CH, Zorning HD, Leke R, Taylor DW, Deloron P, Hviid L: 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 2001, 184:618-626.
13. Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnott DE, Hviid L, Theander TG: Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Mol Microbiol 2003, 49:179-191.
14. Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Taylor DW, Deloron P, Hviid L, Klinkert MQ: Antibodies that protect against Plasmodium falciparum isolates. Malar J 2004, 3:21.
15. Duffy PE, Fried M: Antibodies that inhibit Plasmodium falciparum adhesion to chondroitin sulphate A are associated with increased birth weight and the gestational age of newborns. Infect Immun 2003, 71:6620-6623.
16. Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnott DE, Hviid L, Theander TG: Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Mol Microbiol 2003, 49:179-191.
17. Staalsoe T, Shulman CE, Buhner JN, Kariuki S, Wilson ME, Billewicz WZ: Variable surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated Plasmodium falciparum malaria. Lancet 2004, 363:283-289.
18. Khattab A, Reinhardt C, Staalsoe T, Fievet N, Kremsner PG, Deloron P, Hviid L, Klinkert MQ: Antibodies that protect against Plasmodium falciparum isolated. Malar J 2004, 3:21.
19. Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Taylor DW, Deloron P, Hviid L, Klinkert MQ: Antibodies that protect against Plasmodium falciparum isolated. Malar J 2004, 3:21.
26. Chaiyaroj SC, Angkasukwina P, Buranakit A, Looareesuwan S, Roger- 
sion SJ, Brown GV: Cytoadherence characteristics of Plasmo-
dium falciparum isolates from Thailand: evidence for chondroitin sulfate A as a cytoadherence receptor. Am J Trop Med Hyg 1996, 55:76-80.

27. Beeson JG, Brown GV: Plasmodium falciparum-Infected Eryth-
rocytes Demonstrate Dual Specificity for Adhesion to 
Hyaluronic Acid and Chondroitin Sulfate A and Have Dis-
tinct Adhesive Properties. J Infect Dis 2004, 189:169-179.

28. Rogersen SJ, Chaiyaroj SC, Ng K, Reeder JC, Brown GV: Chondroit-
in sulfate A is a cell surface receptor for Plasmodi-
unum falciparum-infected erythrocytes. J Exp Med 1995, 182:15-20.

29. Buffet PA, Gama B, Scheidig C, Baruch D, Smith JD, Hernandez-
Rivas R, Pouvelle B, Oishi S, Fujiu N, Fusi T, Parzy D, Miller LH, Gysin J, Scherf A: Plasmodium falciparum domain mediating adhesion 
to chondroitin sulfate A: a receptor for human placental infection. Proc Natl Acad Sci U S A 1999, 96:12743-12748.

30. Trager W, Jensen JB: Human parasites in continuous culture. Science 1976, 193:673-675.

31. Scherf A, Hernandez-Rivas R, Buffet P, Bottius E, Benatar C, Pouvelle B, Gysin J, Lanzer M: Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmo-
dium falciparum. Embo J 1998, 17:5418-5426.

32. Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L: Detection of antibodies to variant antigens on Plasmodium falci-
parum-infected erythrocytes by flow cytometry. Cytometry 1999, 35:329-336.

33. Reeder JC, Cowman AF, Davern KM, Beeson JG, Thompson JK, Rog-
gerson SJ, Brown GV: The adhesion of Plasmodium falciparum-
infected erythrocytes to chondroitin sulfate A is mediated by 
P. falciparum erythrocyte membrane protein 1. Proc Natl Acad Sci U S A 1999, 96:5198-5202.

34. Lekana Douki JB, Troare B, Costa FT, Fusai T, Pouvelle B, Sterkers Y, 
Scherf A, Gysin J: Sequestration of Plasmodium falciparum-
infected erythrocytes to chondroitin sulfate A, a receptor for matern al malaria: monoclonal antibodies against the native parasite ligand reveal pan-reactive epitopes in placent al isolates. Blood 2002, 100:1478-1483.

35. Reeder JC, Hodder AN, Beeson JG, Brown GV: Identification of 
glycosaminoglycan binding domains in Plasmodium falci-
parum erythrocyte membrane protein 1 of a chondroitin sul-
 fate A-adherent parasite. Infect Immun 2000, 68:3923-3926.

36. Costa FT, Fusai T, Parzy D, Sterkers Y, Torrentino M, Douki JB, 
Troare B, Petres S, Scherf A, Gysin J: Immunization with recom-
binant duffy binding-like-gamma3 induces pan-reactive and adhesion-blocking antibodies against placental chondroitin sulfate A-binding Plasmodium falciparum parasites. J Infect Dis 2003, 188:153-164.

37. Fried M, Wendler JP, Mutabingwa TK, Duffy PE: Mass spectromet-
ric analysis of Plasmodium falciparum erythrocyte membrane protein-1 variants expressed by placental malaria parasites. Proteomics 2004, 4:1086-1093.

38. Gamain B, Smith JD, Avrill M, Baruch DI, Scherf A, Gysin J, Miller LH: Identification of a 67-amino-acid region of the Plasmodium falciparum variant surface antigen that binds chondroitin sulfate A and elicits antibodies reactive with the surface of placental isolates. Mol Microbiol 2004, 53:445-455.

39. Creasey AM, Staalsoe T, Raza A, Arnott DE, Rowe JA: Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of Plasmodium falciparum isolates implicated in malaria during pregnancy. Infect Immun 2003, 71:4767-4771.

40. Chai W, Beeson JG, Kogelberg H, Brown GV, Lawson AM: Inhibi-
tion of adhesion of Plasmodium falciparum-infected erythro-
cytes by structurally defined hyaluronic acid didecasaccharides. Infect Immun 2001, 69:420-425.

41. Flick K, Scholeran C, Chen Q, Fernandez V, Pouvelle B, Gysin J, 
Wahlgren M: Role of nonimmune IgG bound to PfEMP1 in pla-
cental malaria. Science 2001, 293:2098-2100.

42. Pouvelle B, Buffet PA, Lepolard C, Scherf A, Gysin J: Cytoadhesion of Plasmodium falciparum ring-stage-infected erythrocytes. Nat Med 2000, 6:1264-1268.

43. Andrews KT, Lanzer M: Maternal malaria: Plasmodium falci-
parum sequestration in the placenta. Parasitol Res 2002, 88:715-723.