A single member of the *Plasmodium falciparum* var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A

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In high-transmission regions, protective clinical immunity to *Plasmodium falciparum* develops during the early years of life, limiting serious complications of malaria in young children. Pregnant women are an exception and are especially susceptible to severe *P. falciparum* infections resulting from the massive adhesion of parasitized erythrocytes to chondroitin sulphate A (CSA) present on placental syncytiotrophoblasts. Epidemiological studies strongly support the feasibility of an intervention strategy to protect pregnant women from disease. However, different parasite molecules have been associated with adhesion to CSA. In this work, we show that disruption of the *var2csa* gene of *P. falciparum* results in the inability of parasites to recover the CSA-binding phenotype. This gene is a member of the var multigene family and was previously shown to be composed of domains that mediate binding to CSA. Our results show the central role of var2CSA in CSA adhesion and support var2CSA as a leading vaccine candidate aimed at protecting pregnant women and their fetuses.

Keywords: CSA; malaria; placenta; *Plasmodium*; var

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INTRODUCTION

Plasmodium falciparum causes the most severe form of human malaria, with more than two million deaths per year. Whereas

adults in endemic areas usually develop immunity to clinical malaria, women, during their first pregnancy (primigravidae), become particularly susceptible to infection (Brabin, 1983). The pathologies are associated with massive sequestration of *P. falciparum*-infected erythrocytes (IE) in the placenta. Pregnancy-associated malaria (PAM) is mainly linked to a subpopulation of IE that adhere to chondroitin sulphate A (CSA) expressed by syncytiotrophoblasts in the placenta (Fried & Duffy, 1996). Placental isolates are functionally distinct because they do not bind to CD36, but instead bind to CSA (Fried & Duffy, 1996) and, to some extent, to hyaluronic acid (Beeson *et al*, 2000). With successive pregnancies, women develop broadly strain-transcendent antibodies to the IE surface (Fried *et al*, 1998), suggesting that a vaccine against PAM is feasible. Although the search for the CSA ligands resulted in the identification of several different var gene products, conflicting data have emerged on their validity (reviewed by Rowe & Kyes, 2004).

The var multigene family consists of approximately 60 distinct members per haploid genome that encode *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1; Gardner *et al*, 2002). Expression of the var genes is mutually exclusive, allowing the expression of only one PfEMP-1 on the surface of each IE, mediating sequestration in different microvasculature sites (Chen *et al*, 1998; Scherf *et al*, 1998). Binding studies using recombinant PfEMP-1 domains have shown interactions with various host receptors, such as CD36, ICAM-1 and CSA (reviewed by Smith *et al*, 2001).

A large number of epidemiological studies on pregnant women in malaria endemic areas strongly support the concept that an anti-disease vaccine that blocks the adhesion to CSA and thereby protects pregnant women is possible (reviewed by Duffy & Fried, 2003). To this end, it is crucial to define with certainty which parasite CSA-binding ligands are expressed on the surface of IE during pregnancy. To assess the repertoire of CSA-binding ligands, we generated disruption mutants of the *var2csa* gene that was previously reported to possess several CSA-binding domains, and to be upregulated in placental parasites (Salanti *et al*, 2003;

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Gamain *et al*, 2005). The FCR3Δ*var2csa* mutant parasites did not recover the CSA-binding phenotype, suggesting that a single member of the *P. falciparum* *var* gene family determines cytoadhesion to CSA.

RESULTS

Targeted disruption of the *var2csa* gene in FCR3 parasites

It has been reported that *var2csa* is transcriptionally upregulated and expressed at the surface of CSA-binding parasites (Salanti *et al*, 2003, 2004; Gamain *et al*, 2005). To investigate the role of *var2csa* in *P. falciparum* IE adhesion to CSA, we established parasite lines with a disruption in the *var2csa* gene.

The pHTK-*var2csa* vector contains the *hDHFR* gene flanked by the *var2csa* DBL3-X and DBL5-ε sequences (Fig 1A). Insertional disruptant mutants were generated by double-crossover homologous recombination of the pHTK-*var2csa* transfection construct, resulting in the replacement of the *var2csa* DBL4-ε domain with the *hDHFR* expression cassette (Fig 1A). FCR3 parasites were transfected with pHTK-*var2csa* and selected on WR99210 and ganciclovir to obtain FCR3Δ*var2csa* mutants. After selection of the FCR3Δ*var2csa* population for knob-positive parasites using gelatin flotation, the mutants were cloned by limiting dilution and genetically characterized.

Clones were screened by PCR analysis for the disruption of the *var2csa* gene as well as for the absence of contaminating wild-type *var2csa* and the presence of the *HRP1* (*KAHRP*) gene (data not shown). The presence of the *HRP1* gene, taken together with the enrichment by gelatin flotation, argues for the presence of knobs on the surface of the FCR3Δ*var2csa* IE. To confirm that pHTK-*var2csa* had integrated into *var2csa*, Southern blots were performed using genomic DNA previously digested with *Bam*HI, *Eco*RV or *Pvu*II derived from parental FCR3 or recombinant parasites, and were hybridized with *var2csa* DBL3 or DBL5 radiolabelled probes (Fig 1B). These hybridizations showed bands of the expected size, indicating that the integration occurred at the predicted site within the *var2csa* gene (Fig 1A,B). Pulsed-field gel electrophoresis (PFGE) was performed to further support the integration of the selectable marker cassette within the *var2csa* locus on chromosome 12 (Fig 1B). A clathrin heavy chain probe was used as a chromosome-12-specific marker. After the complete characterization of several mutant clones by PCR, and Southern blotting of both restriction enzyme digests and size-fractionated chromosomal DNA (Fig 1B,C), two clones (1F1 and 2A5) were selected for further analysis.

FCR3Δ*var2csa* clones cytoadhere to CD36

To test the ability of the FCR3Δ*var2csa* mutants to cytoadhere, adhesion of the FCR3Δ*var2csa* mutants to CSA and CD36 was examined (Fig 2A). Equal numbers of erythrocytes infected with trophozoites of the FCR3Δ*var2csa* 1F1 and 2A5 mutant clones or control parasites were seeded on Petri dishes coated with different molecules. FCR3-CSA and FCR3-CD36 were used as controls. Whereas FCR3-CSA IE bound in high numbers to CSA but not to CD36, no adhesion to CSA was observed for 1F1, 2A5 and FCR3-CD36 IE. In contrast, 1F1, 2A5 and FCR3-CD36 IE adhered strongly to CD36. These results show that the FCR3Δ*var2csa* mutants are still able to mediate binding to another host receptor. No cytoadhesion to BSA and chondroitin sulphate C was observed (data not shown).

Total RNA was isolated from ring- and trophozoite-stage parasites to investigate *var* gene expression in the FCR3Δ*var2csa* and the parental FCR3 parasites selected for a CSA- or CD36-binding phenotype. Whereas a full-length *var2csa* transcript (~13 kb) was observed in the FCR3-CSA parasites, a non-functional truncated transcript (~7 kb) was detected in the mutant clones 1F1 and 2A5 (Fig 2B).

Using a semiconserved *varT11.1* exon II probe, larger transcripts of around 9 kb were identified in ring-stage RNA of FCR3-CD36 and in the two mutant clones, showing that full-length *var* genes are transcribed in the CD36-binding FCR3Δ*var2csa* parasites. This result, taken together with the presence of a nonfunctional *var2csa* truncated transcript, shows that mutually exclusive transcription of *var* genes can be overcome under certain conditions. As blots were washed using high-stringency conditions, and because of the divergence in the *var2csa* exon II sequence, the *var2csa* transcript was not detected with the exon II probe in FCR3-CSA parasites. Sterile exon II transcripts were detected at the trophozoite stages for all the parasite clones (Su *et al*, 1995). These results show that full-length *var* genes mediating CD36 binding are transcribed in the FCR3Δ*var2csa* clones 1F1 and 2A5 and that disruption of the *var2csa* locus does not interfere with IE cytoadhesion to receptors such as CD36.

No adhesion of FCR3Δ*var2csa* clones to CSA

To determine the ability of the FCR3Δ*var2csa* mutants to recover cytoadherence to CSA, the parasites were re-selected on CSA using different systems. Switching of *var* genes occurs in *in vitro*-cultured parasites, and variants that are able to adhere to a large number of different host receptors have been isolated using receptor-specific panning assays (Roberts *et al*, 1992; Scherf *et al*, 1998). FCR3Δ*var2csa* IE (clones 1F1 and 2A5) were first selected on recombinant human thrombomodulin-coated plastic dishes (Parzy *et al*, 2000). After four pannings, no specific enrichment was observed (Fig 3A). However, FCR3-CD36 wild-type parasites could be selected for binding to CSA.

In addition, FCR3Δ*var2csa* IE were selected on *Saimiri* brain microvasculature endothelial cell clone Sc1707, which was previously described to express exclusively the adhesion receptor CSA and to be a useful cell system for selecting CSA-binding parasites (Pouvelle *et al*, 1997). No adhesion of the FCR3Δ*var2csa* mutants to the Sc1707 cells was observed after five rounds of selection, whereas wild-type FCR3-CD36 population began to adhere to CSA after only one round of selection (Fig 3B). Parasites selected on Sc1707 were renamed FCR3-CSA¹⁷⁰⁷, FCR3-CD36¹⁷⁰⁷, 1F1¹⁷⁰⁷ and 2A5¹⁷⁰⁷. Similar results were obtained by selecting the four parasite lines on CHO-K1 cells (data not shown).

The adhesion phenotype of the Sc1707-selected parasites was examined on adhesion receptors coated to plastic Petri dishes. Whereas FCR3-CD36¹⁷⁰⁷ bound strongly to CSA, FCR3Δ*var2csa* clones 1F1¹⁷⁰⁷ and 2A5¹⁷⁰⁷ maintained the CD36-binding phenotype that had already been observed before the selection procedure (Fig 3C,D). No CSA-specific adhesion was detected after five pannings of the FCR3Δ*var2csa* clones 1F1 and 2A5 on Sc1707. Taken together, our experiments suggest that the *var2CSA* protein is essential for cytoadhesion of late-stage FCR3-IE to CSA, as no other protein emerged to compensate *var2CSA* loss.

Fig 1 | Targeted gene disruption of *Plasmodium falciparum* var2csa. (A) Schematic representation of the disruption of var2csa by double-crossover integration. The pHTK-var2csa plasmid contains the thymidine kinase gene, hDHFR, and the sequences corresponding to DBL3-X and DBL5-ε of var2csa. The DBL4-ε region has been deleted and replaced by the hDHFR gene. The different Duffy binding-like (DBL) domains and the transmembrane (TM) domain, and carboxy-terminal cytoplasmic domain (ATS) of var2csa are shown. Homologous target sequences are shown in dark grey. Sizes of DNA fragments are shown in kilobases (kb). Restriction enzyme sites and the expected restriction fragments are indicated. Hybridization probes are indicated as black bars. (B) Knockout of var2csa by a double-crossover event. Southern blot analysis of genomic DNA from representative mutant clones 1F1 and 2A5 and the parental FCR3 strain using BamHI, EcoRV and PvuII restriction enzymes. Hybridization was carried out with DBL3-X- and DBL5-ε-specific probes. The positions of the probes are shown in (A). (C) Insertion of pHTK-var2csa in chromosome 12. Southern blot analysis of chromosomal DNA derived from FCR3 wt (wild type) and the representative disrupted mutant clones 1F1 and 2A5. Chromosomes were separated by pulsed-field gel electrophoresis, then transferred onto a nylon membrane and hybridized with probes specific for DBL5-ε, hDHFR and clathrin heavy chain. The position of chromosome 12 is indicated.

Fig 2 | FCR3Δvar2csa clones cytoadhere to CD36 and express a *var* gene that is different from *var2csa*. (A) Cytoadhesion of the *var2csa* disruption mutants to receptors coated to plastic Petri dishes. Erythrocytes infected with the *Plasmodium falciparum* FCR3-CSA, FCR3-CD36 and the FCR3Δvar2csa clones 1F1 and 2A5 were analysed for cytoadhesion to CSA and CD36. Data are the mean ± s.e.m. of IE per square millimetre (IE/mm²) adhering to CSA-coated (left panel) and CD36-coated (right panel) plastic Petri dishes, as determined in three independent assays. (B) Northern analyses of total RNA isolated from ring-stage (R) and trophozoite-stage parasites (T) FCR3-CSA, FCR3-CD36 and the representative FCR3Δvar2csa clones 1F1 and 2A5. The membrane was hybridized with a probe specific for *var2csa* DBL3-X and semiconserved *varT11.1* exon II.

DISCUSSION

In FCR3Δvar2csa mutant parasites, transcription of the full-length *var2csa* and the expression of the protein on the surface of the IE are prevented. Disruption of *var2csa* did not, however, affect the ability of the mutants to switch expression to another *var* gene, and show adhesive phenotypes. This is illustrated by the CD36-binding phenotype and the transcription of *var* transcripts in both FCR3Δvar2csa mutant clones. Similar conclusions were reached by previous studies, showing that targeted disruption of *var* genes only affected expression of this particular gene, but not that of other variants (Horrocks *et al*, 2002; Andrews *et al*, 2003).

The finding that the *var2csa* promoter remains active in both mutant clones, leading to the truncated *var2csa* transcripts, might be explained by the presence of the active hDHFR expression cassette that is introduced in the *var2csa* coding region. As each *var* gene represents a transcriptional unit, the

active promoter of the selectable marker could disturb this silencing mechanism, with the silencer being either located in the *var* intron (Deutsch *et al*, 2001) or, alternatively, in a specific chromatin environment that leads to *var* gene repression (Freitas-Junior *et al*, 2005). Importantly, it shows that the mutually exclusive transcription of *var* genes can be overcome under certain conditions, which are potentially related to the specific transcription mechanism of telomere-associated genes (Duraisingh *et al*, 2005; Ralph *et al*, 2005).

Our FCR3Δvar2csa mutant clones 1F1 and 2A5 did not recover the CSA-binding phenotype, after selection on different CSA-expressing cells or recombinant human thrombomodulin. In previous reports, several *var* gene products other than var2CSA have been linked to cytoadhesion of IE to CSA, var-CS2 and var1CSA (Scherf *et al*, 1998; Buffet *et al*, 1999; Reeder *et al*, 1999; Degen *et al*, 2000; Andrews *et al*, 2003). The presence

Fig 3 | FCR3Δvar2csa mutants show no chondroitin sulphate A (CSA)-specific cytoadhesion after selection on CSA-expressing cell lines and recombinant human thrombomodulin. Mean ± s.d. of IE adhering per square millimetre (IE/mm²) for four different fields is shown (A,B). (A) Selection of FCR3Δvar2csa mutants and parental FCR3 parasites on recombinant human thrombomodulin. Erythrocytes infected with FCR3-CSA, FCR3-CD36, 1F1 and 2A5 were selected four times on recombinant human thrombomodulin-CSA coated to Petri dishes. (B) Selection of FCR3Δvar2csa mutants and parental FCR3 parasites on Sc1707. Trophozoite-stage *Plasmodium falciparum* clones FCR3-CSA, FCR3-CD36, 1F1 and 2A5 were subjected to repeated rounds of selection over Sc1707 cells, followed by evaluation of adhesion to Sc1707. (C,D) Adhesion profiles of *P. falciparum* IE after selection on Sc1707 cells. Trophozoite-stage *P. falciparum* parasites FCR3-CSA, FCR3-CD36, 1F1 and 2A5 were subjected to repeated rounds of selection over Sc1707 cells, followed by evaluation of adhesion to CSA-coated (C) and CD36-coated (D) plastic Petri dishes. Adhesion after selection is shown for FCR3-CSA¹⁷⁰⁷, FCR3-CD36¹⁷⁰⁷, 1F1¹⁷⁰⁷ and 2A5¹⁷⁰⁷. Data are the mean ± s.e.m. of IE per square millimetre adhering to CSA- and CD36-coated plastic Petri dishes, as determined in three independent assays.

and integrity of the *var1csa* and *var-CS2* genes in the genome of the FCR3Δvar2csa mutants was shown by PCR analysis (supplementary Fig S1 online). As *var1csa* is also transcribed in the mutant parasites, our results indicate that *var1csa* and *var-CS2* are not involved in CSA-specific adhesion of late-stage IE. Doubts on a functional role of *var1CSA* in cytoadhesion to CSA were raised earlier, because of the unusual transcription pattern of *var1csa* in CSA- and CD36-binding parasites (Kyes *et al*, 2003). Also, in contrast to our FCR3Δvar2csa mutants, *var1csa* disruption mutants recovered the CSA-binding phenotype after selection on CSA (Andrews *et al*, 2003), leading to the transcription of *var2csa* (Gamain *et al*, 2005). However, experimental evidence strongly supported a role for *var1CSA* in cytoadhesion to CSA (reviewed by Rowe & Kyes, 2004). First, *var1CSA* possesses a combination of a CSA-binding DBLγ domain and a non-CD36-binding CIDR1

(Gamain *et al*, 2002). Second, antibodies raised against the *var1CSA* DBL3γ domain label placental parasite isolates and inhibit IE cytoadhesion to CSA (Costa *et al*, 2003). These antibodies also recognize the *var2CSA* protein on the surface of the IE (Andrews *et al*, 2003). At this stage of our knowledge, we assume that crossreactivity of the antibodies between *var1CSA* and *var2CSA* may be explained by similar three-dimensional structures in the CSA-binding domains of both genes. This hypothesis is supported further by the presence of significant homologies between the *var1CSA* DBLγ minimal binding domain and the *var2CSA* DBL3-X binding domain (Gamain *et al*, 2004). Even if the *var1csa* gene product possesses all the characteristics that would make it a CSA-binding ligand, its biological role remains uncertain. It is possible that the protein is not exported to the surface of the mature asexual-stage IE or is

expressed at another developmental stage of the parasite, such as the sporozoites.

To test the ability of the FCR3Δvar2csa parasites to be recognized by plasma antibodies from malaria-exposed pregnant women, flow cytometry using intact IE was performed (supplementary Fig S2 online). Whereas FCR3-CSA IE are recognized by sera of malaria-exposed multigravidae, FCR3-CD36 and FCR3Δvar2csa IE are not (see the supplementary information online). In contrast, sera of malaria-exposed men do not recognize any of these IE. These results confirm the previously published data that only var2csa-expressing parasites are recognized in a parity-dependent manner (Salanti et al, 2004).

In conclusion, we show that a single member of the var repertoire is required for binding to CSA in FCR3 parasites. Given that FCR3Δvar2csa disruptant mutants do not recover this binding phenotype, even after several rounds of panning selection, we conclude that no other parasite gene can compensate for the loss of function under the experimental CSA selection conditions of our work. Thus, our demonstration of the central role of var2CSA in CSA adhesion is important for the future design of a vaccine against the complications of malaria during pregnancy.

METHODS

Parasites and cells. *P. falciparum* FCR3 clones were cultivated according to standard conditions (Trager & Jensen, 1976). Knob-positive parasites were routinely selected by gelatin flotation using Plasmion (Fresenius Kabi, France). *Saimiri* brain microvasculature endothelial cell clone Sc1707 was cultured, as described earlier (Pouvelle et al, 1997).

Plasmids and transfection. Fragments corresponding to the DBL3-X and DBL5-ε domains of var2csa were PCR amplified from FCR3 genomic DNA using the primer combinations DBL3-F/DBL3-R and DBL5-F/DBL5-R (primer sequences are shown in supplementary Table S1 online). These PCR fragments were sequentially cloned into pHTK (Duraisingh et al, 2002) using the *SacII/Spel* sites, as well as the *EcoRI/AvrII* sites, to derive pHTK-var2csa.

Ring-stage FCR3 parasites were transfected with 100 μg plasmid DNA and selected with 2.5 nM WR99210 (Jacobus Pharmaceutical Co. Inc., Princeton, NJ, USA) and 4 μM ganciclovir (Sigma, Saint Quentin, Fallavier, France), as described previously (Duraisingh et al, 2002).

Pulsed-field gel electrophoresis and Southern blot. Genomic DNA was digested and size fractionated on 0.8% agarose gels. PFGE and Southern blot were performed, as described previously (Hernandez-Rivas & Scherf, 1997). The chromosome-12-specific probe for clathrin heavy chain was obtained by PCR amplification using the primers CHC-F and CHC-R. The *hDHFR* probe was obtained by restriction of pHTK with *Bam*HI and *Hind*III. Var2csa probes were used, as described in the Plasmids and transfection. Membranes were hybridized at high-stringency conditions at 60 °C overnight and washed twice with 0.2 × SSC and 0.1% SDS at 60 °C for 30 min.

Northern blot analysis. Total RNA was prepared from synchronized parasite cultures approximately 10 and 30 h after invasion. RNA preparation, electrophoresis, membrane transfer and hybridization were carried out, as described previously (Kyes et al, 2000). Membranes were hybridized at high-stringency conditions at 60 °C overnight and washed twice with 0.5 × SSC and 0.1% SDS at 60 °C for 30 min. Radiolabelled probes for FCR3 var2csa

DBL3-X or FCR3 var1csa DBL3-γ, or a probe based on the var semiconserved exon II (varT11.1 gene, 7,930–9,147 base pairs; GenBank accession number U67959) were generated, as described previously (Gamain et al, 2005).

***P. falciparum* adhesion assays and pannings.** Trophozoite-stage IE were purified using gelatin flotation and selected for CSA binding on Sc1707 cells and on recombinant human thrombomodulin carrying CSA chains, as described previously (Pouvelle et al, 1997). For Sc1707, the number of IE bound per square millimetre was determined for four different fields and the mean ± s.d. was calculated. Cytoadhesion assays on receptors immobilized on plastic Petri dishes were carried out, as described previously (Baruch et al, 1999; Buffet et al, 1999). The average number of adherent IE (± standard error of the mean (s.e.m.)) for four different fields was determined in three independent experiments.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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REFERENCES

- Andrews KT et al (2003) Recovery of adhesion to chondroitin-4-sulphate in *Plasmodium falciparum* varCSA disruption mutants by antigenically similar PfEMP1 variants. *Mol Microbiol* **49**: 655–669
- Baruch DI, Ma XC, Pasloske B, Howard RJ, Miller LH (1999) CD36 peptides that block cytoadherence define the CD36 binding region for *Plasmodium falciparum*-infected erythrocytes. *Blood* **94**: 2121–2127
- Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV (2000) Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* **6**: 86–90
- Brabin BJ (1983) An analysis of malaria in pregnancy in Africa. *Bull World Health Organ* **61**: 1005–1016
- Buffet PA 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
- Chen Q, Fernandez V, Sundstrom A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M (1998) Developmental selection of var gene expression in *Plasmodium falciparum*. *Nature* **394**: 392–395
- Costa FT, Fusai T, Parzy D, Sterkers Y, Torrentino M, Douki JB, Traore B, Petres S, Scherf A, Gysin J (2003) Immunization with recombinant duffy binding-like-γ3 induces pan-reactive and adhesion-blocking antibodies against placental chondroitin sulfate A-binding *Plasmodium falciparum* parasites. *J Infect Dis* **188**: 153–164
- Degen R, Weiss N, Beck HP (2000) *Plasmodium falciparum*: cloned and expressed CIDR domains of PfEMP1 bind to chondroitin sulfate A. *Exp Parasitol* **95**: 113–121
- Deitsch KW, Calderwood MS, Wellem TE (2001) Malaria. Cooperative silencing elements in var genes. *Nature* **412**: 875–876
- Duffy PE, Fried M (2003) *Plasmodium falciparum* adhesion in the placenta. *Curr Opin Microbiol* **6**: 371–376
- Duraisingh MT, Triglia T, Cowman AF (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *Int J Parasitol* **32**: 81–89
- Duraisingh MT, Voss TS, Marty AJ, Duffy MF, Good RT, Thompson JK, Freitas-Junior LH, Scherf A, Crabb BS, Cowman AF (2005) Perinuclear silencing and locus repositioning are linked to regulation of virulence genes in *Plasmodium falciparum*. *Cell* **121**: 13–24
- Freitas-Junior LH, Hernandez-Rivas R, Ralph SA, Montiel-Condado D, Ruvalcaba-Salazar OK, Rojas-Meza AP, Mancio-Silva L, Leal-Silvestre RJ, Shorte S, Scherf A (2005) Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* **121**: 25–36
- Fried M, Duffy PE (1996) Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**: 1502–1504

- Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE (1998) Maternal antibodies block malaria. *Nature* **395**: 851–852
- Gamain B, Gratepanche S, Miller LH, Baruch DI (2002) Molecular basis for the dichotomy in *Plasmodium falciparum* adhesion to CD36 and chondroitin sulfate A. *Proc Natl Acad Sci USA* **99**: 10020–10024
- Gamain B, Smith JD, Avril M, Baruch DI, Scherf A, Gysin J, Miller LH (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
- Gamain B, Trimmell AR, Scheidig C, Scherf A, Miller LH, Smith JD (2005) Identification of multiple chondroitin sulfate A (CSA)-binding domains in the var2CSA gene transcribed in CSA-binding parasites. *J Infect Dis* **191**: 1010–1013
- Gardner MJ et al (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498–511
- Hernandez-Rivas R, Scherf A (1997) Separation and mapping of chromosomes of parasitic protozoa. *Mem Inst Oswaldo Cruz* **92**: 815–819
- Horrocks P, Pinches R, Kyes S, Kriek N, Lee S, Christodoulou Z, Newbold CI (2002) Effect of var gene disruption on switching in *Plasmodium falciparum*. *Mol Microbiol* **45**: 1131–1141
- Kyes S, Pinches R, Newbold C (2000) A simple RNA analysis method shows var and rif multigene family expression patterns in *Plasmodium falciparum*. *Mol Biochem Parasitol* **105**: 311–315
- Kyes SA, Christodoulou Z, Raza A, Horrocks P, Pinches R, Rowe JA, Newbold CI (2003) A well-conserved *Plasmodium falciparum* var gene shows an unusual stage-specific transcript pattern. *Mol Microbiol* **48**: 1339–1348
- Parzy D, Fusai T, Pouvelle B, Torrentino M, Eustacchio F, Lepolard C, Scherf A, Gysin J (2000) Recombinant human thrombomodulin(csa+): a tool for analyzing *Plasmodium falciparum* adhesion to chondroitin-4-sulfate. *Microbes Infect* **2**: 779–788
- Pouvelle B, Meyer P, Robert C, Bardel L, Gysin J (1997) Chondroitin-4-sulfate impairs *in vitro* and *in vivo* cytoadherence of *Plasmodium falciparum* infected erythrocytes. *Mol Med* **3**: 508–518
- Ralph SA, Scheidig-Benatar C, Scherf A (2005) Antigenic variation in *Plasmodium falciparum* is associated with movement of var loci between subnuclear location. *Proc Natl Acad Sci USA* **102**: 5414–5419
- Reeder JC, Cowman AF, Davern KM, Beeson JG, Thompson JK, Rogerson SJ, Brown GV (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
- Roberts DJ, Craig AG, Berendt AR, Pinches R, Nash G, Marsh K, Newbold CI (1992) Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* **357**: 689–692
- Rowe JA, Kyes SA (2004) The role of *Plasmodium falciparum* var genes in malaria in pregnancy. *Mol Microbiol* **53**: 1011–1019
- Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnot DE, Hviid L, Theander TG (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
- Salanti A et al (2004) Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J Exp Med* **200**: 1197–1203
- Scherf A, Hernandez-Rivas R, Buffet P, Bottius E, Benatar C, Pouvelle B, Gysin J, Lanzer M (1998) Antigenic variation in malaria: *in situ* switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in *Plasmodium falciparum*. *EMBO J* **17**: 5418–5426
- Smith JD, Gamain B, Baruch DI, Kyes S (2001) Decoding the language of var genes and *Plasmodium falciparum* sequestration. *Trends Parasitol* **17**: 538–545
- Su XZ, Heatwole VM, Wertheimer SP, Guinet F, Herrfeldt JA, Peterson DS, Ravetch JA, Wellems TE (1995) The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**: 89–100
- Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. *Science* **193**: 673–675