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Designing a VAR2CSA-based vaccine to prevent placental malaria[☆]

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Abstract

Placental malaria (PM) due to *Plasmodium falciparum* is a major cause of maternal, fetal and infant mortality, but the mechanisms of pathogenesis and protective immunity are relatively well-understood for this condition, providing a path for vaccine development. *P. falciparum* parasites bind to chondroitin sulfate A (CSA) to sequester in the placenta, and women become resistant over 1–2 pregnancies as they acquire antibodies that block adhesion to CSA. The protein VAR2CSA, a member of the PfEMP1 variant surface antigen family, mediates parasite adhesion to CSA, and is the leading target for a vaccine to prevent PM. Obstacles to PM vaccine development include the large size (~350 kD), high cysteine content, and sequence variation of VAR2CSA. A number of approaches have been taken to identify the combination of VAR2CSA domains and alleles that can induce broadly active antibodies that block adhesion of heterologous parasite isolates to CSA. This review summarizes these approaches, which have examined VAR2CSA fragments for binding activity, antigenicity with naturally acquired antibodies, and immunogenicity in animals for inducing anti-adhesion or surface-reactive antibodies. Two products are expected to enter human clinical studies in the near future based on N-terminal VAR2CSA fragments that have high binding affinity for CSA, and additional proteins preferentially expressed by placental parasites are also being examined for their potential contribution to a PM vaccine.

Keywords

Plasmodium falciparum; Placental malaria; Vaccine

1. Introduction

Placental malaria (PM) leads to poor outcomes for pregnant women and their babies, and is caused by *Plasmodium falciparum* sequestration in the intervillous spaces of placenta and the ensuing inflammation. Severe maternal anemia, low birth weight delivery and fetal loss are common sequelae, resulting in 10,000 maternal deaths and 200,000 infant deaths annually in Africa by some estimates, and causing a third of perinatal mortality in the

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absence of preventive measures [1]. The poor outcomes of PM have been associated with the inflammatory infiltrates and levels of inflammatory cytokines observed in the placenta [2–6]. Adult residents of malaria endemic areas enjoy immunity that protects them from severe disease; women become susceptible to infection and disease again during first gestation, then regain immunity over successive pregnancies. *P. falciparum* infected erythrocytes (IE) bind chondroitin sulfate A (CSA) on the syncytiotrophoblast surface and in intervillous spaces; unlike placental IE, IE in non-pregnant individuals bind receptors like CD36 and ICAM-1 but not CSA to sequester in other vascular beds [7].

With increasing parity, women acquire specific antibody against CSA-binding parasites, including that which inhibits IE adhesion, and this antibody is associated with heavier babies and higher maternal hemoglobin levels [8–10]. These naturally acquired antibodies are broadly active to placental IE collected around the world, indicating that the target epitopes are conserved [11]. This model of pathogenesis and immunity provides a framework to develop PM vaccines, and also predicts that vaccine-induced immunity should be naturally boosted when pregnant women are exposed to malaria. Other malaria parasite species such as *Plasmodium vivax* infect pregnant women, but disease sequelae are less severe [12], and these species do not sequester in placenta [13] hence the path to a vaccine against these forms of pregnancy malaria is not clear.

P. falciparum IE adhesion to CSA is mediated by the large (~350 kD) protein called VAR2CSA (Fig. 1), a member of the PfEMP1 variant surface antigen family. PfEMP1 proteins including VAR2CSA are encoded in the genome of *P. falciparum* but not that of other human malaria parasites. VAR2CSA has extracellular, transmembrane, and intracytoplasmic regions, and its extracellular region is uniquely structured among PfEMP1 family members. The extracellular region of VAR2CSA includes an N-terminal sequence, 6 cysteine-rich Duffy binding like (DBL) domains, and inter-domain (ID) regions that increasingly appear to play a key role in adhesion and immunogenicity of recombinant VAR2CSA protein fragments. VAR2CSA is preferentially expressed by placental parasites and isolates selected to bind CSA [14,15], and is currently the leading candidate for a vaccine to prevent malaria during pregnancy. The high molecular weight, multiple extracellular domains, and extensive sequence variation of VAR2CSA pose unique challenges in designing a vaccine that will mimic the broadly neutralizing activity of naturally acquired immunity. The most challenging step is to define the domain or domain combination and boundaries that can elicit potent pan-reactive antibody. Currently, the first 2 candidate VAR2CSA-based products derived from the protein N-terminus region are entering clinical trials evaluation.

We review here the approaches to design VAR2CSA immunogens that can be used in a vaccine to prevent PM, either by defining CSA-binding fragments of VAR2CSA, or by defining the domains and epitopes that induce broadly active antibodies in animals or that react to such antibodies from immune women. We conclude with a discussion of the antigens other than VAR2CSA that are preferentially expressed by placental parasites, as these may also contribute to a protective vaccine.

2. CSA-binding domains of VAR2CSA

PM vaccine development is currently based on mimicking naturally acquired functional antibodies that block parasite adhesion to CSA. Because these antibodies are likely to target epitopes within the binding domains of VAR2CSA, several approaches have been applied to identify CSA binding regions of the protein. Initial *in vitro* studies examined binding to CSA of 6 VAR2CSA recombinant DBL domains of 3D7 allele and the DBL1, DBL2 and DBL3 of the A4 allele: DBL2 and DBL6 domains of 3D7 and DBL2 and DBL3 of A4 bound immobilized CSA [16,17]. In another study, DBL3 of both 3D7 and A4 alleles bound CSA, but the 3D7 form demonstrated stronger binding than the A4 form [18]. Following these observations, two crystallography studies using recombinant DBL3 protein of the A4 allele confirmed binding to CSA [19,20].

In another approach to identify CSA binding motifs, a phage library of *var2csa* gene fragments was panned on CSA-expressing cells or soluble CSA, and identified CSA-binding peptides derived from the first five domains (DBL1 through DBL5) and not DBL6 [21]. Based on these results, peptides representing the sequences of the phage clones were synthesized. One of the peptides from DBL2 domain bound to CSA and anti-peptide antibodies recognized native VAR2CSA [21].

In vitro binding studies have shown that DBL domains bind specifically to CSA but not to the closely related glycosaminoglycan chondroitin sulfate C. However, similar results have been obtained with DBL from other PfEMP1s expressed by IE that do not bind CSA [22]. This raises questions about data interpretation and complicates identification of specific binding domains and vaccine targets. After the full-length extracellular region of VAR2CSA was expressed as recombinant protein, individual DBL domains or domain combinations were re-evaluated using the full-length protein as a bench mark for strength of binding. Full length VAR2CSA binds CSA at a nanomolar affinity [23,24]. Kinetic analysis showed that single domains DBL2, DBL3 and DBL6 bind CSA with affinity in the micromolar range [24,25]. In comparison, recombinant multi-domain DBL1-ID1-DBL2 fragment bound CSA with similar kinetics as the full length protein in one study [25], but did not bind CSA in another study [26]. Instead, the latter study found high affinity binding with DBL1-ID1-DBL2-ID2a domain combination. ID2a and ID2b comprise the so-called cysteine-rich inter-domain region (CIDR) of VAR2CSA, and the evidence suggested that ID2a or a portion of it is required for specific binding to CSA and not to other sulfated glycans [26]. For example, recombinant DBL1-ID1-DBL2-CIDR (ID2a-ID2b) binds to CSA and to heparan sulfate equally, while a recombinant protein truncated after the first 90–100 amino acids of ID2a regained specificity [26]. Interestingly, the binding of DBL1-ID1-DBL2 to decorin was stronger than to CSA [25], a difference that was not seen in binding of intact IE (unpublished data) and that may reflect differences between the native epitopes and the binding epitopes of the recombinant protein. In summary, it appears that the highest binding affinity to CSA was obtained with domain combinations from the N-terminal portion of VAR2CSA, but the specificity of binding is influenced by inclusion or exclusion of different CIDR (ID2a-ID2b) fragments.

3. Naturally acquired antibodies to VAR2CSA domains

Over successive pregnancies women develop specific immunity to the parasites that sequester in the placenta. Antibodies that block parasite adhesion to CSA have been associated with a reduced infection risk and increased newborn birthweight [8,10,11]. Low antibody reactivity with the IE surface was associated with lower hemoglobin level and reduced birthweight [9]. Antibodies that react to the IE surface by flow, or that opsonize or agglutinate CSA-binding IE, also develop in naturally exposed women [9,27–38], although the evidence base is smaller that these antibodies might be associated with clinical outcomes.

Because VAR2CSA is the leading vaccine target, several studies have compared VAR2CSA domains for reactivity to sera of primigravid and multigravid women in order to support vaccine design (Table 1). Tuikue Ndam et al. [39] reported that antibodies to domains DBL5 and DBL6 but not DBL1 measured during the 2nd trimester or at delivery correlated with parity in an area of Senegal with seasonal malaria transmission. In a recent study from Benin, Tuikue Ndam et al. [10] reported that antibody levels to DBL1–ID1–DBL2 and to DBL3 were higher among multigravid than primigravid women both during pregnancy and at the time of delivery, while antibody levels to DBL4 or DBL5 were only higher in multigravid women at delivery or during pregnancy, respectively. Babakhanyan et al. [40] reported that antibody levels to ID1–DBL2–ID2a did not differ between Cameroonian pregnant women and men. Similarly, Gnidehou et al. [41] reported that in Colombia, children, men and pregnant women exposed to malaria have similar antibody levels to ID1–DBL2–ID2a, DBL3 and DBL5 domains. Oleinikov et al. [42] reported significantly higher levels of antibodies to DBL1, DBL3 and DBL6 (but not DBL4 or DBL5) in multigravidae compared to primigravidae living in a malaria holoendemic area of Tanzania. Brolin et al. [33] found that antibodies to DBL5 were higher among multigravid compared to primigravid women, while antibody levels to DBL3 and DBL6 did not differ by gravidity. Several other studies reported parity-dependent serum reactivity to DBL5 domain [14,43,44]. In summary, parity-dependent acquisition of VAR2CSA-specific antibody appears to be a robust correlate of protective immunity, but in general it does not appear to discriminate well between different candidate VAR2CSA immunogens. Further, studies differ as to whether a particular domain specificity is elevated in multigravid women, possibly due to differences in recombinant antigens, in assay format such as ELISA *versus* Luminex platform, or in study populations.

More data are emerging on the relationships between antibodies to DBL domains and birthweight or maternal anemia. Tutterrow et al. [45] examined antibody levels to DBL1, DBL1–ID1–DBL2, DBL3, DBL4, DBL5, and DBL6 of 3 alleles over the course of pregnancy in Cameroon, and concluded that the number of domains and allelic types recognized by naturally acquired antibodies during second trimester predicted a reduction in placental infection status at delivery. Among Kenyan women with acute or chronic malaria infection, higher antibody levels to DBL5 were associated with reduced risk of low birthweight delivery and with increased birthweight [14]. In Mozambique, antibody levels to VAR2CSA domains, to merozoite antigens (EBA-175 and MSP1), and to IE surface were lower among uninfected pregnant women compared to pregnant women with acute, chronic

or past infection. Among women with at least one malaria infection during their pregnancy, high antibody levels to VAR2CSA domains and other proteins not unique to placental parasites like AMA-1, positively correlated with birthweight and gestational age [46]. In a recent study in Benin, high antibody levels to the full recombinant VAR2CSA and DBL3 domain at enrollment (before gestational week 24) were associated with a reduction in the risk of infection in the period between enrollment and delivery; reduced placental malaria risk was associated with higher antibody levels to DBL3, although a similar trend was also observed with an unrelated VSA [10], and high antibody levels to DBL1–ID1–DBL2 were associated with a reduction in low birthweight deliveries [10].

4. VAR2CSA immunization of animals to elicit anti-adhesion antibodies

VAR2CSA is a large molecular weight protein that cannot be commercially manufactured as a full-length recombinant molecule with existing technologies. Identifying smaller fragments that elicit functional antibodies similar to those induced by natural infection has probably been the greatest challenge for VAR2CSA vaccine development. Multiple studies have examined functional activity of antibodies raised in animals to DBL domains of VAR2CSA, as an approach to identify the optimal VAR2CSA-based immunogen(s) for inducing cross-reactive and/or functional antibodies. An important caveat for these studies is that different groups use different assay formats to measure anti-adhesion activity of antibody, and therefore it is not possible to compare activity measurements between studies.

Multi-domain fragments NTS–DBL1–ID1–DBL2, DBL3–DBL4 and DBL5–DBL6 of FCR3 allele induced antibodies that reacted with the surface of homologous parasites at higher levels than did antibodies induced by the six single DBL domains of FCR3 allele [47]. However, antibodies to the single DBL4 domain almost completely inhibited the binding of homologous and 2 heterologous IE to CSA [47,48] while antibodies to other single or multi-domain immunogens only reduced homologous parasite binding to CSA by 50–60% [47]. Anti-adhesion antibodies induced by immunization with DBL4 domain of FCR3 allele were species-dependent: anti-adhesion activity was observed in samples collected from immunized rats but not from rabbits [48]. Antibodies raised to all DBL domains of 3D7 allele type reduced the adhesion of heterologous FCR3 IE by 50–65% compared to control, with the exception of antibodies to DBL1 that almost completely inhibited FCR3 binding to CSA [49]. Antibodies to DBL4 of 3D7 allele as well as that of a placental parasite sample reduced the binding of heterologous maternal parasites by more than 50% [50]. Of the DBL domains of HB3 allele, only DBL3 induced anti-adhesion antibodies that almost completely inhibited the binding of FCR3 IE to CSA [49]. Other studies reported that recombinant DBL5 domain and the multi-domain DBL5–DBL6 of several allelic forms inhibited the binding of FCR3 IE by more than 60% [51] and the binding of culture-adapted isolates from pregnant women [50]. The N-terminal region of VAR2CSA, NTS–DBL1–ID1–DBL2, elicited antibodies that almost completely inhibited the binding of homologous FCR3 and heterologous HB3 IE [52], consistent with an earlier study reporting anti-adhesion activity by antibodies raised against multidomain immunogens including NTS–DBL1–ID1–DBL2 [47]. Smaller fragments of the N-terminal region of VAR2CSA like ID1–DBL1–ID2a and ID1–DBL2–ID2a–ID2b also elicited strong homologous anti-adhesion antibodies [26].

Antibody anti-adhesion activity against IE collected from pregnant women has also been induced by the FCR3 allelic forms of DBL4 and of N-terminal multi-domain fragments of VAR2CSA. DBL4 domain of FCR3 inhibited 5/13 isolates from binding to CSA by >50% [53]. An extended form of DBL4 with an additional C-terminal 40 amino acids had broader anti-adhesion activity, inhibiting 10/13 maternal isolates by >50% [53]. In separate studies, antibodies raised in mice and rats to DBL4 failed to inhibit the binding of maternal isolates [52,54]. A recent analysis of the cross reactivity of this form showed minimal activity against heterologous isolates selected for CSA binding [55]. DNA vaccination with NTS–DBL1–ID1–DBL2 elicited antibodies that blocked the binding of the majority of the isolates to CSA by >50% (FCR3 allele: 12/15; 3D7 allele: 13/18; FCR3 allele: 11/18) [52,56]. The smaller fragment ID1–DBL2 reduced the binding to CSA of 6/8 maternal isolates, of which the reduction was significant for 2 isolates [57].

Notably antibodies to the full length VAR2CSA inhibited the binding of homologous parasite isolate [23,58] but not heterologous parasites [56,58]. Previously we showed that plasma from multigravid women inhibited the binding of maternal isolates in a strain independent manner [11]. Thus, it is not clear whether immunization with multiple allelic types is required to induce broadly reactive antibodies, or whether animal immunization with recombinant protein induces a response to different and possibly more variant epitopes than does natural infection, resulting in a strain-dependent response.

5. Epitope mapping

Immune multigravid women develop strain-independent functional antibodies against placental parasites, and these antibodies are associated with clinical protection. Identifying epitopes targeted by protective antibodies can facilitate development of a broadly reactive vaccine to prevent pregnancy malaria. Several approaches have been applied to identify protective epitopes, including screening of peptide libraries, producing nanobody, and developing monoclonal antibodies from B cells of immune women.

For screening peptide libraries, Pepscan analysis was performed on a peptide array corresponding to the extracellular portion of VAR2CSA [59]. Pooled plasma from immune women recognized multiple peptides across all VAR2CSA domains, except for a small reduction in reactivity with peptides corresponding to DBL2 domain; most reactivity was not reduced after depleting the plasma on native VAR2CSA protein [59].

Antibodies to the DBL4 domain raised in rats had adhesion blocking activity against homologous and heterologous parasites [47,53]. Using a DBL4 peptide array, the reactivity of anti-DBL4 antisera to 3 regions within DBL4 was associated with adhesion blocking activity [60]. This region was also recognized by sera from 4/6 immune women, with reactivity directed to a conserved sequence in DBL4 [60]. However, antibodies raised to these peptides neither reacted with VAR2CSA expressed on the IE surface nor inhibited parasite adhesion to CSA. One possible explanation is that the linear epitopes did not mimic the conformational epitopes required to induce functional antibodies [60]. Recombinant DBL4 mutated in the region corresponding to the peptides recognized by native sera induced antibodies with lower adhesion blocking activity than the wild type form. The loss of

potency may have resulted from a change in a specific B-cell epitope, or alternatively from a change in the overall protein folding resulting in a less immunogenic protein [60].

Nanobody technology has been used as another approach to identify vaccine targets in VAR2CSA. This technology is based on camelid heavy-chain only antibody in which the variable heavy chain (VHH) alone comprises the antigen binding site. Recombinant forms of the VHH are called nanobodies. Using this approach, nanobodies that reacted with the recombinant DBL domains ID1–DBL1–ID2a, DBL4, DBL5, and DBL6 [61] and DBL1, DBL4, DBL5, and DBL6 [62] were produced. Several nanobodies to DBL1 and ID1–ID2a reduced parasite adhesion to CSA while nanobodies to DBL4, DBL5 and DBL6 had no inhibitory activity [61,62]. In one study, the majority of nanobodies reacted to DBL1 domain [62], while the other study did not identify any nanobodies to this domain [61], making it difficult to determine the utility of this information for designing a vaccine.

Finally, B cells from immune African women were immortalized as a source of specific reagents, and using this approach 8 monoclonal antibodies that reacted with the surface of CSA-selected isolates were identified [63]. Of those, 3 monoclonal antibodies reacted with recombinant DBL3 domain and 4 with DBL5 domain of VAR2CSA; the 8th monoclonal antibody named PAM1.4 did not react with recombinant DBL domains and reacted poorly with full length VAR2CSA [63,64]. Monoclonal antibody PAM1.4 recognized the surface of placental parasites and inhibited their adhesion to CSA, while it only moderately inhibited the adhesion to CSA of the laboratory isolate FCR3-CSA [64]. These results raised an important issue that maternal parasites may express additional proteins mediating adhesion to CSA that are targeted by natural immunity and are not expressed in model laboratory isolates selected for CSA binding phenotype.

Taken together, these different approaches to epitope mapping have provided insights into domains or epitopes that react to functional antibody. However, these studies have not definitively identified a single VAR2CSA fragment that is the ideal target for a vaccine.

6. Invariant proteins expressed by placental parasites

In addition to VAR2CSA, several conserved proteins expressed by placental parasites have been identified in transcriptomic and proteomics analysis of placental parasites. While gene knockout studies have demonstrated that VAR2CSA is required to maintain the CSA-binding phenotype [65], other genes and proteins are upregulated in placental parasites and therefore might contribute to the binding phenotype or to placental malaria pathogenesis as well. Comparative transcriptome analyses identified 11 genes that were upregulated in placental parasites compared to children's parasites [66] and 38 genes in placental parasites compared to the reference parasite line 3D7 [67]. Two comparative proteomics analyses of placental parasites identified 17 and 53 conserved proteins respectively that were expressed exclusively or at higher levels in placental parasites than in parasites collected from children [68,69]. Because these novel genes and proteins are upregulated in VAR2CSA-expressing parasites, studies should be undertaken to determine whether deletion of VAR2CSA may influence their expression or localization.

Two conserved proteins PFB0115w and PFI1785w were common between three (PFB0115w) or four (PFI1785w) comparative studies. PFB0115w is expressed at higher levels in placental parasites compared to children's isolates, while PFI1785w is exclusively expressed by placental parasite. PFI1785w is a member of the Plasmodium helical interspersed subtelomeric family, and is annotated as an exported protein. Antibodies to a recombinant form of PFI1785w reacted with membrane protein extracted from placental parasites but not with membrane proteins extracted from children's isolates or FCR3 parasites selected to bind CSA [68]. Antibody levels to recombinant PFI1785w are significantly higher in plasma from pregnant women than men [67]. Antibody to recombinant PFI1785w expressed in *Escherichia coli* failed to react with the surface of live IE, suggesting that the antibody recognizes non-conformational epitopes [68]. Further studies with protein expressed in a eukaryotic system that better reflects native structure could be valuable for studies of localization and adhesion to CSA.

Of 5 conserved genes that were upregulated in transcriptomic studies of placental parasites [66], one of the proteins named PfCSAL binds to CSA and co-localizes with VAR2CSA on the surface of maternal IE (Keitany, Duffy, et al., unpublished data). This protein is currently being evaluated as an additional placental malaria vaccine candidate.

7. Summary

The rational design of a vaccine to prevent malaria during pregnancy is based on the observations that a distinct form of the parasite that infects the placenta is associated with disease and death in mothers and newborns, and that women become resistant to pregnancy malaria as they develop antibodies against proteins expressed on the surface of this parasite form. The identification of a single human monoclonal antibody (PAM1.4) from immune multigravid women that recapitulates anti-adhesion activity in plasma from multigravidae [64], together with the observation that plasma from multigravidae have strain-transcending functional activity [11], strongly suggest that a limited number of epitopes can be targeted by an effective PM vaccine.

Currently, VAR2CSA is the leading pregnancy malaria vaccine candidate. Different research approaches have been taken to identify the targets that should be included in a VAR2CSA-based vaccine, including mapping the CSA-binding sites, mapping antibody epitopes, defining DBL domains preferentially recognized by immune sera, and assessing functional activity by antibodies raised against recombinant DBL domains. While multiple VAR2CSA domains bind CSA in *in vitro* assays, an N-terminal fragment (ID1-DBL2-ID2a-ID2b) binds CSA with similar kinetics as the full length protein [26]. Antibody levels to N-terminal fragments of VAR2CSA are similar between men, primigravid and multigravid women in Africa [40]. However, antibodies induced in animals to the NTS-DBL1-ID1-DBL2-ID2a multi-domain have heterologous functional activity, and the serum activity induced by this recombinant form has been broader than that induced by the smaller ID1-DBL2 fragment [52,56].

In addition to VAR2CSA, several conserved proteins expressed by placental parasites have been identified [66-69]. These conserved proteins may play an independent role in IE

adhesion, or may interact with VAR2CSA to form conformational epitopes targeted by naturally acquired immunity. One example is the invariant protein PfCSAL that interacts with VAR2CSA (Keitany, Duffy, et al., unpublished data). Further studies of PFI1785w, an exported protein exclusively expressed by placental parasites, are required to determine whether it also has a role in inducing protective immunity.

The development of a placental malaria vaccine is based on naturally occurring immunity. Based on this model, the vaccine is predicted to induce antibodies in nulligravidae that will be boosted by malaria exposures during subsequent pregnancies, and similarly should boost functional antibodies that have been acquired by multigravidae in endemic areas. The first field trials will soon begin to assess whether individual VAR2CSA immunogens may be sufficient for inducing functional antibodies that have been associated with protection from placental malaria, or whether additional components may be required for an effective vaccine that protects pregnant women.

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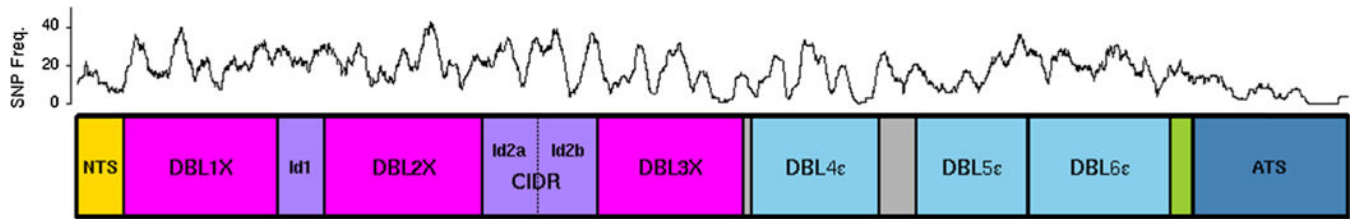


Fig. 1. Domain architecture of VAR2CSA

Schematic organization of VAR2CSA protein, showing its N-terminal sequence, 6 cysteine-rich Duffy binding like (DBL) domains, inter-domain (ID) regions, and intracytoplasmic tail (acidic terminal sequence, ATS). Prevalence of sequence variability along the extent of the protein is graphically displayed as the number of single nucleotide polymorphisms (SNPs) per 100 nucleotides, measured as a moving average along the sequence. Analysis does not include insertion/deletion differences. SNP data obtained from PlasmoDB. Figure and sequence analysis prepared by Robert Morrison.

Table 1

Studies of acquired antibody to VAR2CSA DBL domains in African women, indicating the relationship between antibody levels and maternal parity. Timing of sample collection during pregnancy or delivery is indicated.

| DBL domain | Significant increase with parity | No difference with parity |
|-------------------|--|---|
| DBL1 | Oleinikov [42] (Delivery) | Tuikue Ndam [39] (Enrollment ^a & Delivery) |
| DBL1–DBL2 | Tuikue Ndam [10] (Enrollment ^a & Delivery) | |
| ID1–ID2a | | Babakhanyan ^b [40] |
| DBL3 | Oleinikov [42] (Delivery) Tuikue Ndam [10] (Enrollment ^a & Delivery) | Brolin [33] (3rd trimester) |
| DBL4 | Tuikue Ndam [10] (Delivery) | Oleinikov [42] (Delivery) Tuikue Ndam [10] (Enrollment ^a) |
| DBL5 | Brolin [33] (3rd trimester) Gangnard [44] (During pregnancy) ^c Gnidehou [43] (2nd and 3 rd trimester) Salanti [14] (Delivery) Tuikue Ndam [39] (Enrollment ^a & Delivery) Tuikue Ndam [10] (Enrollment ^a) | Oleinikov [42] (Delivery) Tuikue Ndam [10] (Delivery) |
| DBL6 | Oleinikov [42] (Delivery) Tuikue Ndam [39] (Enrollment ^a & Delivery) | Brolin [33] (3rd trimester) Tuikue Ndam [10] (Enrollment ^a & Delivery) |

^aSamples collected at enrollment at any time during the first 6 months of gestation.

^bSamples collected during the 1st, 2nd and 3rd trimester.

^cSamples collected during pregnancy, but timing is not specified.