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EDITED BY
Sara Healy,
National Institutes of Health (NIH),
United States

REVIEWED BY
Augustina Frimpong,
University of Ghana, Ghana
Benjamin Mordmüller,
Radboud University Medical Centre,
Netherlands

*CORRESPONDENCE
Bernard N. Kanoi,
bkanoi@mku.ac.ke

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Towards identification and development of alternative vaccines against pregnancy-associated malaria based on naturally acquired immunity

Alex K. Rotich[1], Eizo Takashima[2], Stephanie K. Yanow[3], Jesse Gitaka[1] and Bernard N. Kanoi[1]*

[1]Centre for Research in Infectious Diseases, Directorate of Research and Innovation, Mount Kenya University, Thika, Kenya, [2]Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Japan, [3]School of Public Health, University of Alberta, Edmonton, AB, Canada

Pregnant women are particularly susceptible to *Plasmodium falciparum* malaria, leading to substantial maternal and infant morbidity and mortality. While highly effective malaria vaccines are considered an essential component towards malaria elimination, strides towards development of vaccines for pregnant women have been minimal. The leading malaria vaccine, RTS,S/AS01, has modest efficacy in children suggesting that it needs to be strengthened and optimized if it is to be beneficial for pregnant women. Clinical trials against pregnancy-associated malaria (PAM) focused on the classical VAR2CSA antigen are ongoing. However, additional antigens have not been identified to supplement these initiatives despite the new evidence that VAR2CSA is not the only molecule involved in pregnancy-associated naturally acquired immunity. This is mainly due to a lack of understanding of the immune complexities in pregnancy coupled with difficulties associated with expression of malaria recombinant proteins, low antigen immunogenicity in humans, and the anticipated complications in conducting and implementing a vaccine to protect pregnant women. With the accelerated evolution of molecular technologies catapulted by the global pandemic, identification of novel alternative vaccine antigens is timely and feasible. In this review, we discuss approaches towards novel antigen discovery to support PAM vaccine studies.

KEYWORDS

***Plasmodium falciparum*, VAR2CSA, pregnancy-associated malaria, vaccines, antigen discovery**

Background on pregnancy-associated malaria

Plasmodium falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi are the five major species of *Plasmodium* that cause malaria in humans. Malaria remains a global public health threat despite decades of intense control efforts (1). According to the World Health Organization (WHO)-2020 malaria report, there were 241 million malaria infections and 627,000 fatalities globally, with 14 million infections and 69,000 more deaths than in 2019. During the pandemic, nearly two-thirds of these excess deaths (47,000) were attributable to interruptions in malaria prevention, diagnosis and treatment (2). *P. falciparum* causes the majority of malaria infections and fatalities with most occurring in sub-Saharan Africa. The major disease burden is in children less than 5 years of age, pregnant women, and individuals with immunodeficiencies.

Despite immunity acquired after years of exposure, the risk of infection is the same for women of all gravidities (3). However, the risk of poor birth outcomes is increased in primigravid and secundigravid women because they lack protective antibodies against placental malaria parasites; such outcomes include maternal anemia, spontaneous abortions, premature delivery (gestation of less than 37 weeks) and intrauterine growth retardation (IUGR) (4–7). This is primarily driven by the accumulation of infected erythrocytes (iRBCs) in the vascular spaces of the placenta (8).

Immunopathogenesis of malaria in pregnancy

The sequestration of iRBCs in the vascular portion of the placenta causes placental malaria (9). These parasites express a surface ligand that is unique to placental malaria called VAR2CSA. VAR2CSA is a member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family and binds to chondroitin sulfate A (CSA), a glycosaminoglycan found in the placenta and other cells that mediates sequestration of iRBCs in pregnancy-associated malaria (PAM) (10, 11). These parasites bind to CSA but not to other major surface ligands, notably CD36 and ICAM-1, whereas most parasite isolates from non-pregnant women do the contrary (12, 13). Parasite sequestration in the placenta triggers a transition in the cell-mediated immune response, often from Th2 to Th1, which is marked by an increase in production of pro-inflammatory cytokines (14). Although Th1-type responses are important for the elimination of the parasite through stimulation of cellular immune responses, overproduction of cytokines alters pregnancy (15). In addition, increased number of maternal phagocytic cells and fibrin in the intervillous space together with deposition of hemozoin in phagocytic leucocytes induce placental inflammation that is associated with low birth weight and maternal anemia (8).

Protective antibodies against PAM

There is a strong negative association between gravidity and poor birth outcomes from PAM (16). This suggests that protective immunity develops against PAM and is directed against a target that is pregnancy-specific and highly immunogenic (17). VAR2CSA meets these criteria and antibodies to this protein are associated with protection against poor pregnancy outcome (18). In a recent study in Benin, high antibody responses to the N-terminal region of VAR2CSA were associated with lower risk of infection and low birth weight highlighting the protective role of these antibodies in immunity to placental malaria (19). Furthermore, in another cohort, anti-VAR2CSA antibody responses involved cytophilic immunoglobulin G (IgG) 1 and IgG3 subclasses, with prevalence ranging from 28% IgG3 to 50% IgG1. During pregnancy, higher levels of VAR2CSA-specific total IgG and cytophilic IgG3 were consistently linked to higher birth weights, whereas high levels of IgG4 were linked to a lower risk of placental infections, an indicator that both cytophilic and non-cytophilic antibodies offer protection (20).

In a similar study in Kenya, plasma from pregnant women exposed to malaria inhibited more than 35% of parasite adhesion to CSA (21). As expected, only one of 47 primigravid women had anti-adhesion antibodies, whereas anti-adhesion activity was higher in secundigravid women. This was associated with increased newborn birth weight and gestational age but not maternal hemoglobin. In a different study, IgG levels but not IgM against parasite lysate were positively correlated with newborn weight increase (22). In a study conducted in Mozambique, IgG levels against placental and pediatric isolates, as well as recombinant VAR2CSA (DBL2X, DBL3X, DBL5 and DBL6 domains) and other blood-stage antigens correlated with normal neonatal weight and gestational age in women with PAM (23). In contrast, a recent systematic review suggests that anti-VAR2CSA antibodies may instead be markers of infection rather than correlates of protection (24).

Taken together, VAR2CSA appears to be an important target of protective immunity to PAM (25), but additional targets, and protective mechanisms could also exist. The majority of studies, including vaccine trials, have focused on understanding and/or evaluating the role played by VAR2CSA; this is the only antigen under consideration as a PAM vaccine currently.

Current status of PAM vaccines

Surface expression of VAR2CSA is restricted to CSA-adhering iRBCs, prompting development of vaccines targeting this protein (26). The leading VAR2CSA-based vaccines, PAMVAC and PRIMVAC (27), are designed to generate functional antibodies capable of blocking the binding of infected cells to CSA on placental syncytiotrophoblast as well as opsonizing iRBCs expressing VAR2CSA. Such a vaccine would protect pregnant women, especially primigravids, from adverse outcomes in PAM.

PAMVAC is derived from the VAR2CSA subunit spanning ID1-DBL2-ID2a of the FCR3 variant (28) while PRIMVAC is based on CSA-binding DBL1x-2x region of the 3D7-VAR2CSA allele (29). The clinical trials for PAMVAC (under the registration EudraCT 2015-001827-21; ClinicalTrials.gov NCT02647489) were carried out in Germany and in Benin. The vaccine was well tolerated and immunogenic, and elicited IgG antibodies that recognized the homologous variant of VAR2CSA and blocked adhesion of these parasites to CSA *in vitro* (30).

The clinical trial for PRIMVAC (registered with ClinicalTrials.gov, NCT02658253) was conducted in two phases, the first in 18 to 35-year-old women in France and the second in women who were naturally exposed to *P. falciparum* and nulligravid in a research center in Burkina Faso using the same formulations as for PAMVAC (31). Similarly, the vaccine was safe, immunogenic and elicited antibodies with high reactivity toward parasites expressing the VAR2CSA variant homologous to the one in the vaccine. However, there was no recognition or anti-adhesion activity against heterologous variants of VAR2CSA (31).

This poor recognition of antibodies to the PAM vaccines against heterologous VAR2CSA alleles is a significant limitation that must be overcome to generate a broadly effective vaccine against PAM. In a recent analysis of the *var2csa* gene in more than 2,000 *P. falciparum* field isolates across 23 geographically distant countries (32), Benavente et al. reported high nucleotide and haplotype diversity of VAR2CSA in African parasites compared to South East Asian parasites. Significant differences in the frequency of extra VAR2CSA copies was also observed in Oceania, West Africa, East Africa and Southeast Asia isolates at 88%, 35%, 29% and 21%, respectively. Surprisingly, insertion and deletions were observed within the DBL-2x domain, a major component of both PAMVAC and PRIMVAC vaccines (33). Further investigations are needed to shed light on whether such indels could impact the protein structure necessary for binding to CSA and/or its ability to induce the immune response. High polymorphism in *var2csa* was also observed in a strain from the Democratic Republic of Congo (34). These studies have clearly demonstrated genetic variation in *var2csa*, cementing the need to develop broad multivalent vaccine candidates that could be efficacious across different variants.

While most vaccines against PAM focus on VAR2CSA, vaccines that prevent liver stage infection would also have a profound impact on pregnant women. One vaccine, the radiation attenuated *P. falciparum* NF54 sporozoites (PfSPZ Vaccine), which demonstrated significant protection in African adults (35), is now being assessed in Mali for safety, immunogenicity, and potential protective efficacy in women of childbearing potential (clinicaltrials.gov; NCT03989102).

Identification of targets of broadly cross-reactive antibodies

A critical feature of a PAM vaccine is to elicit antibodies that mimic naturally acquired immunity and recognize diverse alleles of *var2csa*. Natural immunity may be mediated by a repertoire of antibodies to diverse epitopes (36) or increased affinity to conserved epitopes that are shared across variants. Doritchamou et al. revealed conserved epitopes in a single variant of VAR2CSA and these epitopes are targets of neutralizing antibodies, a promising discovery to develop a vaccine using a few selected variants (37). Moreover, VAR2CSA has several globally shared, cross-reacting polymorphic epitopes (38).

The choice of construct for a domain specific VAR2CSA vaccine is one that elicits antibodies that are broadly cross-reactive. The VAR2CSA domain NTS-DBL1-Id1-DBL2X elicits rabbit antibodies that completely inhibit the FCR3 strain of *P. falciparum* from binding to CSA to the same extent as antisera against full-length extracellular VAR2CSA (NTSDBL1X to DBL6) (39). Epitope mapping revealed that Id1-DBL2X in the N-terminal region of VAR2CSA contains an epitope that induces highly inhibitory antibodies (40). However, some domains (e.g. the VAR2CSA DBL6-epsilon domain expressed in HEK293) induce limited cross-reactive and blocking antibodies to CSA binding parasites (41). Immunization with the DBL5 recombinant protein elicits broadly cross-reactive antibodies against many parasite strains and clinical isolates from pregnant women whereas antibodies to DBL1 had limited breadth and only reacted with three or four parasite strains (42). Using flow cytometry to measure antibody recognition of the native protein, DBL3 and to a lesser extent DBL5, generated antibodies that are cross-reactive to different CSA-binding parasite lines whereas DBL6 antibodies were highly strain-specific (43). However, even antibodies to the full length VAR2CSA do not always inhibit binding of different placental cell lines to CSA (44). We believe that identification of other conserved antigens form novel targets can be investigated as potentially cross-protective vaccines (45).

One alternative approach is to identify conserved epitopes that are shared across different proteins that contain DBL domains. These epitopes may be related structurally and present in proteins with diverse biological functions. For example, antibodies to *P. vivax* Duffy Binding Protein (PvDBP) elicited during natural infection with *P. vivax* or following animal immunization with PvDBP, can cross-react with and inhibit adhesion of strains expressing different variants of VAR2CSA (46). Although these proteins are functionally unrelated, they have a common homologous ancestry and share conserved epitopes that can be exploited in vaccine design.

Identification of targets of complement fixing antibodies in PAM

Antibodies can activate the complement system to mediate effector functions against myriad developmental stages of the parasite life cycle (47) that are linked to protection against clinical malaria (48). New evidence suggests that these mechanisms may also contribute to vaccine-induced immunity. For example, the RTS,S vaccine can induce complement-fixing antibodies against the central repeat and C-terminal regions of CSP (49).

Antibodies from pregnant women naturally exposed to malaria also fix complement. Sera from a longitudinal cohort of pregnant women in Papua New Guinea identified VAR2CSA-DBL5 and DBL3 antibodies that fixed C1q and C3 (50). C1q protein mediated complement enhanced the ability of VAR2SCA antibodies to inhibit iRBC binding to CSA. Protective associations between antibody-mediated complement fixation and the risk of placental malaria infection was weaker for C1q-fixation of DBL3 when compared with C1q or C3 fixation of the DBL5 domain or placental-binding iRBCs, highlighting the immuno-dominant nature of these antigens (50). IgG1 and IgG3 subclasses are the dominant antibodies to placental-binding iRBCs and to recombinant VAR2CSA (51) with the ability to fix and activate human complement in PAM (52). However, the proportion of VAR2CSA-specific IgG in plasma that is involved in complement fixation relative to other effector functions remains to be determined.

Importantly, Opi et al. observed higher C1q and C3 fixation on genetically modified CS2 *P. falciparum* infected iRBCs (that lack surface expression of PfEMP1) among multigravida compared to primigravida (50). This demonstrated that lack of PfEMP1 expression did not eliminate complement fixation on iRBCs suggesting that other surface proteins such as other variable surface antigens (repetitive interspersed family (RIFIN), subtelomeric variable open reading frame (STEVOR), surface-associated interspersed gene family (SURFIN), could also be important targets of complement-fixing antibodies. Evaluation of these molecules warrant investigation to establish if they have a role in parasite sequestration in the placenta as they do in other microvascular spaces (53, 54). Importantly, antibodies to RIFIN can clear parasites in malarial infection (55). In summary, these studies have demonstrated that elevated complement-fixing antibodies are associated with a reduced risk of placental parasitemia probably by enhanced inhibition of iRBC adhesion to CSA, thus the role of complement in protection against PAM deserve further investigations.

Identification of new vaccine targets other than VAR2CSA

Recent studies by genome-wide transcript analysis of *P. falciparum* isolates freshly collected from placenta identified other proteins that are upregulated during placental malaria (56). Thus, other parasite proteins beside VAR2CSA may contribute to the pathogenesis of malaria during pregnancy (26) and acquisition of antibodies against these proteins may contribute to protective immunity. For example, *P falciparum* chondroitin sulfate A ligand (PfCSA-L) is a highly conserved novel molecule expressed on the surface of iRBCs that binds placental CSA and is peripherally associated with the outer surface of knobs through specific protein-protein interactions with VAR2CSA (57). Discovery and characterization of additional antigens that interact with VAR2CSA is an area that deserves prioritization for vaccine development.

While direct detection of PfEMP1 on the surface of placental and CSA-adhering iRBCs by mass spectrometry has proved difficult owing to the low quantity of PfEMP1 in the iRBC membrane (58), several peptides were identified that corresponded to a highly conserved protein named PF3D7_0936900 (or PFI1785w) (59). PF3D7_0936900 and four other conserved genes including PFD1140w were identified in the transcription profiling study of placental parasites (60, 61). Antibodies to PF3D7_0936900 were significantly higher in *P. falciparum*-exposed women than men while seroreactivity profiles of PF3D7_0424000 (PFD1140w) were similar to those of VAR2CSA (62). Thus, these proteins are immunogenic and may be important for parasite development or adhesion in the placenta. It is therefore worth evaluating whether antibodies to these antigens can inhibit placental malaria infection (Table 1).

TABLE 1 List of proteins over-expressed in placental malaria associated parasites.

Gene ID	Gene name	Significance during pregnancy associated malaria	References
PF3D7_0936900	Plasmodium exported protein (PHISTb)	Peptides from this non-PfEMP1 conserved protein (PFI1785w), were exclusively identified in placental iRBC samples. Antibodies to this protein were observed to be significantly higher in *P. falciparum*-exposed women than men.	(59)
PF3D7_0202400	Translation-enhancing factor	Studies reveals a critical role this protein plays as a translation -enhancing factor in the production of VAR2CSA. Surface expression of this protein is unknown.	(63)
PF3D7_1001000	*P falciparum* chondroitin sulfate A ligand	Systematic immunoassay of various groups in East Africa revealed a significant seropositivity of the anti-PfCSA-L antibodies especially amongst multigravidas that inhibited this ligand from binding CSA.	(57)
PF3D7_0424000	Plasmodium exported protein (PHISTc)	Forms complex with PfEMP1, and seroreactivity profile is similar to that of VAR2CSA.	(62)
PF3D7_1201000	Plasmodium exported protein (PHISTb)	Localizes to the surface of iRBCs.	(64)

Immuno-epidemiological, functional studies and clinical trials in PAM vaccine discovery

A recent a systematic review of different epidemiological studies that assessed the relationship between pregnancy-specific malarial immunity and risk of malaria in pregnancy observed that prospective studies as well as standardized immunoassays are lacking (24). The analysis strongly suggested that to identify novel pregnancy-specific antigens, prospective cohort studies evaluating immune responses to many proteins in different epidemiological settings and throughout the gestational period using high-throughput, but standardized assays are needed. Antibodies to the identified antigens will then need to be evaluated using functional assays such as erythrocyte and CSA binding inhibition (anti-adhesion assay), iRBC cell-agglutination, phagocytosis, and complement fixation. The agglutinating and anti-adhesion assays are associated with reduced odds of placental infection (21), and the capacity of antibodies in pregnant women to opsonize phagocytosis CSA-binding iRBCs *in vitro* was observed (65) and can be explored for other antigens.

Flow cytometry-based assays have also been established with CSA-coated channels, CSA-expressing cell lines or primary trophoblast. In these studies, iRBCs flow through these channels and attach in settings that closely mimic blood vessels *in vivo* (66). Blocking these attachments correlate with the functional activities of the antibodies. Similarly, to recapitulate human placental organ physiology and function, the use of stem cell organoids and bioengineered placenta-on-a-chip models to construct biomimetic *in vitro* three-dimensional (3D) tissue or organ models warrant more consideration (67, 68). In this model, trophoblast cells and human umbilical vein endothelial cells are cultured on the opposite sides of a porous polycarbonate membrane allowing both maternal and fetal investigations. However, it remains unclear if these PAM-specific functional assays would correctly predict protective immunity in vaccine trials. In addition, the design of effective well-controlled studies with clear and globally acceptable endpoints need to be carefully considered, as recently proposed (69).

Leveraging wheat germ cell-free system and the mRNA-based vaccine approach to advance PAM vaccine research

The discovery of antigens that induce strain-transcending antibodies as well as new targets of complement fixing/activation antigen in PAM has been limited partly due to a lack of robust high-throughput screening platforms. This is hampered mainly by challenges associated with production of functional recombinant malaria antigens (70). However, the eukaryotic wheat germ cell-free protein system (WGCFS) overcomes these problems, as summarized by Kanoi et al., 2021 (71). In validation studies, WGCFS was used to generate more than 2500 recombinant proteins from various *P. falciparum* life cycle stages (72). This includes the synthesis of all high molecular weight VSAs including PfEMP1, SURFINs, RIFINs and STEVORs (73, 74). Similarly, more than 300 P. *falciparum* proteins were functionally annotated following generation of antibodies in immunized rabbits and mice, suggesting immunogenicity of the produced proteins (75).

The WGCFS technology has also been used to study vivax malaria in Thailand, Brazil and Papua New Guinea towards development of markers of recent infection. Antibody responses to 307 P. *vivax* recombinant proteins were measured at the onset of infection and at several points during the follow-up period (76). Antibodies with relatively longer-lived responses were

observed in PNG where individuals had greater past exposure to *P. vivax*. In subsequent independent studies, eight proteins with specificity for detecting recent infections were identified (77). These findings further support WGCFS as well as high-throughput screening platforms as rational tools for production, screening and characterization of functional recombinant proteins that can be explored for induction of protective antibodies against falciparum and non-falciparum malaria during pregnancy. Thus, coupled with the rapid advancement of bioinformatics tools, WGCFS could support identification of potential placenta-specific or placental-binding ligands that induce antibodies that abrogate parasites sequestration in the placenta (Figure 1).

We note that robust bioinformatics and artificial intelligence (AI) tools have been developed to support antigen selection, utilizing either sequence-dependent, structure-based, or ligand-based approaches (78). Such approaches, which have been applied in COVID-19 vaccine studies (79), combine multiple immuno-informatics approaches in integrated frameworks that include supervised machine learning. Recently, Rawal et al. used tools such as PsortB, WoLF PSORT, BLAST, HMMTop, ProtParam, FungalRV, NetCTL, VaxiJen 2.0, or IEDB tools to identify potentially immunogenic, stable and cross-protective antigens against Trypanosoma species (80). Other sequence-independent methods use auto cross-covariance (ACC), a mining method that transforms protein sequences into uniform equal-length vectors (81, 82). This has been extended to develop servers such as VaxiJen for the prediction of protective antigens (83). Such statistical approaches can be exploited in PAM to enhance the search for novel antigens.

Concurrently, numerous mRNA vaccine platforms have been developed and evaluated for immunogenicity (84–86). The ability to genetically modify the RNA sequences has hastened the production of translatable non-toxic synthetic mRNA and delivery systems (87). This approach was applied recently to the production of coronavirus vaccines by Moderna and Pfizer-BioNTech (88). These examples raise optimism for adoption of this technology in the production of vaccines for PAM.

Critical appraisal and summary

Understanding the complexity of naturally acquired immunity against PAM will inform antigen discovery and vaccine development. It is now clear that VAR2CSA is not the only antigen that is involved in PAM but there are other antigens that may be targets of protective immunity. With this knowledge, it is plausible to further query the plasmodium genome using emerging bioinformatics tools such as machine learning and artificial intelligence to identify possible conserved antigens that can be interrogated for development of broadly neutralizing vaccines. Application of eukaryotic WGCFS for generation of the recombinant proteins that induce immunogenic and protective antibodies need to be granted critical consideration. In addition, the advent of mRNA-based vaccine development platforms comes with high flexibility and rapid development while still inducing robust immune responses against specific targets. The mRNA technological approach is a great milestone for rapid *in vivo* production of full-length proteins and multivalent or multi-antigen vaccines. This has renewed the hope of quickly developing effective vaccines.

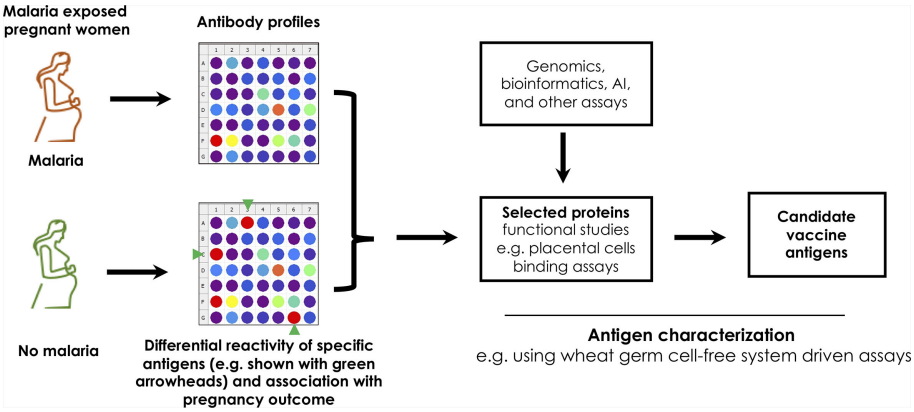

FIGURE 1
An illustration of an immunoscreening pipeline to identify targets of potentially protective antibodies from pregnant women with and without PAM. Unique proteins are represented by colored dots. The antigens may also be identified using complex genomic assays, statistical approaches such as artificial intelligence (AI), machine learning and expressed using robust platforms such as the wheat germ cell free system for functional studies.

Author contributions

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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