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RESEARCH ARTICLE

Targeting Plasmodium falciparum chondroitin sulfate a ligand: A highly conserved malaria antigen with potential for pregnancy-associated malaria vaccine development

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Abstract

Background

Pregnant women in malaria-endemic regions exhibit heightened susceptibility to *Plasmodium falciparum* infection due to accumulation of infected red blood cells (iRBCs) in the placenta. This is aided by the variant antigen 2 chondroitin sulfate A (VAR2CSA) protein which remains a target for pregnancy-associated malaria (PAM) vaccine studies. However, polymorphism in this antigen presents a significant challenge in developing broadly efficacious vaccines. This study explored PfCSA-L, a protein co-expressed and co-localized with VAR2CSA, as a potential alternative vaccine target due to its hypothesized role in PAM pathogenesis.

Methods

Sera and dried blood spots were collected from pregnant women attending antenatal care clinic at Webuye County Hospital, Western Kenya. *P. falciparum* infection status was confirmed by PCR. Recombinant PfCSA-L, expressed using a eukaryotic wheat germ cell-free system (WGCFS), was used to assess IgG antibody responses via ELISA.

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Results

We observed a statistically significant increase in IgG levels as gestation advanced, suggesting potential exposure-driven antibody maturation against PfCSA-L. Primigravida women exhibited a trend toward higher anti-PfCSA-L antibody levels compared to multigravida during the second visit, possibly reflecting a more vigorous immune response during the first pregnancy. Genetic analysis of field parasite isolates revealed a high conservation of PfCSA-L at both DNA and protein levels. B-cell epitope prediction identified potential targets on the PfCSA-L surface within the conserved region.

Conclusion

These findings, coupled with the observed IgG response, further positions PfCSA-L as a promising vaccine candidate for PAM. However, further investigations are warranted to elucidate the functional role of anti-PfCSA-L antibodies and definitively validate PfCSA-L and/or the identified epitopes as potential PAM vaccine targets.

Plain language summary

This is a research study that aims to provide more insights into understanding how a potential vaccine candidate termed PfCSA-L is (1) conserved at the protein level among *Plasmodium falciparum* isolates in Western, Kenya, (2) immunoreactive in sera collected from pregnant women from this region who were malaria positive. This is a strategy towards prioritizing this antigen as vaccine for pregnant women infected with malaria. This targeted antigen is a protein that *P. falciparum* parasite uses to adhere to molecules in the placenta and thus accumulate there, causing challenges for the mother and the child. To achieve these objectives, the protein was produced in the lab, purified, and used to test whether it could react with serum samples from this malaria-endemic region. It was expected that the sera from these women would potentially react with PfCSA-L, though it was not clear how reactivity for women who were pregnant for the first time compared to those who had more than one pregnancy would raise antibodies to PfCSA-L. Generally, this study reports that PfCSA-L is highly conserved and immunoreactive in serum samples from this region and, thus, can be studied further for vaccine development.

Keywords

Malaria in pregnancy, PfCSA-L, B-cell epitope, pregnancy associated malaria (PAM)

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Introduction

Individuals frequently exposed to *Plasmodium falciparum* infections develop semi-protective naturally acquired immunity (NAI), which reduces the risk of severe and symptomatic malaria disease[1]. However, pregnancy-associated malaria (PAM) remains a significant contributor to maternal and fetal morbidity and mortality in these malaria endemic areas, making pregnant women a considerable exception to the accrued benefits of the NAI[2]. According to WHO's 2023 World malaria report[3], various issues including climate change are impeding the global effort to eradicate malaria in pregnancy (MiP) with estimated cases standing at 12.7 million as of 2022. PAM is characterized by placental accumulation of infected red blood cells (iRBCs) which bind to chondroitin sulfate A (CSA)[4]. The pathogenesis of PAM is facilitated by a 350 kDa variant protein, VAR2CSA, expressed on the surface of infected red blood cells. VAR2CSA is a member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family and promotes parasite sequestration in the placenta by binding to placental chondroitin sulfate A[5–8]. Exposure to VAR2CSA triggers production of antibodies that prevent iRBCs adhesion to placental CSA and marking them for clearance by the immune system[9–11]. Antibodies specific to VAR2CSA are typically low in primigravid women but levels increase with gravidity[12–14]. Thus, PAM is less severe in subsequent pregnancies, suggesting that VAR2CSA remains a potential PAM vaccine candidate[15]. The ongoing VAR2CSA-based vaccine candidates (PAMVAC and PRIMVAC) for PAM are significant milestones for mitigating the adverse effects of placental malaria infection[16–18]. While both vaccine candidates illicited anti-VAR2CSA antibodies that effectively inhibited homologous VAR2CSA-expressing IRBCs from adheing to CSA, the antibody response was ineffective against parasite strains that were heterologous[19,20].

Emerging evidence suggests that the pathogenesis of PAM may involve parasite proteins beyond VAR2CSA. This is supported by the upregulation of multiple invariant genes in placenta infecting parasites[21–24]. Indeed, Keitany *et al.* identified a protein, *P. falciparum* chondroitin sulfate A ligand (PfCSA-L; PlasmoDB: PF3D7_1001000), co-expressed with VAR2CSA on the surface of infected red blood cells[25]. Notably, PfCSA-L exhibits nanomolar affinity for both placental CSA and VAR2CSA. Furthermore, they demonstrated that antibodies to this antigen may provide protection especially in multigravida women. Despite this reported immunological protection, acquisition of anti-PfCSA-L antibodies during gestation and in malaria exposed women of different gravidity remain inconclusive. Moreover, the mechanisms and timing of protective immunity conferred by antibodies targeting iRBC surface proteins, including PfCSA-L, in the context of placental malaria are poorly understood[26]. This knowledge, as well as the genetic diversity of any target molecule[27,28], will be critical for development of effective PAM vaccines.

This study aimed to characterize the antigenicity of PfCSA-L in the context of naturally acquired immunity against *P. falciparum* infection in pregnant women residing in a malaria-endemic region. By longitudinally assessing PfCSA-L IgG antibody responses across pregnancy trimesters, we demonstrated a progressive increase in antibody levels in the majority of study participants. Furthermore, our findings indicate a high degree of sequence conservation within the *pfcsa-l* gene among circulating parasite isolates (https://www.ebi.ac.uk/ena/browser/view/PRJEB46180).

Methods

Study design and ethics statement

This longitudinal cohort study was conducted among pregnant women aged 18–40 years undergoing routine antenatal care at Webuye County Hospital, Bungoma County, western Kenya between 2020 and 2021. Participants were enrolled in week 12–20 of gestation and followed approximately every 4 weeks until delivery. Venipuncture was performed with serum tubes (Becton Dickinson, Franklin Lakes, NJ) to collect 4 ml blood samples, which were subsequently centrifuged to obtain serum. Serum samples were stored at -80°C. Additionally, duplicate blood samples were collected on Whatman 903 filter paper (GE Healthcare, Cardiff, UK) as dried blood spots (DBS), desiccated, and stored at -80°C for future analysis[29]. The women who tested malaria positive with rapid diagnostic testing were treated in line with Kenya national malaria management guidelines[30,31]. Out of a total of 300 pregnant women enrolled at this site, samples from 80 randomly selected participants were analyzed in the current study.

Ethical clearance to conduct the study was obtained from the scientific and institutional review committee of Mount Kenya University (approval number 083 and 1099 dated 20th Aug 2019 and 10th Dec 2021, respectively). All study participants provided written informed consent before donating samples. Regarding the *P. falciparum* parasites sequencing, the study's approval was obtained from the Kenyatta National Hospital - University of Nairobi (KNH-UoN) ethical review committee (P609/10/2014 dated 21st Jun 2019). Written consent was obtained from all the participants or guardians, and malaria cases were treated per the national malaria guidelines.

DNA extraction

DNA extraction from DBS on filter paper was carried out according to the protocol by Simon *et al.*[32]. Briefly, 3 mm diameter size punched filters were soaked in 1 ml of phosphate-buffered saline (PBS, catalog number 20012027, Thermo Fisher Scientific™), incubated for 40 min at room temperature in a 1.5-ml tube and centrifuged at 10,600g for 3 minutes. After centrifugation, the supernatant was discarded, and 50 µl of chelex 20% (catalog number 11139-85-8, Sigma-Aldrich™) was added. The mixture was first incubated at 99 °C for 10 min, and centrifuged at 5000g for 2 minutes. The genomic DNA supernatant was transferred into a 1.5 ml tube and kept at −20 °C for further analysis.

Polymerase chain reaction (PCR) for *Plasmodium* species identification

Nested PCR was performed using rPLU5/rPLU6, and rFAL1/rFAL primer pairs (Table 1) to determine the *Plasmodium*

Table 1. Primers for nested PCR of 18S rRNA and *pfcsa-l* gene in malaria parasites.

Species	Primer	Sequence (5'-3')	Product size
18S rRNA _*outer*	rPLU5	CTTGTTGTTGCCTTAAACTTC	1200 bp
	rPLU6	TTAAAATTGTTGCAGTTAAAACG	
18S rRNA *inner*	rFAL 1	TTAAACTGGTTTGGGAAAACCAAATATATT	205 bp
	rFAL 2	ACACAATGAACTCAATCATGACTACCCGTC	
Pfcsa-l	Pfcsal_F	GGCGGATATCTCGATGTTGTTCTCTTTCAAATATATATATTTTTCGTC	693bp
	Pfcsal_R	CGGTACCCGGGGTTATATATCTATCTTCGTATTTTAAAAAATAAAAAGAATC	

genus and *P. falciparum* species, respectively. In both cases, the PCR mixture had a total volume of 25 µl, containing 1X buffer, 200 µM dNTPs, 10 µM of each primer, 2.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 2 µl of DNA solution. The thermo-profile consisted of an initial denaturation step at 94 °C for 5 min, followed by 25 cycles at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Amplicons were subjected to electrophoresis for approximately 40 min at 100 V on standard 2% agarose gels (catalog number 1613102, Bio-Rad™).

Production of PfCSA-L recombinant protein

Recombinant full-length PfCSA-L protein was synthesized using wheat germ cell-free system (WGCFS) as described[33]. Briefly, PfCSA-L sequence was amplified from *P. falciparum* 3D7 parasite isolates using specific primers (Table 1). The amplified DNA was restricted with XhoI and NotI restriction enzymes, ligated into the pEU-E01-HIS vector, and expressed as an N-terminal HIS fusion protein using WGCFS. The proteins were affinity purified using the nickel sepharose columns, separated in a 12.5% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue R-250 (CBB) (catalog number 6104-59-2, Sigma-Aldrich).The protein resolved at 27 kDa position relative to the protein marker), which was consistent with the expected band size as described elsewhere[25]. The protein was used for enzyme-linked immunoassays (ELISAs) assays.

Quantification of IgG levels by ELISA

Measurement of antibodies against PfCSA-L was performed as previously described with slight modification[34]. Briefly, 96-well enzyme-linked immunosorbent assay plates were coated with 50µl of 100 times diluted antigen per well in coating buffer (20 mM boric acid, pH 8.9, catalog number 10043-35-3, Sigma-Aldrich) and incubated at 4°C overnight. The plates were blocked with 2% casein (catalog number 9005-46-3, Sigma-Aldrich) in the coating buffer. The sera were diluted (1:600) in phosphate-buffered saline with 0.1% Tween 20 (PBS-T, pH =7.2) in duplicates and incubated for 1 h at 37°C. After the plates were washed, they were incubated with 100µl of 1:3000-diluted HRP-conjugated goat anti-human IgG (catalog number A18805, Thermofisher, polyclonal) in PBS-T for 1 h at 37°C. After that, they were incubated with 100µl of azino-bis-3-ethylbenthiazoline-6-sulfonic acid (catalog number 002024, Thermofisher™). The reaction was stopped with 0.1 M citric acid (catalog number 110450050, Thermofisher) and optical densities (ODs) were measured at 415 nm against a reference wavelength of 490nm using a precision microplate reader (Molecular Devices, Sunnyvale, CA).

B-Cell epitope prediction

Linear B-cell epitopes of the PF3D7_1001000 PfCSA-L protein were predicted using BepiPred 2.0 in IEDB (http://www.cbs.dtu.dk/services/BepiPred/; BepiPred 2.0, Immune Epitope Database and Analysis Resource, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) with a threshold of 0.55 (corresponding specificity > 0.817 and sensitivity < 0.292). Only the epitopes with more than 5 residues were considered for subsequent antigenicity analysis. Antigenicity was evaluated via the VaxiJen v2.0 (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) server online tool (The Jenner Institute, Oxford, UK).

Genetic diversity

The samples analyzed here for genetic diversity, involving *pfcsa-l, pfama-1, pfadsl and var2csa* genes, were a subset of samples obtained in a previous study in western Kenya[35,36]. These sequences are archived at the DDBJ BioProject, Accession number PRJDB12148, where up to 26 sequences of field isolates were analyzed. The isolates had passed quality checks as previously described elsewhere[35]. Briefly, the parasite DNA was extracted from archived whole blood samples from patients recruited for a drug resistance surveillance study in local hospitals on four selected islands (Mfangano, Takawiri, Kibuogi, and Ngodhe) in Lake Victoria, a coastal mainland (Ungoye) between **July 2014 and July 2016**[37,38].

Read mapping and coverage

Sequence reads were aligned against the *P. falciparum* 3D7 reference genome (version 8.1) (https://plasmodb.org/common/downloads/release-46/Pfalciparum3D7/fasta/data/) using Burrows-Wheeler Alignment tool (BWA)[39] (http://bio-bwa.sourceforge.net) with default parameters. The resulting alignment was further processed with Samtools (http://www.htslib.org/)[39] and Picard v1.66 (https://broadinstitute.github.io/picard/)[40] to

remove duplicates. SNPs were called using Genome Analysis Toolkit (GATK) (https://gatk.broadinstitute.org/hc/en-us) HaplotypeCaller with the following parameters – genotyping mode DISCOVERY–output mode EMIT_VARIANTS_ONLY, –stand_emit_conf 10 and –stand_call_conf 30. To improve the quality of variant calling, this study further discarded genotyping calls with coverage of <5 reads. The resulting variant call format (VCF) files were then merged into one file using VCF tools (https://vcftools.github.io/)[41].

Variant calling and analysis

The VCF file containing twenty-six samples that passed the quality test from read mapping analysis was used as the input file in VCF tools for variant analysis. High-quality SNPs in *pfcsa-l* were functionally annotated in the SNPEFF tool[42]. ARTEMIS software further analyzed Called variants[43]. MUSCLE tool was used to perform multiple sequence alignment and translation of nucleotide sequences to amino acid sequences. Gene variants were identified by aligning the amino acid sequence reads to their corresponding 3D7 reference gene sequence. To test the sensitivity of the above approach, the sequences were analyzed at different variant calling parameters to assess the impact of these settings on downstream analysis. Additionally, before variant calling, base quality score recalibration was performed to adjust the base quality scores of sequences reads as well as local realignment around indels to reduce false-positive variant calls resulting from alignment artifacts. The aligned sequences by MUSCLE software were subjected to genetic diversity determined by parameters, such as segregating sites (S), the average number of nucleotide differences at each position between two sequences (Pi), the number of haplotypes and TajimaD values. Tajima D and **Pi (π)** values were calculated in AlignStatPlot software[44]. D and π values were plotted against the mid-point of the window length. AlignStatPlot, An R package and online tool for robust sequence alignment

Results

Characteristics of study population

The recruited and sampled participants (n=78) were either primigravida (46%), secundigravida (44%), or tertigravida (10%) women (Table 2). All deliveries were term at 39–40 weeks of gestation. All neonates were born alive and in good health. Serum samples assayed were those collected at three time points, each approximately coinciding with a trimester. The number of assays performed varied by visit and gravidity, depending on the availability of sufficient sample volume. Malaria infection was detected in 23% of the pregnant women by PCR with previous history of malaria exposure.

Anti-PfCSA-L antibodies levels increased with developing pregnancy

To assess if PfCSA-L was naturally immunogenic in the study population, we evaluated the immunoreactivity of human antibodies to recombinant PfCSA-L using serum samples from the study region. In general, antibodies to PfCSA-L were significantly (P<0.05) elevated in pregnant women as the pregnancy developed (Figure 1). There was a significant increase in anti- PfCSA-L antibodies in all periods corresponding to first, early second and third trimesters of pregnancy, respectively, (P<0.05) suggesting that there was an increased exposure to parasites expressing this protein.

To further understand how immunoreactivities to PfCSA-L associated with gravidity, antibodies levels in women of different gravida across the follow up period were assessed. In the first visit (representing first trimester), we observed high levels of antibodies in secundigravida women compared to primigravida women (P>0.05), with non-significant lower levels in tertigravida (Figure 2A). On the second visit, primigravida women exhibited non-significantly higher antibody levels compared to other populations, while no difference was observed among all groups in the third visit (Figure 2B, C). This suggested that, although all women responded to possible exposure the PfCSA-L, primigravida women had a stronger humoral response as compared to other groups.

Trends in IgG levels per visit, individual, and gravidity

To understand acquisition or boost of immunity during PAM, we assessed the kinetics of PfCSA-L IgG antibody responses. The analysis revealed a general pattern of increasing immunoreactivity during the first, second and third visits for all the gravida (P<0.05) except for the tertigravida women (P>0.05) (Figure 3). This could suggest immune boost effect following natural infections.

pfcsa-l is highly conserved as compared to *var2csa*

Since PfCSA-L is immunogenic, with antibodies potentially offering protection, making it a suitable target for vaccine development[25], we investigated the genetic polymorphism

Table 2. The demographic characteristics of the assessed samples.

Characteristics	Primigravid (n=36)	Secundigravid (n=34)	Tertigravid (n=8)	*P* value*
Age, years median (IQR)	25(6)	24.5 (7.25)	27 (3)	0.5033
PCR (x/n) positive	7/25	5/26	(1/5)	
Hemoglobin, median (IQR), g/dL	13.2 (1.1)	12.6 (3.125)	14.6 (2.25)	0.07757
Anti- PfCSA-L at enrolment (Mean, sd*)	0.5501 (0.3757)	0.6788 (0.3932)	0.6577 (0.7782)	0.3409

*Statistically significant between the groups (p < 0.05; Kruskal-Wallis followed by Bonferroni–Dunn post hoc test)

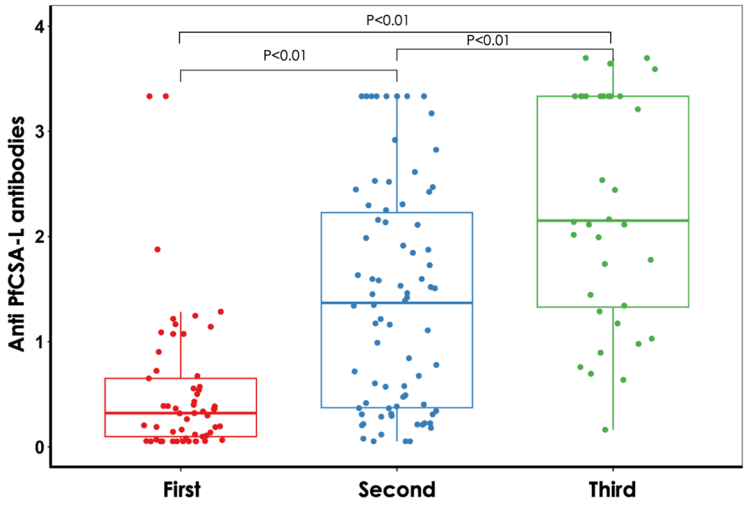


Figure 1. Levels of antibodies to PfCSA-L across trimester 1, 2 and 3 in the gestation period.

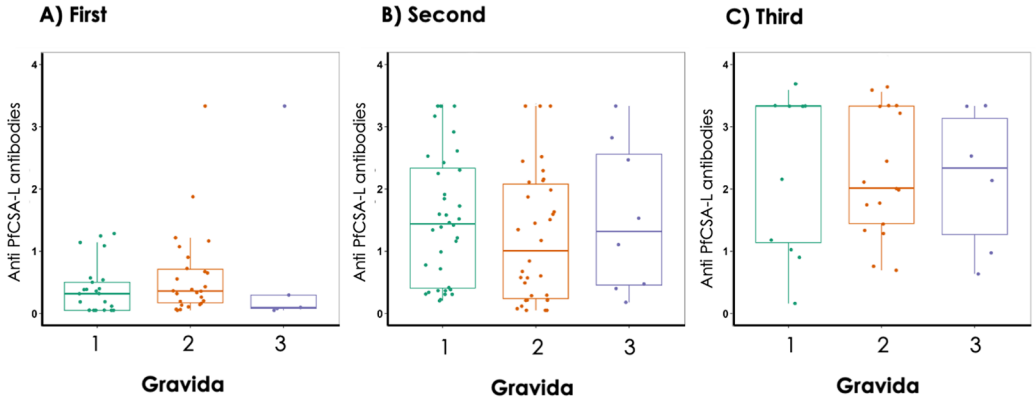


Figure 2. PfCSA-L antibody levels in first-, second-, and third-time pregnant women during each pregnancy trimester.

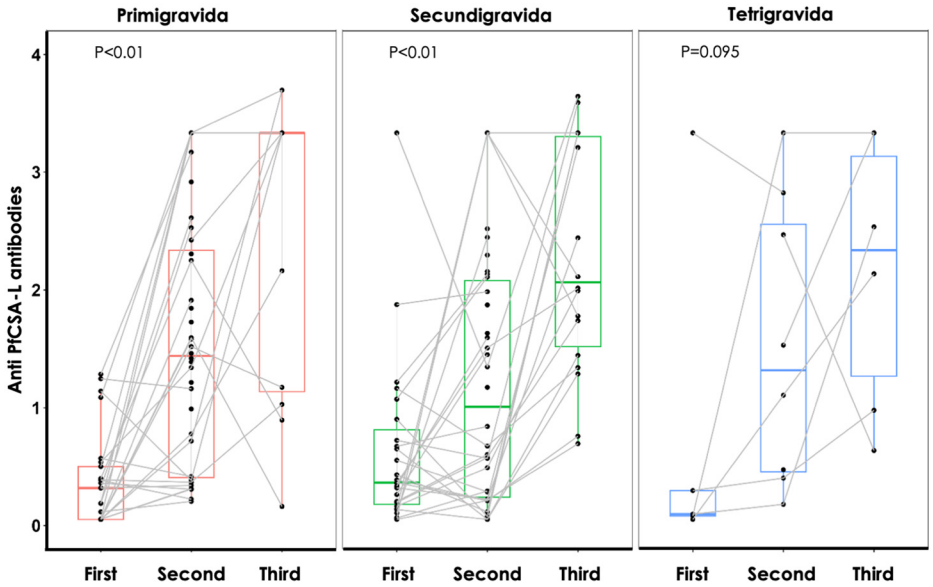


Figure 3. Antibody response trends in primigravida, secundigravida, and tertigravida women across pregnancy trimesters.

of *pfcsa-l* in parasites circulating within Western Kenya, a region highly endemic for malaria. Field isolate sequences of *pfcsa-l* and other control genes were successfully mapped to the reference genome of *P. falciparum* 3D7. Following quality control, 26 nucleotide sequences were analyzed for genetic polymorphisms. To assess diversifying selection, the neutrality measure was calculated using a sliding window approach for nucleotide diversity (π) and Tajima's D. We observed a negative neutrality summary statistic for *pfcsa-l*, with a Tajima's D of -1.73363 and a π of 0.00033 (Table 3) with only 2 singleton mutations across its 696bp sequence. This indicates that the *pfcsa-l* gene is well conserved compared to the highly polymorphic gene *pfama1* and the target of the most advanced PAM vaccine candidate, *var2csa*. In comparison to *pfcsa-l,* and *pfads-l*, a conserved house-keeping gene, *var2csa* displayed the least conservation, with a Tajima's D summary value of 0.27733 and a π of 0.06084 (Figure 4 and Figure 5). While Fu & Li's statistics were significant for the *pfcsa-l* gene ($p<0.05$), they were not significant for *var2csa.* These findings suggest that PfCSA-L is either not under strong immune pressure from the human host or is conserved due to its essential role as a ligand for parasite development. Analysis of secondary sequences retrieved from the PlasmoDB database (https://plasmodb.org/plasmo/app) using predefined selection criteria identified no single nucleotide polymorphisms (SNPs) within the 349 deposited Plasmodium isolates.

B cell epitope prediction and antigenicity

Following the observation that PfCSA-L induces a strong IgG response and is highly conserved in field parasite isolates, suggesting its importance in naturally acquired immunity, we sought to identify B-cell epitopes that can be targeted for vaccine development. An exploratory *in silico* analysis was conducted using online open-source software bepiPred v2.0 () with a threshold score of 0.55 to predict the B cell linear epitopes; the generated peptides with higher scores above the threshold were considered. The input file was a FASTA file sequence of PF3D7_1001000 strain of PfCSA-L. Leveraging the software capabilities, this study only predicted linear epitopes, acknowledging that antibodies can react with both linear and conformational epitopes. Those that met the threshold and had more than five (5) amino acid residues were considered for further antigenicity analysis as described elsewhere[45]. Six (6) epitopes that resided within the conserved region of the gene were predicted with their corresponding positions in the protein sequence shown in Table 4. The antigenicity of these epitopes was predicted using vaxijen v2.0 http://www.jenner.ac.uk/VaxiJen, and a default 0.5 threshold setting yielded two epitopes (Table 4).

Discussions

Despite multiple studies demonstrating acquisition of immunity against malaria during pregnancy, the specific mechanisms

Table 3. Fu & Li's neutrality tests statistics based on the Lake Victoria sample population.

Gene	No. of samples	S	Fu and Li's D*	Fu and Li's F*
pfcsa-l	26	3	-2.58495*	-2.70880*
Pfadsl	26	3	- 0.2162*	3.98709
var2csa	26	530	0.53538	0.53259

S- Segregating sites, Statistical significance, Asterisk* indicates significance, P < 0.05

Table 4. B cell epitope prediction/Antigenicity.

Protein position	Epitope	Antigenicity
25–30	KYIQNF	-0.5663
38–63	**NEKCSSKYIRLISEQTSDESTSKSIK**	**0.8129**
66–165	**KCVNKRNNKNEDNHELQSKSRSLKGTELDVTSYNKSKHTQQNNLGDSLK RTKNNITNISSKKNETYLFDENTNEYKLRNTLRLMNERYADSIVDTMDLND**	**0.7173**
182–193	ENPDKQRKLYEQ	0.3859
198–203	IEKYKK	0.2006
215–227	NIYDSFYFLKYED	0.4868

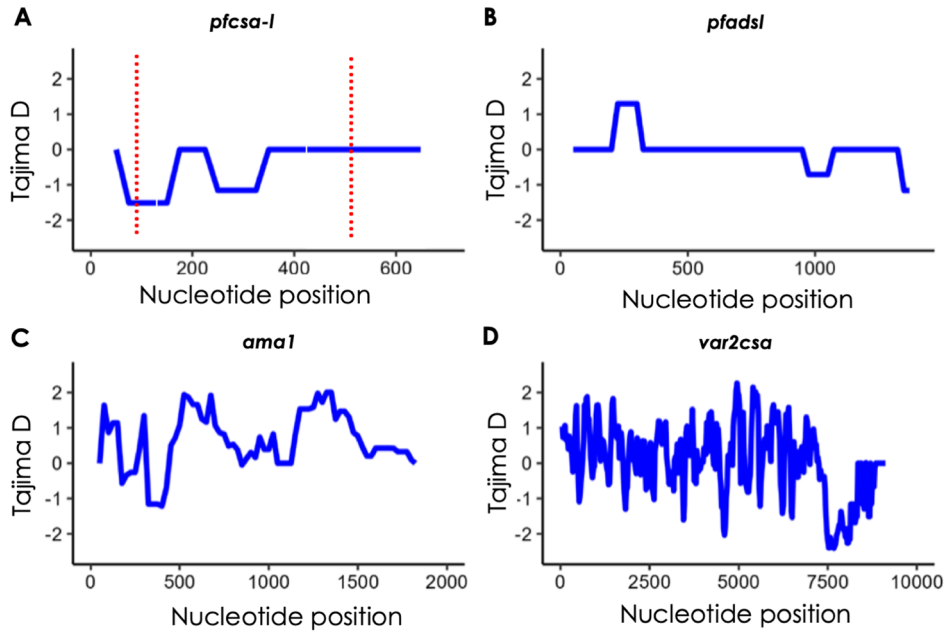


Figure 4. Tajima's D sliding window analysis of study genes. PfCSA-L: highlighted region indicates identified epitope.

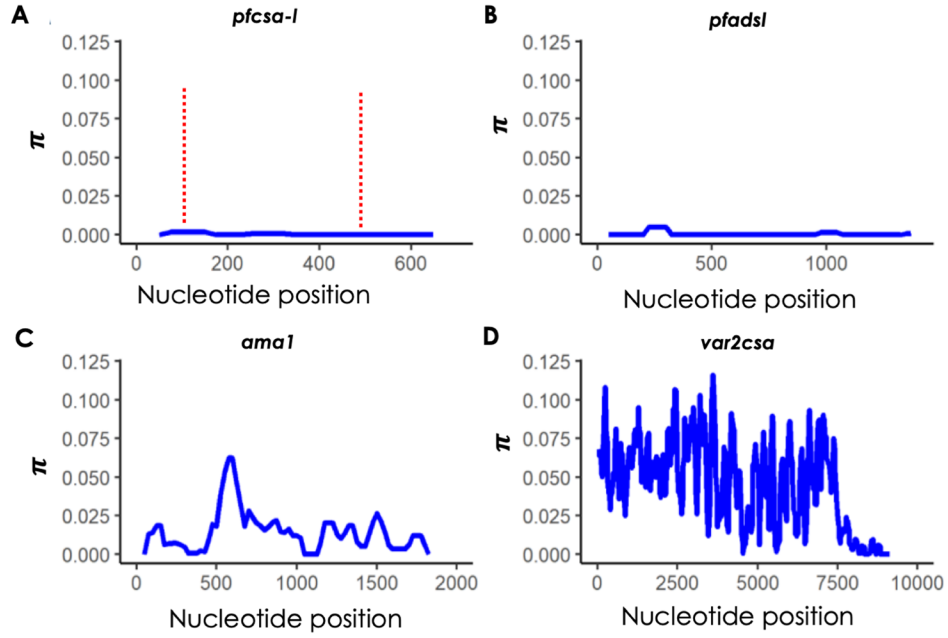


Figure 5. Nucleotide diversity (π) analysis comparing genetic diversity of three genes in Lake Victoria samples.

against adverse pregnancy outcomes, and the key targets of protective antibodies remain unresolved[46–48]. Emerging evidence suggests that the pathogenesis of PAM may involve parasite proteins beyond the classical VAR2CSA. Recently, a ligand PfCSA-L was identified as co-expressed with VAR2CSA on the surface of infected red blood cells with anti-PfCSA-L antibodies blocking parasite cyto-adherence. This puts PfCSA-L as potential vaccine candidate. This study, aimed at understanding, the critical gap as to when and how acquisition of anti-PfCSA-L antibodies occur during gestation and in women

of different gravidity. Consistent with previous studies, we observed that PfCSA-L is highly immunogenic in malaria natural infections. We also observed a significant increase in antibodies against PfCSA-L in primigravidae during the second visit corresponding to the second trimester. Consistent with other studies targeting PfEMP1 domains[49], primigravida women had a robust humoral response as compared to other groups. Other studies have also shown that both primigravidae and multigravidae have antibodies at term, though levels increases with increasing gravidity, a phenomena which has been attributed to the gravidity-dependent protection against the development of severe placental malaria[50–52]. A further comparison of this data[50–53], however, showed that a significant number of primigravidae had high levels of anti-C4S adhesion antibodies. This might escalate the unknown reason why primigravidae are more susceptible to placental malaria.

Polymorphisms in vaccine candidates have been a major hurdle in developing effective malaria vaccines. This has consistently resulted in strain-specific immune protection and failed field trials[54]. Here, PfCSA-L is demonstrated as a highly conserved, with only limited polymorphisms observed in Kenya field isolates here and other settings. Specifically, *pfcsa-l* gene displayed low nucleotide diversity and a negative Tajima's D value, suggesting an excess of rare variants and potential balancing selection against polymorphisms[55]. Similar negative values were observed for Fu and Lis' D* statistics. These findings are consistent with previous observations investigating leading and conserved blood-stage vaccine targets, PfP113 and PfRipr[55]. This makes the molecule highly susceptible to cross-strain neutralizing naturally acquired and vaccine-induced antibodies. The population-specific investigations reported here, with other studies from different malaria transmission settings, will inform design of effective vaccine for Kenyan population.

When selecting an antigen for inclusion in a multi-component malaria vaccine, it is crucial to consider the location of epitopes within the protein, particularly in conserved regions or intrinsically unstructured regions (IURs), as IURs are key targets for the host immune system, including antibody recognition[56,57]. A significant finding of this study is the identification of B-cell epitopes within PfCSA-L conserved regions. Future studies are therefore needed to assess the inhibitory activity of antibodies to these epitopes through functional assays to determine their biological and immunological relevance or as potential candidates for a PfCSA-L-associated multi-component vaccine. Estimating the decay rates of antimalarial antibody responses especially before and during pregnancy need to assessed[58].

In summary, the data presented herein demonstrate the high conservation of PfCSA-L among field isolates, the presence of multiple B-cell epitopes within conserved regions, and its immunogenicity in natural malaria infections, particularly during the first trimester of pregnancy. These findings suggest that PfCSA-L could be a promising vaccine candidate for protecting primigravid women, who are at heightened risk of malaria-related complications. While further research is needed to validate its efficacy and safety, the results of this study support the continued investigation of PfCSA-L for inclusion in malaria vaccine strategies aimed at preventing maternal and fetal morbidity.

Data availability

Underlying data

Zenodo: Targeting *Plasmodium falciparum* chondroitin sulfate a ligand: A highly conserved malaria antigen with potential for pregnancy-associated malaria vaccine development, **https://doi.org/10.5281/zenodo.13868368**

This project contains following data:
PfCSA-L_Raw_Data.xlsx

Data is available under the license CC4.0

Accession numbers

European Bioinformatics Institute (EBI), Sequence Read Archive (SRA): Genomic data for *Plasmodium falciparum* from Lake Victoria; Accession number PRJEB46180; https://www.ebi.ac.uk/ena/browser/view/PRJEB46180.

Software availability

All statistical analyses were performed with R version 4.3.3. Optical densities (ODs) were normalized with a 5-parameter logistic curve relative to the standard curve generated on the same plate. Continuous variables were compared using the non-parametric Kruskal–Wallis test.

Author contributions

BK and JG conceived and designed the study; JG provided the analyzed whole genome sequences, facilitated and supervised the design of protocols for participants' enrollment; AR performed the experiments, including the bioinformatics and statistical analysis. ET provided the recombinant proteins, other reagents and technical advice. AR and BK wrote the first draft of the manuscript, and the final version accommodated edits from all authors. All authors read and approved the final manuscript.

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?

Jeroen Bok
Radboud University Medical Center, Nijmegen, The Netherlands

In this manuscript, the authors investigate the potential of PfCSA-L as a target for a pregnancy-associated malaria vaccine. They present serological data from pregnant women in malaria-endemic Western Kenya and analyze the protein's nucleotide sequence using bioinformatics methods. Their findings indicate increasing levels of immunoreactivity over the course of a pregnancy and demonstrate a high degree of conservation of PfCSA-L at both the DNA and protein levels. These findings are interesting and relevant, but I do have several comments. As my expertise does not extend to bioinformatics, my remarks are limited to the sero-epidemiological aspects of this study. The following points require attention before the manuscript can be approved.

Major points

1. PCR positivity

The manuscript reports that malaria infection was detected by PCR in 23% of the pregnant women. However, Table 2 suggests that PCR analysis was conducted on only a subset of the 78 participants. The authors are encouraged to clarify the availability of samples and specify which samples were analyzed (i.e. at which timepoints they were collected). Additionally, it would be valuable to further investigate the immune response by comparing PCR-positive and PCR-negative individuals.

2. Data presented in figures

It would enhance the utility of Figures 1, 2, and 3 if the authors could include the corresponding numerical data in the main text—either as means with standard deviations or medians with interquartile ranges—for each of the depicted antibody levels.

3. Immunoreactivity in relation to gravidity

The authors present a general trend of increasing immunoreactivity over the course of pregnancy trimesters. However, an increase in immunoreactivity with gravidity— as generally observed for VAR2CSA—was not noted. While this point is briefly addressed in the discussion, a more detailed exploration would add strength to the manuscript.

Could the authors elaborate on gravidity-dependent protection against placental malaria in light of the current findings? Additionally, it remains unclear how elevated levels of anti-C4S adhesion antibodies are associated with increased susceptibility to placental malaria in primigravidae. Further clarification on this point would be appreciated.

4. General discussion and study limitations
The discussion would benefit from a more thorough evaluation of the functional activity of PfCSA-L antibodies. While the manuscript demonstrates that PfCSA-L is highly immunogenic during pregnancy and references a study showing molecular interaction with CSA, it remains unclear whether there is any direct evidence that PfCSA-L antibodies can inhibit the binding of Plasmodium falciparum-infected erythrocytes to CSA —i.e. whether they possess functional blocking activity.

Furthermore, the role of PfCSA-L as a vaccine candidate should be discussed in the context of existing VAR2CSA-based vaccine candidates. Would PfCSA-L serve as a potential replacement, or could it be used in combination with VAR2CSA to enhance vaccine efficacy?

Finally, a dedicated paragraph addressing the limitations of the study should be included in the discussion section to provide a more balanced interpretation of the findings.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Placental malaria, clinical epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 07 May 2025

https://doi.org/10.21956/openreseurope.20096.r53353

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?

Prasun Kundu
Department of Clinical Medicine, Cambridge Institute for Medical Research, Cambridge, UK

This manuscript presents a study on Pregnancy-Associated Malaria, aiming to evaluate PfCSA-L as a potential vaccine candidate. The authors assessed antibody responses to recombinantly expressed PfCSA-L using sera from pregnant women aged 18–40. They report that PfCSA-L is conserved at the nucleotide levels and use in silico tools to predict potential epitope regions. Their findings suggest a positive correlation between antibody levels and gravidity, indicating the immunological relevance of this antigen in naturally exposed individuals.

Here are some points that will need addressing before it may get indexed.

Major Points

1. Signal Peptide in Construct Design

The gene sequence of *PF3D7_1001000* includes a predicted signal peptide at the N-terminus. Based on the information provided, it appears that the primers used were designed from the start of the coding region, meaning the signal peptide was likely retained in the expressed construct. Could the authors clarify the rationale for this design choice? In many expression systems, inclusion of the native signal peptide can affect protein folding, secretion, or expression levels, and it would be helpful to understand whether this was intentional or overlooked.

2. Clarification on Reverse Primer Design

Could the authors please clarify the origin and purpose of the bolded sequence in the reverse primer (*Pfcsal_R*: **CGGTACCCGGG**GTTATATATCTATCTTCGTATTTTAAAAAATAAAAAGAATC)? This region does not appear to match the *PfCSA-L* genomic sequence, and its inclusion requires explanation—particularly whether it represents a cloning site, adapter sequence, or linker added for expression purposes.

3. Protein Purity Assessment

It would strengthen the manuscript if the authors could include an SDS-PAGE image showing the purification profile of recombinant PfCSA-L. This would allow readers to assess the purity and integrity of the expressed protein, which is important for interpreting the downstream antibody binding and immunogenicity data.

4. Epitope Prediction

The epitope prediction section appears to be the weakest part of the manuscript, as it relies entirely on in silico predictions without any experimental validation. Additionally, using only a single prediction tool (BepiPred) limits the robustness of the analysis. While the authors acknowledge the limitation of focusing solely on linear epitopes, it would have strengthened their

claims if they had at least mapped the predicted epitopes onto the AlphaFold structural model of PfCSA-L to assess whether these regions fall within well-modeled and surface-exposed areas. Incorporating predictions from multiple tools or structural mapping would add credibility to this analysis.

5. ELISA Controls and Quantification
It is unclear how the authors accounted for background signal in the ELISA assays. From the Methods section, it appears that neither positive nor negative control sera were included. Could the authors clarify how they quantified antibody levels in the absence of reference standards, and what criteria were used to define a positive response? Additionally, it would be important to understand whether any steps were taken to assess or rule out cross-reactivity with other *Plasmodium* antigens or unrelated proteins, which could impact the interpretation of the antibody binding results.

6. Figure Legends
The current figure legends are very brief and lack sufficient detail. It would be helpful if the authors could expand the legends to provide more context and clearly explain what each figure is showing, including relevant experimental conditions, sample information, and key findings. This would enhance clarity and allow the figures to stand on their own for the reader.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plasmodium vivax Blood-stage malaria, Vaccine target screening and prioritisation, Protein biochemistry, Structural biology, Genetic editing using CRISPR/Cas9 in Plasmodium knowlesi

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.