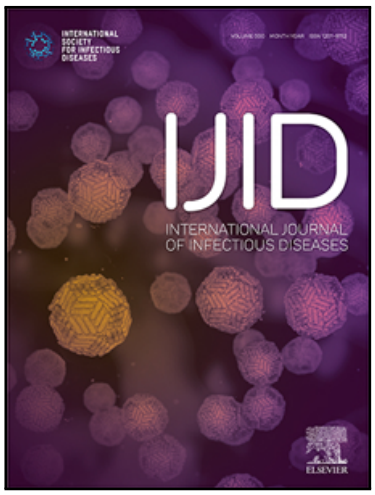


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# Structural insights into global mutations in the ligand-binding domain of VAR2CSA and its implications on placental malaria vaccine

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## Highlights

- Plasmodium falciparum VAR2CSA adheres to CSA on placenta causing placental malaria
- Three-dimensional structure of CSA-VAR2CSA complex revealed 23 CSA binding residues
- Analysis of VAR2CSA global field isolates shows polymorphisms in binding regions
- Structural mapping of mutations suggests major alterations in CSA binding surfaces
- The variants highlight concerns for vaccine development based-on VAR2CSA

## Abstract

Placental malaria is a public health burden particularly in Africa as it causes severe symptoms and results in stillbirths or maternal deaths. *Plasmodium falciparum* protein VAR2CSA drives placental malaria (PM) in pregnant women by adhering to chondroitin sulfate A (CSA) on the placenta. VAR2CSA is a primary vaccine candidate for PM with two vaccines based

on it already under clinical trials. The first cryo-EM three-dimensional structure of *Pf* CSA-VAR2CSA complex revealed crucial interacting residues considered to be highly conserved across *P. falciparum* strains. In the current study, we have conducted a global sequence analysis of 1,114 VAR2CSA field isolate sequences from more than nine countries across three continents revealing numerous mutations in CSA-binding residues. Further, structural mapping has revealed significant polymorphisms in the ligand binding surfaces. The variants from this limited set of 1,114 sequences highlight the concerns that are vital in current considerations for development of vaccines based-on VAR2CSA for placental malaria.

**Keywords:** Placental malaria; VAR2CSA; Placental malaria vaccine; field isolates, sequence analysis, structural mapping



Placental malaria affects susceptible pregnant women and is a major health problem especially in sub-Saharan Africa (World Health Organization, 2019). Among all Plasmodium spp., *Plasmodium falciparum* possesses the exceptional ability to sequester in the placenta of pregnant women wherein infected red blood cells bind to the syncytiotrophoblast (cells that line the maternal blood spaces of the placenta) causing maternal anaemia, stillbirth or low birth weight in offspring (Desai et al., 2007, Steketee et al., 2001). The parasite protein VAR2CSA, a member of PfEMP1 family (erythrocyte membrane protein 1) mediates this sequestration process by adhering to chondroitin sulfate A (CSA), a glycosaminoglycan which is heavily expressed on syncytiotrophoblasts (Duffy & Fried 2003, Clausen et al.,

2012). As women acquire immunity against placental malaria in subsequent pregnancies, VAR2CSA is a primary candidate for a vaccine for placental malaria (Duffy & Fried 2003, Gamain et al., 2021). Structurally, VAR2CSA ectodomain is comprised of an N-terminal sequence (NTD), six Duffy-binding-like (DBL) domains and variable interdomain (ID) regions (Fig 1a-b) (Ma et al., 2021). Two vaccines derived from VAR2CSA - PRIMVAC and PAMVAC that span the CSA-binding regions (DBL1X, ID1, DBL2X, ID2a) are presently in phase I/II clinical trials and their safety and immunogenicity has been reported (Gamain et al 2021). PRIMVAC, based on 3D7 strain, spans DBL1X, ID1 and DBL2X and PAMVAC, based on FCR3 spans ID1, DBL2X and ID2a – making ID1 and DBL2X as the shared region between them (Gamain et al., 2021).

The first cryo-EM structure of *Pf* VAR2CSA from N54 strain in complex with CSA has revealed 22 residues that are involved in the receptor-ligand interactions (PDB ID 7JGH) (Ma et al., 2021). Interestingly, these residues span across NTS, DBL2X and DBL4ε domains, and maximum interactions of CSA were observed with DBL2X (13 of 23), followed by DBL4ε (7) and NTS (3). In earlier studies DBL2X, DBL3X and ID1 and ID2 have been considered the major interacting domains (Clausen et al., 2012; Gamain et al., 2021) and also, DBL4ε domain is not considered in the current VAR2CSA-based vaccines (Gamain et al., 2021). Sequence comparison of 14 *P. falciparum* strains of VAR2CSA (including NF54) suggested that CSA-binding residues spanning the binding region are highly conserved (Ma et al., 2021).

This work expands on the sequence analysis from 14 strains to 1,114 field isolates and reveals that the inherent sequence variations in CSA binding residues of VAR2CSA may be significant. Field isolates collected worldwide from pregnant woman and placental samples

are a valuable resource to further elucidate and validate the conservation of CSA-binding residues. We searched the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/protein) using the keyword 'VAR2CSA' and this resulted in 2,255 VAR2CSA sequences of field isolates from studies conducted worldwide in the past. The final dataset consists of 1,114 DBL2X partial sequences with or without NTS domain containing at least one or more CSA interacting residues, from independent studies conducted in more than nine countries across three continents in the past two decades (Doritchamou et al., 2015, Rajwani et al., 2017, Verity et al., 2018, Salanti et al., 2003, Patel et al., 2017, Doritchamou et al, 2019; Bockhorst et al., 2007, Rovira-Vallbona et al., 2013, Sander et al., 2009, Fried et al., 2018, Huynh et al., 2011, Madanitsa et al., 2011, Magistrado et al., Tuikue et al., 2008). The blood samples were collected from pregnant women and placental isolates during delivery and from children up to 5 years of age. It is noticeable that no sequences were available for DBL4ε; also, both the current VAR2CSA-based vaccines under development do not include DBL4ε (Gamain et al., 2021). An earlier study had analysed data of field isolate gene sequences from Malaria Genomic Epidemiology Network (MalariaGEN; www.malariagen.net) and displayed sequences clusters into clades, with differing representation of populations and geographical regions (Benavente et al., 2018). The objective of the present study is to focus on understanding the CSA-binding potential of VAR2CSA with respect to amino acid mutations that are found in field isolates.

Sequence analysis of CSA-binding residues [16 residues across NTS and DBL2X confirmed by PISA (www.ebi.ac.uk/pdbe/pisa/), PLIP (www.plip-tool.biotec.tu-dresden.de/plip-web/plip/index) and PDBsum (http://www.ebi.ac.uk/pdbsum/)] was done using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) and structural analysis was depicted using Pymol (www.pymol.org). Sequence analysis results clearly show that 4 residues (K48, N557,

K835 and K561) out of these 16 undergo variation in both field isolate(s) and parasite strains (NF54, FCR3, 7G8, HB3, CD01, Dd2, GA01, GB4, KE01, KH01, ML01, SN01, TG01 and GN01) (Ma et al., 2021) (Fig 1-3) Additionally, 7 out of 16 residues exhibit unique variation exclusively in field isolate(s) (Fig. 1-3). Furthermore, structural mapping of these mutations revealed that four crucial DBL2X residues and their mutations which are seen only in field isolates (R846Q, K828M, K562Q, Q832K) can significantly alter interaction with CSA by disrupting H-bonds and salt bridges (Fig. 2a-d) as well as by changing the electrostatic surfaces of the binding site (Fig. 2e). Thus these four mutations (Fig. 2a-e) may potentially effect the binding affinity. The binding site is further altered by other mutations present either both in the above 14 strains and field isolate(s) – K48D, N557I/K/Y, K835M, K561G (Fig 1-3) or a different mutation in field isolate(s) – Y44S, A822P (Fig 1a, 3). Further, with reference to Benavente et al 2018, variant analysis of data from MalariaGEN Pf3k project (www.malariagen.net/parasite/pf3k) and MalariaGEN *Plasmodium falciparum* Community Project (www.malariagen.net/apps/pf/4.0/) revealed two mutations in DBL4ε - H1782R/N in the East Africa region. Since H1782 makes a h-bond with CSA, the mutation of H-to-N has the potential also to affect binding affinity.

Geographical distribution of mutations shows that at least two or more of these NTS and DBL2X mutations are prominent in three continents including eight in Africa (Benin, Congo, Ghana, Malawi, Mali, Mozambique, Senegal and Tanzania) (Doritchamou et al., 2015, Verity et al., 2018, Salanti et al., 2003, Patel et al., 2017, Doritchamou et al., 2019, Rovira-Vallbona et al., 2013, Sander et al., 2009, Fried et al., 2018, Huynh et al., 2011, Madanitsa et al., 2011, Magistrado et al., Tuikue et al., 2008), northern tip of South America (Colombia) (Rajwani et al., 2017) and from undisclosed Asian location (from South Asia) (Bockhorst et al., 2007) (Fig 3a-b). Concurrent presence of at least two or more of these mutations in a single field

isolate(s) suggests a wider disruption of the CSA-binding site. High frequency variation, defined as a mutation seen overall in more than 5% of the total field isolates in the dataset, was observed in 9 out of total 11 mutations within NTS and DBL 2X CSA-binding residues in field isolate(s). In addition, a higher rate of mutation was observed in interacting residues NTS Y44, K48 and DBL2X N557, K828, K835, R846 and K561 (Fig. 3a-b). Mutations in K828, Q832 and R846 were observed exclusively in Benin, a malaria endemic country with high mortality in children under 5 years of age (WHO, 2018); while K562Q mutation was observed in Senegal and Tanzania (Sander et al., 2009, Tuikue et al., 2008, Magistrado et al., 2008). It is interesting to note that three out of four important mutations co-exist in field isolate(s) from Benin reaffirming that the CSA-binding site may be significantly altered (Fig 2a-e) (Doritchamou et al., 2015).

VAR2CSA DBL2X contains five cysteine disulphide bonds which are highly conserved in *Pf* strains and play an important structural role (Higgins 2008, Singh et al., 2006, Chitnis and Sharma 2008, Gill et al., 2009, Yogavel et al., 2018). Further analysis of field isolate(s) showed variation in C643, C745 and C769 (Fig 4), thus 2 out of 5 disulphides – C643-C745 and C769-C901 are not conserved exclusively in field isolate(s) (Fig. 4). Interestingly, C745S was a common mutation from the seven locations in six studies (Benin, Congo, Malawi and Benin, Mozambique, Senegal and Tanzania) where mutations in cysteines were identified (Fig. 4). Benin and Mozambique showed mutation in at least 2 cysteines (C745 and C643/C769) thus altering both 2 disulphides – C643-C745 and C769-C901. Further, in reference to the Benavente et al 2018, variant data analysed from MalariaGEN *Plasmodium falciparum* Community Project (www.malariagen.net/apps/pf/4.0/) revealed mutations in cysteines in DBL4ε - C1902Y, C1670R, C1614Y/R – which can alter three disulfides (C1891-C1902, C1670-C1777 and C1558). Variability in disulphides in DBL2X and DBL4ε

core structure suggests a possibility of conformational flexibility which could then have implications on the full-length structure. It is reasoned that "major conformational change is not prerogative for CSA-binding" as overall structural similarity has been shown between at least two strains - NF54 and FCR3 (Ma et al., 2021). However, given the mutations in structure-stabilizing cysteine residues, conformational changes may indeed be at play in some variants. Earlier studies have shown that DBL1X to ID2a is sufficient for binding to CSA, although full-length VAR2CSA (ectodomain) binds to CSA with higher affinity (Clausen et al., 2012, Srivastava et al., 2010). The current vaccine candidates for placental malaria are based on 3D7 and FCR3 strains. The FCR3 strain has shown to have structural similarity with NF54 strain which is isogenic with 3D7 (Ma et al., 2021, Gamain et al., 2021). However, inherent sequence polymorphisms in VAR2CSA raise concern in regards to selecting any one strain as a vaccine candidate.

**Conclusions**

We have conducted the global sequence analysis of VAR2CSA based on 1,114 field isolates from more than nine countries across three continents. Although Benavente *et al* 2018, analysed the global diversity of VAR2CSA but with different objectives, the present study focusses on CSA binding domain and its interactions with CSA based on structure of the receptor-ligand complex. We show that 7 out of 16 CSA-binding residues of NTS and DBL2X for which field isolate sequences were available undergo unique mutations exclusively and these mutations are not seen in 14 parasite strains (NF54, FCR3, 7G8, HB3, CD01, Dd2, GA01, GB4, KE01, KH01, ML01, SN01, TG01, GN01) (Ma et al., 2021). Total of 4 out of 16 residues show similar mutations in both domains. Structural mapping of these mutations on the CSA-VAR2CSA complex further indicates that at least 4 of these mutations (R846Q, K828M, K562Q and Q832K) may alter the binding surfaces (R-to-Q, K-to-M, K-to-

Q and Q-to-K) and have the potential to effect CSA binding/affinity. VAR2CSA-based antigens are leading vaccine candidates for placental malaria and mutations in crucial CSA-interacting residues may have an impact on vaccine development. Similar concerns are also being addressed for COVID-19 vaccines whose efficacy may be altered against new variants having SNPs (Harvey et al., 2021). Similarly, a recent study on *Plasmodium vivax* DBL (Duffy-binding like) protein that binds to DARC (Duffy antigen receptor for chemokines) receptor for invasion into red blood cells showed inherent variability in its sequences worldwide, which may act as a limitation for the development of an effective, universal vaccine (Mittal et al., 2020). Thus, it is clear that region-wise or country-wise polymorphism analysis in the ligand-binding domain of VAR2CSA must be covered for vaccine development. The ultimate goal is to provide an effective vaccine for placental malaria based upon VAR2CSA that can tackle the public health burden of placental malaria in endemic countries. Our sequence and structural analysis have implications for the development of a universal VAR2CSA vaccine for combating placental malaria.



**Ethical approval:** Not required.

**Declaration of interests:** The authors declare that they have no competing interests.

**Author Contributions:** AS designed the study. JG collected and analyzed the data and wrote the paper. SC, PB and AS helped with the manuscript. All authors read and approved the final form of the manuscript.

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**Figure Captions**

**Figure 1. Sequence diversity in CSA-interacting residues of *Plasmodium falciparum* VAR2CSA NTS, DBL2X and DBL4ε domains in field isolate(s)**. Domain diagram of ectodomain *of Plasmodium falciparum* VAR2CSA NF54. The CSA-interacting domains NTS, DBL2X and DBL4ε are coloured yellow, pink and blue, respectively. Non-interacting domains are coloured grey. CSA-interacting residues from PISA, PLIP and PDBSum are listed under NTS, DBL2X and DBL4ε. Residues which are conserved in *P. falciparum* VAR2CSA strains and field isolate(s) are coloured green. Residues which show sequence

variation in both VAR2CSA 14 strains (Ma et al. 2021) and field isolate(s) are coloured orange. Residues which show unique variation exclusively in field isolate(s) are coloured red. DBL4ε field isolate(s) were unavailable and are marked with * and coloured green based upon their conservation in *Pf* strains. b. Three-dimensional cryo-EM structure of *Pf* VAR2CSA NF54 in complex with CSA (PDB ID: 7JGH). The CSA-interacting domains NTS, DBL2X and DBL4ε are coloured yellow, pink and blue, respectively. Non-interacting domains are coloured grey. DBL5ε and DBL6ε not available in the current structure. c. Interaction of CSA with *Pf* VAR2CSA. The CSA-interacting residues of NTS, DBL2X and DBL4ε are coloured yellow, pink and blue respectively and shown as sticks with transparent surface. CSA is shown as cyan-coloured stick.

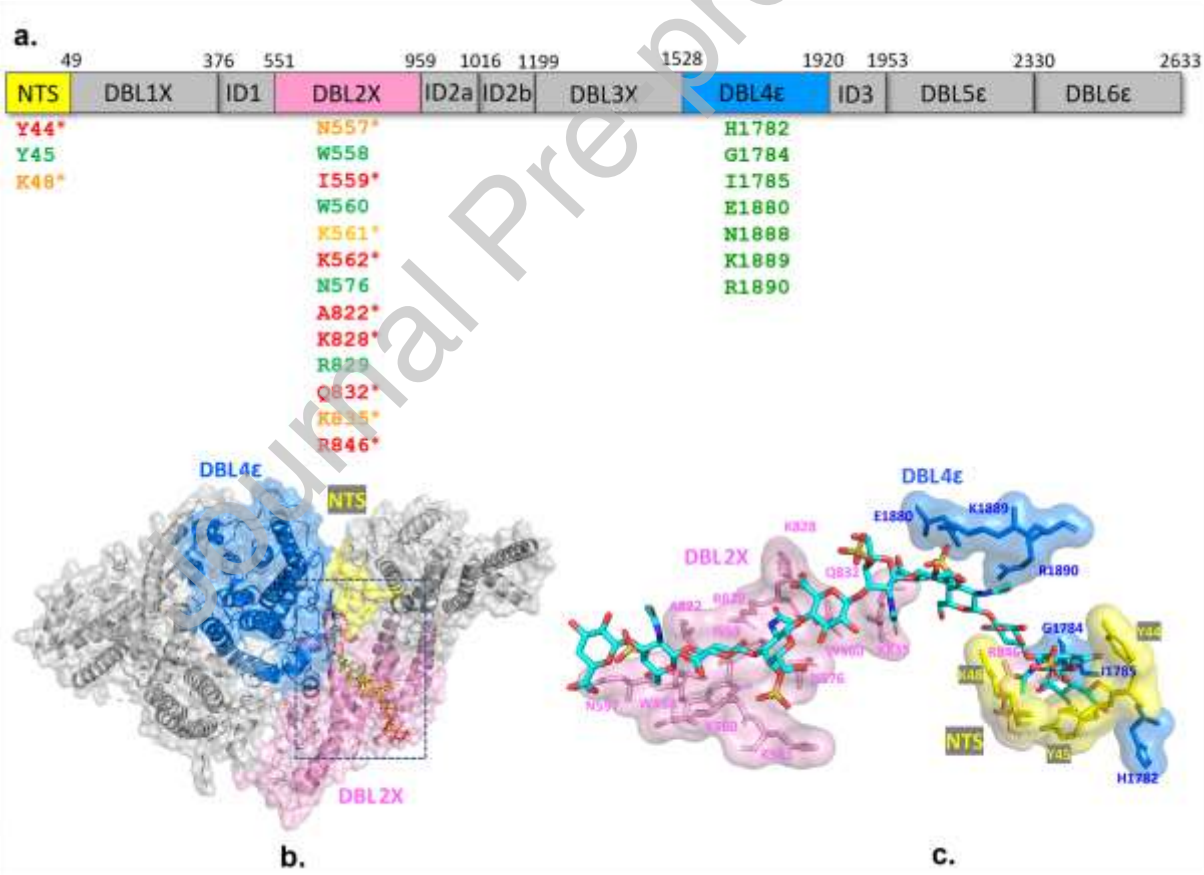



**Figure 2. Structural close-up of sequence variation in crucial CSA-interacting residues of *Pf* VAR2CSA DBL2X in field isolate(s).** a-d. Mutation in DBL2X - R846Q, K828M,

K562Q, Q832K - shows disruption in interaction with CSA. e. Electrostatic surface showing important basic residues on the binding surface before and after mutations.

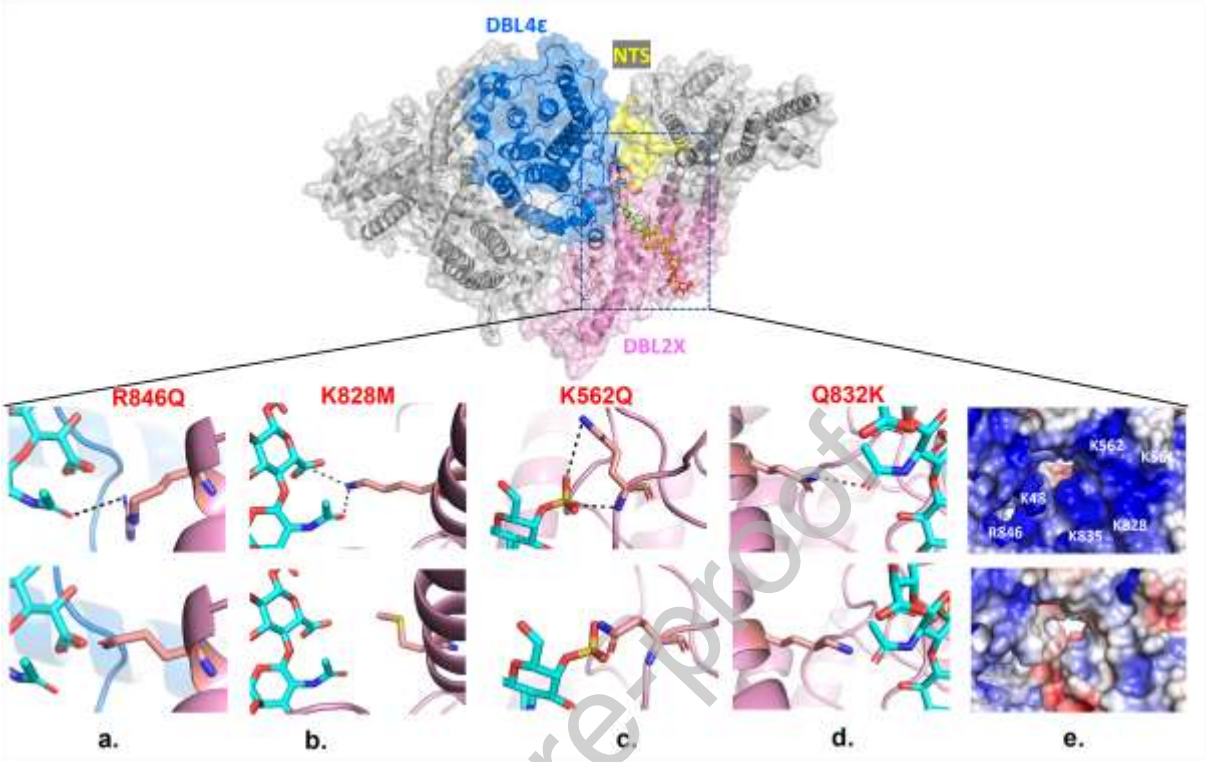



**Figure 3. Sequence diversity in CSA-interacting residues of *Pf* VAR2CSA NTS and DBL2X domains in field isolate(s)**. a. Mutations in NTS residues (colored yellow) and DBL2X residues (colored pink) are listed by country. Mutations seen in both VAR2CSA strains and field isolate(s) are colored orange. Mutations seen exclusively in field isolate(s) are colored red. The total frequency of mutations is shown in percentage. *Various indicates location either from South America/South Asia/Africa. b. Bar graph representation of total frequency of mutations in CSA-interacting residues of NTS and DBL2X seen in *P. falciparum* field isolate(s). NTS and DBL2X residues are colored yellow and pink respectively. Mutations seen in both VAR2CSA strains and field isolate(s) are shown as orange bar. Mutations seen exclusively in field isolate(s) are shown as red bar. Total frequency of mutations in each residue is shown in percentage.

| Country (no. of sequences) | Y44 | K48 | N557 | I559 | K561 | K562 | A822 | K828 | Q832 | K835 | R846 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Benin (90) | Y44D<br>Y44S | K48N | N557I<br>N557K<br>N557Y | I559T | K561Q | - | A822S | K828M | Q832K | - | R846Q |
| Congo (581) | - | - | - | - | - | - | A822S<br>A822T | - | - | K835M | - |
| Colombia (45) | - | - | N557K | - | - | - | A822S | - | - | - | - |
| Ghana (5) | - | - | N557K | - | - | - | - | - | - | K835M | - |
| Mali (4) | - | K48N | N557K | - | - | - | A822S | - | - | K835M | - |
| Malawi & Benin (142) | - | - | N557I<br>N557K<br>N557Y | I559T | K561Q | - | A822S | - | - | - | - |
| Mozambique (201) | - | - | - | - | - | - | A822T<br>A822P | - | - | - | - |
| Senegal & Tanzania (36) | Y44D | K48N | N557I<br>N557K | I559T | - | - | - | - | - | K835M | - |
| Various* (10) | - | - | N557I<br>N557K<br>N557Y | I559T | - | K562Q | A822S | - | - | K835M | - |
| Total Frequency (in %) | 28.1 | 76 | 79.9 | 13.6 | 7.7 | 2.7 | 27.1 | 25.3 | 2.4 | 29.6 | 8.4 |

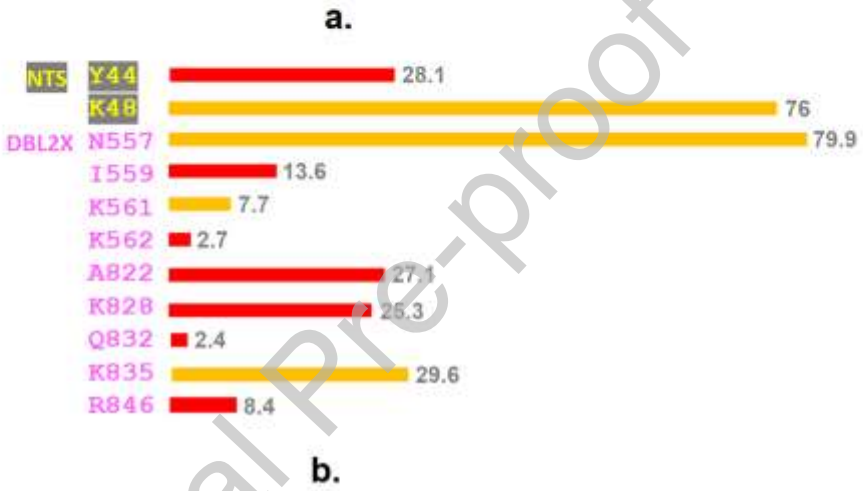
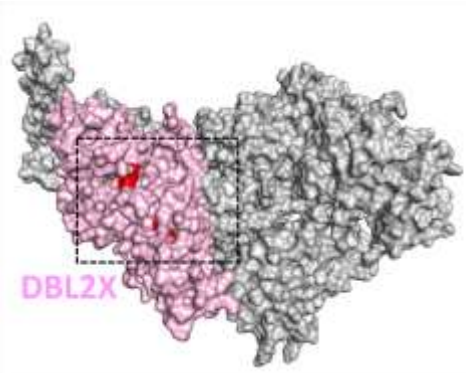



**Figure 4. Sequence variation in cysteines of *Pf* VAR2CSA DBL2X in field isolate(s)**. VAR2CSA DBL2X five cysteine disulphide bonds are listed. The bridges conserved across *P. falciparum* field isolate(s) are coloured green, and those showing mutations are coloured red. Total frequency of mutations in DBL2X residues C643, C745 and C769 in field isolate(s) is shown in percentage.




DBL2X

C783–C801
C797–C957
C805–C955

C643–C745

C769–C901

| Country | C643 | C745 | C769 |
|---|---|---|---|
| Benin | C643Y | C745S | |
| Congo | - | C745S | |
| Malawi & Benin | - | C745S | |
| Mozambique | - | C745S | C769R<br>C769Y |
| Senegal & Tanzania | - | C745S | |
| Various* | - | C745S | |
| Total Frequency (%) | 1.2 | 8.5 | 1.5 |

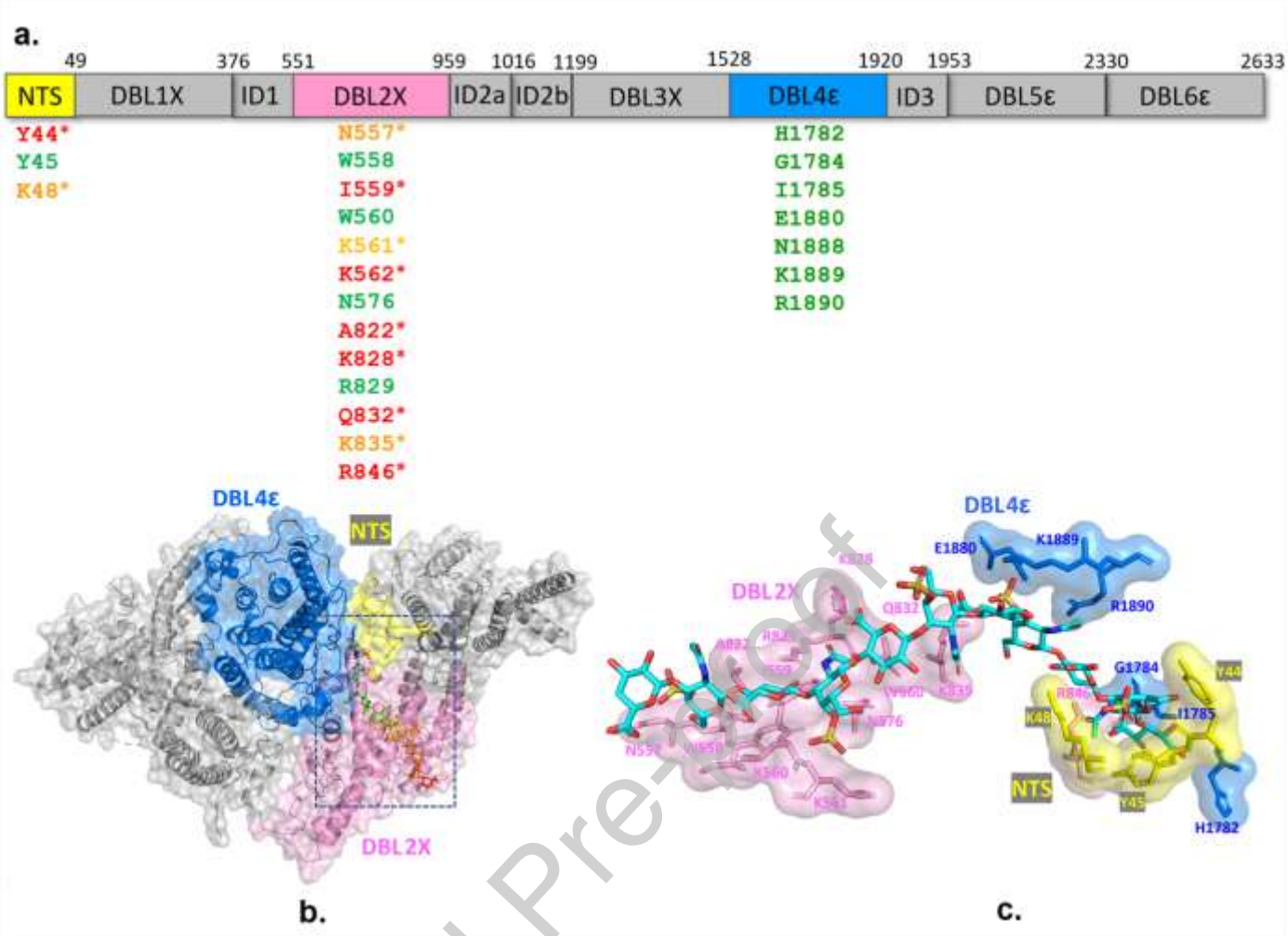



Figure 1

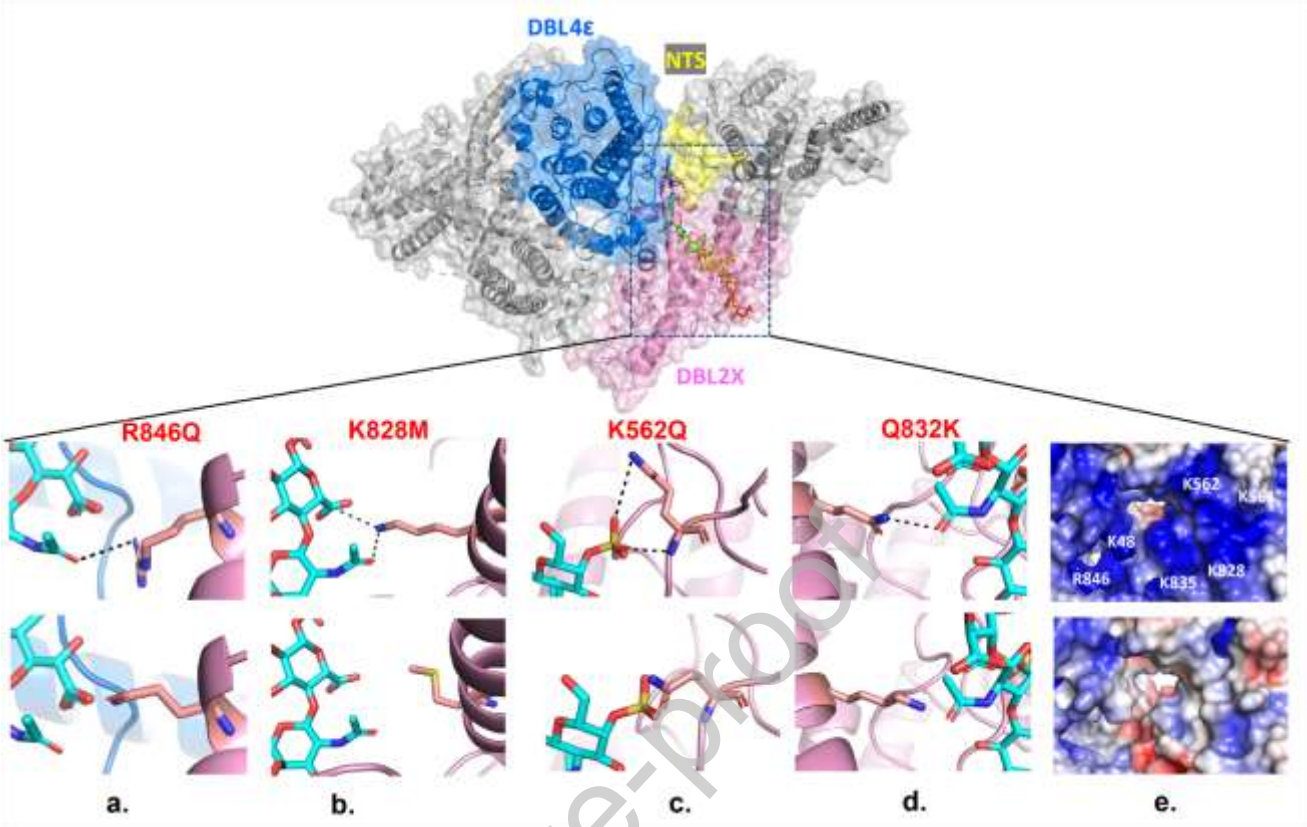



Figure 2

| Country (no. of sequences) | Y44 | K48 | N557 | I559 | K561 | K562 | A822 | K828 | Q832 | K835 | R846 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Benin (90) | Y44D<br>Y44S | K48N | N557I<br>N557K<br>N557Y | I559T | K561Q | - | A822S | K828M | Q832K | - | R846Q |
| Congo (581) | - | - | - | - | - | - | A822S<br>A822T | - | - | K835M | - |
| Colombia (45) | - | - | N557K | - | - | - | A822S | - | - | - | - |
| Ghana (5) | - | - | N557K | - | - | - | - | - | - | K835M | - |
| Mali (4) | - | K48N | N557K | - | - | - | A822S | - | - | K835M | - |
| Malawi & Benin (142) | - | - | N557I<br>N557K<br>N557Y | I559T | K561Q | - | A822S | - | - | - | - |
| Mozambique (201) | - | - | - | - | - | - | A822T<br>A822P | - | - | - | - |
| Senegal & Tanzania (36) | Y44D | K48N | N557I<br>N557K | I559T | - | - | - | - | - | K835M | - |
| Various* (10) | - | - | N557I<br>N557K<br>N557Y | I559T | - | K562Q | A822S | - | - | K835M | - |
| Total Frequency (in %) | 28.1 | 76 | 79.9 | 13.6 | 7.7 | 2.7 | 27.1 | 25.3 | 2.4 | 29.6 | 8.4 |

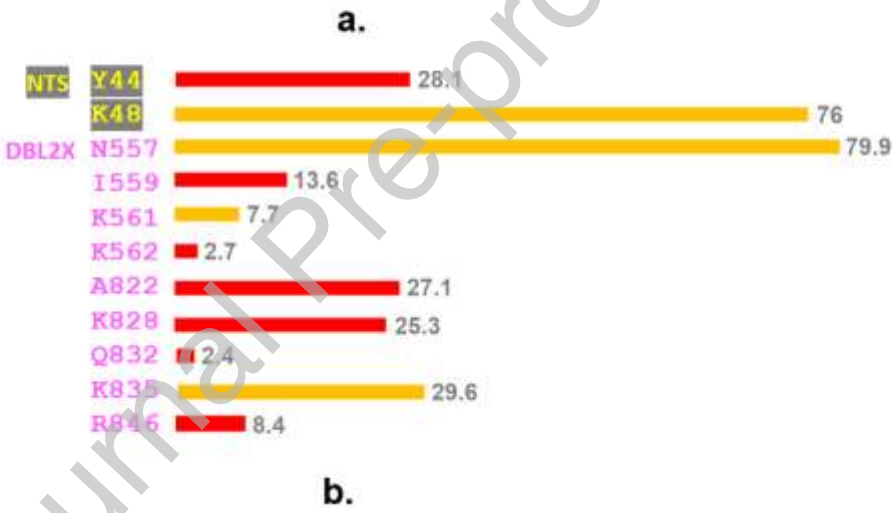



Figure 3


| Country | C643 | C745 | C769 |
|---|---|---|---|
| Benin | C643Y | C745S | |
| Congo | - | C745S | |
| Malawi & Benin | - | C745S | |
| Mozambique | - | C745S | C769R<br>C769Y |
| Senegal & Tanzania | - | C745S | |
| Various* | - | C745S | |
| Total Frequency (%) | 1.2 | 8.5 | 1.5 |

Figure 4