Global genetic diversity of \textit{var2csa} in \textit{Plasmodium falciparum} with implications for malaria in pregnancy and vaccine development

Ernest Diez Benavente$^{1,}$, Damilola R. Oresegun$^{2}$, Paola Florez de Sessions$^{3}$, Eloise M. Walker$^{1}$, Cally Roper$^{1}$, Jamille G. Dombrowski$^{4}$, Rodrigo M. de Souza$^{4,5}$, Claudio R. F. Marinho$^{4}$, Colin J. Sutherland$^{1}$, Martin L. Hibberd$^{1,3}$, Fady Mohareb$^{2}$, David A. Baker$^{1}$, Taane G. Clark$^{1,6}$ & Susana Campino$^{1}$

Malaria infection during pregnancy, caused by the sequestering of \textit{Plasmodium falciparum} parasites in the placenta, leads to high infant mortality and maternal morbidity. The parasite-placenta adherence mechanism is mediated by the VAR2CSA protein, a target for natural occurring immunity. Currently, vaccine development is based on its ID1-DBL2Xb domain however little is known about the global genetic diversity of the encoding \textit{var2csa} gene, which could influence vaccine efficacy. In a comprehensive analysis of the \textit{var2csa} gene in >2,000 \textit{P. falciparum} field isolates across 23 countries, we found that \textit{var2csa} is duplicated in high prevalence (>25%), African and Oceanian populations harbour a much higher diversity than other regions, and that insertions/deletions are abundant leading to an underestimation of the diversity of the locus. Further, ID1-DBL2Xb haplotypes associated with adverse birth outcomes are present globally, and African-specific haplotypes exist, which should be incorporated into vaccine design.

Malaria infection during pregnancy, caused by \textit{Plasmodium falciparum} parasites, is a major public health burden in tropical areas of Africa and South East Asia, being responsible for substantial maternal and infant morbidity and mortality, including increased adverse outcomes such as miscarriage, maternal anaemia and low birth weight. There is an estimated 200,000 infant and 10,000 maternal deaths per year caused by placental malaria (PM)$^{1}$. The increased susceptibility to \textit{P. falciparum} infection during pregnancy, regardless of previously acquired malaria immunity, has been attributed to the sequestration of infected erythrocytes in the placenta$^{2,3}$. \textit{P. falciparum} blood stage parasites accumulate in the placenta by adhering to chondroitin sulfate A (CSA)$^{4}$. The interaction between infected erythrocytes and the placental syncytium receptor is mediated by the parasite protein VAR2CSA$^{5}$. The extracellular region of this ~350 kDa cysteine–rich transmembrane protein is formed by six Duffy-binding-like (DBL) domains and binds with high specificity to the CSA receptor$^{6,7}$. The protein is encoded by the \textit{var2csa} gene (~10 Kbp in length), which is the most conserved member of the \textit{var} family that encodes the variable antigen \textit{P. falciparum} erythrocyte membrane protein-1 (PfEMP1)$^{8}$. VAR2CSA is preferentially expressed by placental parasites$^{9}$. Primigravid women with a lack of immunity against these subpopulation of parasites are the most affected, but become less susceptible in subsequent pregnancies$^{9,10,11}$.

$^{1}$Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom. $^{2}$School of Water, Energy and Environment, Applied Bioinformatics, Cranfield University, Cranfield, United Kingdom. $^{3}$Genomics Institute of Singapore, Biopolis, Singapore. $^{4}$Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. $^{5}$Multidisciplinary Center, Federal University of Acre, Acre, Brazil. $^{6}$Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, United Kingdom. Correspondence and requests for materials should be addressed to T.G.C. (email: taane.clark@lshtm.ac.uk) or S.C. (email: Susana.campino@lshtm.ac.uk)
The full length var2csa gene has been identified on chromosome 12, but additional loci have also been found on other chromosomes (e.g., 1, 5–9) both in laboratory and field isolates. However, links to phenotypic advantage have not yet been established. Within a single genome, multiple var2csa gene copies are not necessarily identical, and a high proportion of parasites from infected pregnant women have been found to possess multiple var2csa variants, suggesting that having multiple copies may be advantageous during disease progression. Parasites with multiple var2csa copies may persist longer during pregnancy by having an increased capacity for antigenic variation and evasion of the maternal immune response.

Intermittent preventive treatment in pregnancy (IPTp) has contributed to a reduction in PM burden, but currently recommended anti-malarial drugs are threatened by high levels of parasite resistance. The development of a placental malaria vaccine is based on naturally occurring immunity, and two VAR2CSA-based candidates are currently in clinical trials. Several studies have compared levels of antibody in the sera of primigravid and multigravid women that recognise specific domains of VAR2CSA, including N-terminal VAR2CSA fragments that have high binding affinity for CSA. The purpose of any vaccine for PM is to induce immunity in nulligravid women that would confer protection against PM during subsequent infection, and similarly should boost the immunity acquired by multigravid in endemic areas. It remains to be established whether individual VAR2CSA immunogens are able to induce PM protective immunity analogous to that of naturally-acquired immunity.

Studies looking at var2csa genetic diversity in field isolates sourced from pregnant infected women, have found that parasites cluster into five different clades based on the CSA-minimal binding site sequence (ID1-DBL2Xb). A clade identified as 3D7-like was associated with the delivery of infants with lower birthweight, suggesting that VAR2CSA diversity affects pathogenicity and, by inference, antigenicity. Thus, the intra- and inter-population genetic diversity of the var2csa gene is likely to affect the efficacy of any vaccine developed based on the VAR2CSA protein, highlighting the need for genetic diversity studies. Therefore, to systematically evaluate the magnitude of this variation, we assess the genetic diversity and structure of the var2csa, and estimate copy number profiles, across more than 2,000 P. falciparum field isolates and laboratory strains spanning 23 countries. We find strong evidence that isolates circulating amongst African populations harbour a much higher diversity in the gene compared to other regions, in both nucleotide variants and structural variants, as well as a significantly higher prevalence of parasites encoding two or more different copies. We report for the first time the global distribution of the different ID1-DBL2Xb clades associated with adverse birth outcomes and an unexpectedly high structural variability of the DBL2x domain across the different populations.

Results
Var2csa gene copy numbers in laboratory strains. We investigated the number of var2csa gene copies present in 21 genomes from laboratory cultured strains sequenced using the PacBio RS-II long-read sequencing platform. These were Dd2 (IndoChina); KH01 and KH02 (Cambodia); D10 (Papua New Guinea); T9/96 and K1 (Thailand); 7G8 and IT (Brazil); HB3 (x2) (Honduras); 3D7 and NF54 (Africa); GA01 (Gabon); GB4 (Ghana); GN01 (Guinea); CD01 (Congol); K01 (Kenya); SD01 (Sudan); TG01 (Togo); SN01 (Senegal) and ML01 (Mali). For each strain, contigs for the var2csa gene were extracted from assemblies of high-quality reads, and aligned to the 3D7 reference, known to have only one copy of var2csa, to generate a phylogenetic tree. Almost all strains have single copies of the var2csa gene, except HB3 (confirming), D10 and KH01, which have 2 copies. The HB3 copies are closely related and were more similar to each other (93% similarity) than the D10 (87% similarity) and KH01 (88%) pairs. Isolates TG01 and ML01 isolates presented with evidence of multiplicity of infection (MOI >1) and the extra var2csa gene copies observed are thought to belong to the different clones in the sample. Investigation of the sequences found 3,401 unique mutations, spanning 2,632 polymorphic sites from a total of 8,011 sites (excluding gaps and without missing data). Also, 601 unique InDels were found, of which 439 were overlapping, leaving a set of 162 non-overlapping InDels.

Global structural analysis of var2csa gene extra copies. We sought to determine the number of var2csa gene copies present in P. falciparum field isolates (n = 3,125; 23 countries; including from the Pf3k project) and laboratory strains (n = 5) with Illumina sequencing data in the public domain. The analytical pipeline is summarised in S2 Fig. After quality control filtering, a total of 2,099 (67.2%) field isolates with non-high multiplicity of infection (clonal for >70% of genome), low numbers of heterozygous single nucleotide polymorphism (SNPs) (<0.015% of total SNPs) and high genome-wide coverage (>30-fold) were retained (S3 Fig.). By comparing the coverage of the larger N-terminal of var2csa exon 2 to the average read coverage across the rest of the resident chromosome (see S4 Fig.), we confirmed the inferred copy numbers for 5 laboratory strains (HB3 and D10 have 2 copies; 3D7, 7G8 and GB4 have 1 copy). The presence of extra and different var2csa gene copies manifests itself in heterozygous or mixed genotype signatures. Therefore we compared the estimated number of var2csa copies to the proportion of mixed calls present in the gene. There was a clear increase in heterozygous calls in the gene for the samples with additional copies, and the mean proportion of heterozygous calls for samples presenting 1 copy is close to zero (S5 Fig.). This approach confirmed the presence of similar and different var2csa copies in HB3 (93% similarity) and D10 (87%), respectively. In the field isolates (n = 2,099) we found geographical differences in the frequency of extra copies, where Oceania (21/24, 88%) was highest, followed by African populations (West Africa (172/489, 35%); East Africa (120/409, 29%), and the lowest frequency was in South East Asia (235/1108, 21%) (test of proportions P < 0.001) (Table S1).

Characterisation of the var2csa gene and genetic diversity in field isolates. Isolates with a single var2csa copy number and low numbers of heterozygous SNPs (<2%) in var2csa (S6 Fig.) were identified (n = 1,647), and raw Illumina reads de-novo assembled using velvet software. A robust multi-software pipeline (see Materials and Methods) led to a high quality multi-alignment of 1,249 sequences, spanning more than 7Kb of the N-terminal end of the var2csa gene (Table 1, Fig. 1A and Table S1). The length of non-missing sequence
| Population  | n   | Number sites without missing data | Number of Haplotypes | Haplotype diversity (Hd) | Nucleotide diversity (π) | Average no. of nucleotide differences (pairwise comparison) |
|------------|-----|----------------------------------|----------------------|--------------------------|--------------------------|-----------------------------------------------------------|
| Burkina Faso | 7   | —                                | —                    | —                        | —                        | —                                                         |
| Cameroon   | 49  | 5317                             | 44                   | 0.995                    | 0.0828                   | 440                                                       |
| Gambia     | 31  | 6700                             | 21                   | 0.963                    | 0.0795                   | 533                                                       |
| Ghana      | 116 | 6037                             | 105                  | 0.998                    | 0.0798                   | 482                                                       |
| Guinea     | 33  | 6553                             | 32                   | 0.998                    | 0.0866                   | 567                                                       |
| Mali       | 15  | 6792                             | 15                   | 1                        | 0.0873                   | 593                                                       |
| Nigeria    | 3   | —                                | —                    | —                        | —                        | —                                                         |
| DRC        | 77  | 6484                             | 71                   | 0.998                    | 0.0815                   | 530                                                       |
| Kenya      | 21  | 6784                             | 19                   | 0.990                    | 0.0821                   | 557                                                       |
| Malawi     | 95  | 5953                             | 63                   | 0.992                    | 0.0819                   | 488                                                       |
| Tanzania   | 30  | 6578                             | 30                   | 1                        | 0.0892                   | 587                                                       |
| Uganda     | 4   | —                                | —                    | —                        | —                        | —                                                         |
| Madagascar | 10  | 6899                             | 10                   | 1                        | 0.0976                   | 673                                                       |
| Bangladesh | 11  | 6830                             | 10                   | 0.982                    | 0.0896                   | 612                                                       |
| Cambodia   | 360 | 4828                             | 46                   | 0.911                    | 0.0696                   | 336                                                       |
| Laos       | 40  | 6776                             | 29                   | 0.979                    | 0.0786                   | 533                                                       |
| Myanmar    | 72  | 6784                             | 27                   | 0.949                    | 0.0836                   | 567                                                       |
| Thailand   | 184 | 6651                             | 47                   | 0.968                    | 0.0809                   | 538                                                       |
| Vietnam    | 68  | 6712                             | 30                   | 0.893                    | 0.0794                   | 533                                                       |
| PNG        | 3   | —                                | —                    | —                        | —                        | —                                                         |
| Brazil     | 3   | —                                | —                    | —                        | —                        | —                                                         |
| Colombia   | 10  | 7045                             | 6                    | 0.889                    | 0.0775                   | 546                                                       |
| Peru       | 6   | —                                | —                    | —                        | —                        | —                                                         |

Table 1. Summary of the *Plasmodium falciparum var2csa* assembled 7Kb fragment and its diversity by country using the field isolates with little evidence of mixed infections (n = 1,249). bolded are African countries, italicised are South East Asian countries, DRC = Democratic Republic of Congo; PNG = Papua New Guinea.

Figure 1. *Plasmodium falciparum var2csa* diversity across a 7 kb region covering the five DBL domains, and 1,249 field isolates. (A) Schematic structure of the var2csa gene including the N-terminal sequence (NTS, blue), 5 Duffy binding like-Domains (DBL, green), 3 Inter-Domains (ID, red) and the Cysteine-Rich Inter-Domain (CIDR, yellow); the lengths in amino acids of the 3D7 reference are presented in parentheses. (B) Accumulation of unique insertions and deletions (InDels) across the var2csa gene. (C) Distribution of nucleotide diversity (π) across the gene and by population. Regions of abnormal (“flat”) nucleotide diversity are highlighted in green (B and C).
The open reading frame (ORF) element upstream of the var2csa gene is highly conserved. The ORF region of the var2csa plays a role in regulating expression of the gene\(^{24}\). Analysis of the complete sequences (n = 1,249; S2 Data) revealed that these elements harbour little diversity and are highly conserved across geographical regions and populations (mean \(\pi = 0.02\)) (Table S3). The clustering of var2csa genes into four clades is not complemented by a similar pattern in the ORF regulatory region, which seems to indicate that the gene expression regulatory function of this region might be conserved across clades.
Discussion

The VAR2CSA protein is a vaccine candidate for PM\textsuperscript{15,16} and is the basis of two vaccines currently in Phase I clinical trials\textsuperscript{15,16}. It is therefore essential to understand the genetic and structural diversity of var2csa in natural populations of P. falciparum parasites, to predict the impact of potential vaccines, and improve the understanding of the mechanisms by which the malaria parasites sequester in the placenta. It has been suggested that parasite populations with multiple copies of the var2csa gene persist longer during pregnancy, hypothesising that this could be due to the ability of these parasites to generate a wider diversity of antigenic variation\textsuperscript{14}. Across global field samples ($n = 2,099$), all populations had evidence of extra copies (27% on average, broadly consistent with\textsuperscript{14}), but the prevalence was higher in African and Papua New Guinean parasites. For the African populations this could be due to the higher immune pressure that this gene might be under in higher transmission settings. Whilst, for PNG it could be due to a founder effect arising from the parasite population’s mixed Asian and African ancestry\textsuperscript{26}. It is possible the degree of copy number variation in the population-based field isolates sourced predominantly from children with malaria could be different to that found in pregnant mothers. However, the var2csa sequences from pregnant mothers included in our analysis overlapped with the sequences and resulting tree clusters observed in the field isolates.

By applying a pipeline validated using Pacbio sequenced strains, 7 kb fragments encoding the VAR2CSA extra-cellular domains were assembled across 1,249 field isolates with little evidence of mixed infections. The diversity was highest towards the N-terminus of the protein, as seen previously\textsuperscript{19} and, in general, higher in African parasite populations compared to South East Asian ones. This result is consistent with African genomes being older and with the higher transmission rates in that continent currently. InDels have been a neglected source of variation and diversity in var2csa studies. The presence of a high number of low frequency InDels, which had the highest density in regions with flat nucleotide diversity, revealed that the level of diversity was underestimated. Comparing across the 5 VAR2CSA domains, the highest density of InDels was found in the DBL2X (part of CSA minimal binding domain), leading to the greatest variability in sequence length, where up to an additional 120 amino acid insertions were present. The impact of small in-frame InDels and short frame shifts on the protein structure of the DBL domain and their binding capabilities are not clear, and structural modelling approaches are difficult to scale-up to the levels of variation observed. A large number of the SNPs and InDels could lead to important changes in the amino acid sequence while conserving the overall gene structure. However, this should be fully explored to understand any impact on antibody binding affinity and CSA-binding in placental malaria. Further phenotypic characterization is required to evaluate the contribution of the diversity observed in the gene at both SNP and InDel level to parasite sequestration in the placenta, which could provide insight into the mechanisms by which the infection causes the associated adverse outcomes during pregnancy.

A recent study in pregnant women in Malawi and Benin identified 5 clades of the ID1-DBL2Xb domain of the var2csa gene and found an association between the infection of parasites harbouring the 3D7-like sequence
and low birthweight\(^1\). Our work suggests that there are four main ID1-DBL2Xb domain clades, including a 3D7-like (Clade 1) and FCR3-like (Clade 2). Two of the previously reported clades appear to be too homogenous to be separated in our much larger dataset. Three of the four remaining clades (1, 2 and 4) were present across all the regions, including Clade 1, which was found in West Africa (41.2%), East Africa (27.5%), South East Asia (23.5%) and South America (21.1%), consistent with previous findings in Malawi (44.8%)\(^9\) and Malawi and Benin (52.1%)\(^9\). Clade 3 isolates appear to be rare (<1% overall) and almost exclusively present in African parasites. Previous studies\(^9\) have focused on the effect of the major clades have on pregnancy outcomes, but these data highlight the presence of Clade 4 isolates across very diverse regions very diverse regions globally, therefore stressing the need to further investigate the relationship between the prevalence of the different parasite clades and the pregnancy outcomes. Countries outside of sub-Saharan Africa such as the South East Asian region where the Clade 1 (3D7-like) has been reported for the first time, are of particular interest given that there is no evidence on the impact that parasites harbouring Clade 1 VAR2CSA protein have on pregnancy in these host populations. Furthermore, studies of the potential impact of the less prevalent Clades 3 and 4 on adverse outcomes in pregnancy are needed.

An analysis of the ID1-DBL2Xb domain haplotypes revealed higher diversity across African populations compared to South East Asian populations. A higher proportion of unique haplotypes in African parasites is likely to be a reflection of the higher transmission intensity. These diversity patterns suggest that introduction of a vaccine based on one single haplotype or a few heterologous haplotypes might be of greater benefit in South East Asian populations, although it is in Africa that the vaccine is most needed. Our work suggests that it would be advisable to consider the four clades of related var2csa haplotypes when testing the efficacy of the vaccine and, if possible, including heterologous haplotypes for the African specific clades found in this study in future vaccine design.

Overall, our study reveals the genetic diversity of the var2csa gene across more than 23 countries, demonstrating that SNPs and InDels are frequent at this locus, and generate considerable haplotype diversity, especially in African parasites. We also report a high frequency of multiple var2csa gene copies in the genome of field isolates. Further molecular and association studies are essential to understand the effect of VAR2CSA variants and extra gene copies on parasite sequestration in the placenta, the outcome of pregnancy and vaccine efficacy. Our findings can be used to support the assessment and development of new preventive tools against placental malaria, including the design of new vaccines that are robust to regional genetic diversity.

**Materials and Methods**

**Samples, sequence data and processing.** High-quality and high molecular weight DNA (20 µg) was purified from laboratory strains (D10, K1, HB3, T9/96 and NF54) using the Qiagen Genomic tip 20/G.

Sequencing data for D10 (Papua New Guinea), T9/96 (Thailand), HB3 (Honduras), K1 (Thailand), NF54 (3D7 Parental line) strains were generated on Pacific Biosciences (PacBio) RS-II long read technology at the Genome Institute Singapore and were supplemented by similar data for 7G8 and IT (Brazil), GB4 (Ghana), GN01 (Guinea), CD01 (Congo), Dd2 (IndoChina), KE01 (Kenya), KH01 and KH02 (Cambodia), GA01 (Gabon), SD01 (Sudan), TG01 (Togo), SN01 (Senegal) and ML01 (Mali) in the public domain (ftp://ftp.sanger.ac.uk/pub/project/pathogens/Plasmodium/falciparum/PF3K/PilotReferenceGenomes/GenomeSequence/Version1/). High quality sequencing reads (range: 33,855–66,185 reads) were assembled using Hierarchical Genome Assembly Process HGAP3 and corrected using Illumina reads when available, implemented in the SMRT Portal software suite and following previously described methods\(^27\). Overlaps between the start and end of large contigs were found using Mummer software\(^26\) and removed using in-house scripts.

Raw sequencing data from Illumina data was available for previously published *P. falciparum* strains (3D7, HB3, D10, 7G8 and GB4)\(^29\)\(^30\) and isolates from East Africa (Kenya (n = 38), Malawi (353), Tanzania (63), Uganda (12), Madagascar (18)), West Africa (Burkina Faso (48), Gambia (63), Ghana (443), Guinea (116), Mali (55), Nigeria (6), Cameroon (127), Central Africa (Democratic Republic of Congo (DRC) (232)), South America (Colombia (15), Peru (9), Brazil (3)), South Asia (Bangladesh (53)) and South East Asia and Oceania (Cambodia (649), Laos (112), Myanmar (134), Papua New Guinea (26), Thailand (326), Vietnam (199))\(^31\). Public accession numbers for raw sequence data analysed are contained in SRA studies ERP000190 and ERP000199, as well as being accessible from the Pf3k project website (https://www.malariagen.net/projects/pf3k). All Illumina short reads were mapped to the 3D7 reference genome (version 3.0) using bwa-mem (version 0.7.17)\(^32\). SNPs and small InDels were called from the alignment files using samtools\(^32\) and bcftools (version 1.5) with default settings\(^32\). Only those variants with quality scores in excess of 30 (indicating an error rate less than 1 per 1000bp) and with minimum coverage of 10 were retained\(^32\). The pipeline is summarised in S2 Fig. In total, the dataset contains 1,649 isolates and 1,513,940 high quality SNPs; 47.3% within genes and 5.2% have a minor allele frequency greater than 1%. A principal component analysis (not shown) based on pairwise SNP differences between isolates did not reveal any geographic outliers. We used the proportion of heterozygous calls per sample (\(>1\)) obtained using estMOI (\(>1\)) to infer copy numbers. In particular, \(>1\) isolates were found in West Africa (41.2%), East Africa (27.5%), South East Asia (23.5%) and South America (21.1%). Consistent with previous findings in Malawi (44.8%)\(^9\) and Malawi and Benin (52.1%)\(^9\).

**Characterisation of the var2csa gene and copy number.** The Pacbio sequencing contigs that contained the var2csa gene were identified, and mapped to the 3D7 reference genome, allowing the direct assessment of copy numbers. Similarly, the var2csa gene was characterised from contigs constructed through de novo assembly of Illumina short reads using velvet software\(^23\). The resulting contigs obtained were aligned and assembly errors were corrected manually using Aliview\(^36\). Once corrected the sequences were trimmed from the N-Terminal to form a 7 kb fragment which was then translated using Orffinder\(^27\) and sequences presenting more than one ORF were excluded from further analysis. The approach was validated by comparing the contigs obtained from PacBio and Illumina platforms, using 3D7, 7G8 and GB4 samples with both sets of data (S8 Fig.). Illumina data were also used to infer copy numbers. In particular, Delly software (version 0.7.7)\(^38\) was used to calculate genomic coverage.
from the alignment bam files, and the Control-FREEC tool (Version 10.6)\textsuperscript{38} was used to estimate copy number based on GC content corrected ratios of coverage using a sliding window of 500 bp and a step size of 100 bp\textsuperscript{39}. We also estimated copy number based on the ratio of the average var2csa gene coverage against the average gene coverage in the rest of the genome, optimised and tested using the HB3, D10, GB4, 3D7, and 7G8 strains.

Genetic diversity within and across populations. The selection of the samples for genetic diversity analysis (n = 1,649) focused on those with the presence of a single copy of the var2csa gene based on coverage (S4 Fig.) and a maximum of 2\% of bases in the var2csa gene presenting heterozygous calls in the gene (S7 Fig.). We then screened the contigs assembled using Blast alignment\textsuperscript{37} to the 3D7 reference of the var2csa gene and retrieved 1,317 isolate sequences with a hit that contained a contig of more than 8 kb in length. We aligned the sequences using Mafft\textsuperscript{40} with default parameters, reverse-complemented when needed and extracted the region corresponding to the var2csa gene and µORF region using AliView\textsuperscript{46}. The alignment was manually curated to remove poly-Ns added by the assembly software as well as obvious assembly errors. The final set of sequences was then translated using orfinder\textsuperscript{47} and a further 72 sequences presenting fragmented translation were excluded. The final dataset consisted of 1,249 sequences spanning 7 Kb of the N-terminal end of the var2csa gene (Table 1, S1 Data).

The DnaSP (version 5)\textsuperscript{41} and the ape and agegene\textsuperscript{42} R libraries were used to compute population genetic parameters at the region and country levels, including nucleotide diversity (\pi), the average number of nucleotide differences per site between any two given sequences and the haplotype diversity (Hd), which is the probability that two randomly sampled haplotypes are different. We also calculated distance matrices using R package seqinr\textsuperscript{48} in order to generate a Principal Component Analysis (PCA) of the nucleotide and protein sequences and the corresponding Neighbour-Joining trees. A k-means approach was used in R to obtain the clusters observed in the neighbour joining trees. The vecg blocks package was used to calculate the rarefaction curves using the ID1-DB2Xb region haplotypes by region and country.

Data Availability
Public accession numbers for raw sequence data analysed are contained in SRA studies ERP000190 and ERP000199, as well as being accessible from the PF3k project website (https://www.malariagen.net/projects/pf3k). Data was complemented by PacBio data in the public domain (ftp://ftp.sanger.ac.uk/pub/project/pathogens/Plasmodium/falciparum/PF3K/PilotReferenceGenomes/GenomeSequence/Version1/).

References
1. WHO. World Malaria Report 2017 (2017).
2. Bray, R. S. & Sinden, R. E. The sequestration of Plasmodium falciparum infected erythrocytes in the placenta. Trans. R. Soc. Trop. Med. Hyg. 73, 716–719 (1979).
3. Nunes, M. C. & Scherf, A. Plasmodium falciparum during pregnancy: a puzzling parasite tissue adhesion tropism. Parasitology 134, 1863–1869 (2007).
4. Duffy, P. E. & Fried, M. Malaria during pregnancy: parasites, antibodies and chondroitin sulphate A. Biochem. Soc. Trans. 27, 478–482 (1999).
5. Salanti, A. et al. Evidence for the Involvement of VAR2CSA in Pregnancy-associated Malaria. J. Exp. Med. 200, 1197–1203 (2004).
6. Srivastava, A. et al. Full-length extracellular region of the var2csa region of the VAR2CSA variant of PfEMP1 is required for specific, high-affinity binding to CSA. Proc. Natl. Acad. Sci. USA 107, 4884–4889 (2010).
7. Khunrae, P. et al. Full-Length Recombinant Plasmodium falciparum VAR2CSA Binds Specifically to CSPG and Induces Potent Parasite Adhesion-Blocking Antibodies. J. Mol. Biol. 397, 826–834 (2010).
8. Salanti, A. et al. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Mol. Microbiol. 49, 179–191 (2003).
9. Tuikue Ndam, N. G. et al. High level of var2csa transcription by Plasmodium falciparum isolated from the placenta. J. Infect. Dis. 192, 331–335 (2005).
10. Fried, M., Nosten, F., Brockman, A., Brabin, B. J. & Duffy, P. E. Maternal antibodies block malaria. Lancet (London, England) 363, 283–289 (2004).
11. Sander, A. F. et al. Multiple var2csa-type PFEMP1 genes located at different chromosomal loci occur in many Plasmodium falciparum isolates. PLoS One 4, e6667 (2009).
12. Brolin, K. J. M. et al. Simultaneous transcription of duplicated var2csa gene copies in individual Plasmodium falciparum parasites. Genome Biol. 10, R117–R117 (2009).
13. Sander, A. F. et al. Positive selection of Plasmodium falciparum parasites with multiple var2csa-type PFEMP1 genes during the course of infection in pregnant women. J. Infect. Dis. 203, 1679–1685 (2011).
14. European Vaccine Initiative. http://www.euvaccine.eu/portfolio/project-index/plasmalvac.
15. European Vaccine Initiative. http://www.euvaccine.eu/portfolio/project-index/primalvac.
16. Kane, E. G. & Taylor-Robinson, A. W. Prospects and Pitfalls of Pregnancy-Associated Malaria Vaccination Based on the Natural Immune Response to Plasmodium falciparum VAR2CSA-Expressing Parasites. Malar. Res. Treat. 2011, 764845 (2011).
17. Patel, J. C. et al. Increased risk of low birth weight in women with placental malaria associated with P. falciparum VAR2CSA clade. Sci. Rep. 7, 7768 (2017).
18. Bode, J. et al. Long read assemblies of geographically dispersed Plasmodium falciparum reveal highly structured subtelomeres. Wellcome Open Res. 3, 52 (2018).
19. Zerbino, D. R. & Birney, E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res 18 (2008).
20. Trimnell, A. R. et al. Global genetic diversity and evolution of var genes associated with placental and severe childhood malaria. Mol. Biochem. Parasitol. 148, 169–180 (2006).
21. Bancells, C. & Deitsch, K. W. A molecular switch in the efficiency of translation reinitiation controls expression of var2csa, a gene implicated in pregnancy-associated malaria. Mol. Microbiol. 90, 472–488 (2013).
22. Tutterrow, Y. L. et al. High Avidity Antibodies to Full-Length VAR2CSA Correlate with Absence of Placental Malaria. PLoS One 7, e40049 (2012).

Malaria as a Disease of Pregnancy: The Development of Plasmodium falciparum as a Human Pathogen.
26. Preston, M. D. et al. A barcode of organellar genome polymorphisms identifies the geographic origin of Plasmodium falciparum strains. *Nat. Commun.* **5**, 4052 (2014).
27. Benavente, E. D. et al. A reference genome and methylome for the Plasmodium knowlesi A1-H.1 line. *Int. J. Parasitol.* [https://doi.org/10.1016/j.ijpara.2017.09.008] (2017).
28. Kurtz, S. et al. Versatile and open software for comparing large genomes. *Genome Biol.* **5**, R12–R12 (2004).
29. Shears, M. J. et al. Characterization of the Plasmodium falciparum and *P. berghei* glycerol 3-phosphate acyltransferase involved in FASII fatty acid utilization in the malaria parasite apicoplast. *Cell. Microbiol.* **19**, e12633 (2017).
30. Miles, A. et al. Indels, structural variation, and recombination drive genomic diversity in Plasmodium falciparum. *Genome Res.* **26**, 1288–1299 (2016).
31. Ravenhall, M. et al. Characterizing the impact of sustained sulfadoxine/pyrimethamine use upon the Plasmodium falciparum population in Malawi. *Malar. J.* **15** (2016).
32. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
33. Li, H. et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
34. Assefa, S. A. et al. *estMOI*: estimating multiplicity of infection using parasite deep sequencing data. *Bioinformatics* **30**, 1292–1294 (2014).
35. Diez Benavente, E. et al. Analysis of nuclear and organellar genomes of Plasmodium knowlesi in humans reveals ancient population structure and recent recombination among host-specific subpopulations. *PLOS Genet.* **13**, e1007098 (2017).
36. Larsson, A. AllView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics* **30**, 3276–3278 (2014).
37. Sayers, E. W. et al. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **39**, D38–51 (2011).
38. Rausch, T. et al. DELLY: structural variant discovery by integrated paired-end and split-read analysis. *Bioinformatics* **28** (2012).
39. Boeva, V. et al. Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. *Bioinformatics* **28**, 423–425 (2012).
40. Katoh, K. & Standley, D. M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability, *Mol. Biol. Evol.* **30**, 772–780 (2013).
41. Librado, P. & Rozas, J. *DnaSPv5*: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452 (2009).
42. Jombart, T. & Ahmed, I. *adegenet* 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics* **27**, 3070–3071 (2011).
43. Charif, D., Thioulouse, J., Lobry, J. R. & Perriere, G. Online synonymous codon usage analyses with the ade4 and seqinR packages. *Bioinformatics* **21**, 545–547 (2005).

Acknowledgements

Sequence data analysis was performed on the MRC UK eMedLab computing resource. TGC is supported by the Medical Research Council UK (Grant no. MR/K000551/1, MR/M01360X/1, MR/N010469/1, MR/R020973/1) and BBSRC (BB/R013063/1). SC is funded by the Medical Research Council UK (Grant no. MR/M01360X/1, MR/R020973/1) and BBSRC (BB/R013063/1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

E.D.B., T.G.C. and S.C. conceived and designed the study; E.M.W. and D.A.B. cultured malaria parasites and E.M.W., C.R., J.G.D., R.M.d.S., C.R.F.M., C.J.S. and D.A.B. contributed parasite DNA for sequencing; P.F.d.S., M.L.H. and S.C. coordinated the sequencing of samples; E.D.B. and D.R.O. performed the statistical analysis, and F.M., T.G.C. and S.C. wrote the first draft of the manuscript. The final manuscript was read and approved by all authors.

Additional Information

Supplementary information accompanies this paper at [https://doi.org/10.1038/s41598-018-33767-3](https://doi.org/10.1038/s41598-018-33767-3).

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.