Genetic diversity of *Plasmodium vivax* isolates from pregnant women in the Western Brazilian Amazon: a prospective cohort study

Jamille Gregório Dombrowski,[a] *Holly Acford-Palmer,*[b] *Monica Campos,*[b] *Erika Paula Machado Separovic,*[a] *Sabrina Epiphanio,*[c] *Taane Gregory Clark,*[b,d] *Susana Campino,*[b,e,**] *and Claudio Romero Farias Marinho*[a,e,*]

[a]Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[b]Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom
[c]Department of Clinical and Toxicological Analyses, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[d]Faculty of Epidemiology and Population Health, London School of Hygiene & Tropical Medicine, London, United Kingdom

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Summary

Background Each year, 92 million pregnant women are at risk of contracting malaria during pregnancy, with the underestimation of the mortality and morbidity burden associated with *Plasmodium vivax*. During pregnancy, *P. vivax* infection is associated with low birth weight, maternal anaemia, premature delivery, and stillbirth. In the State of Acre (Brazil), high transmission leaves pregnant women at greater risk of contracting malaria and having a greater number of recurrences. The study of genetic diversity and the association of haplotypes with adverse pregnancy effects is of great importance for the control of the disease. Here we investigate the genetic diversity of *P. vivax* parasites infecting pregnant women across their pregnancies.

Methods *P. vivax* DNA was extracted from 330 samples from 177 women followed during pregnancy, collected in the State of Acre, Brazil. All samples were negative for *Plasmodium falciparum* DNA. Sequence data for the *Pvmsp1* gene was analysed alongside data from six microsatellite (MS) markers. Allelic frequencies, haplotype frequencies, expected heterozygosity (H_E) were calculated. Whole genome sequencing (WGS) was conducted on four samples from pregnant women and phylogenetic analysis performed with other samples from South American regions.

Findings Initially, the pregnant women were stratified into two groups—1 recurrence and 2 or more recurrences—in which no differences were observed in clinical gestational outcomes or in placental histological changes between the two groups. Then we evaluated the parasites genetically. An average of 18.5 distinct alleles were found at each of the MS loci, and the H_E calculated for each marker indicates a high genetic diversity occurring within the population. There was a high percentage of polyclonal infections (61.7%, 108/175), and one haplotype (H1) occurred frequently (20%), with only 9 of the haplotypes appearing in more than one patient.

Interpretation Most pregnant women had polyclonal infections that could be the result of relapses and/or re-infections. The high percentage of H1 parasites, along with the low frequency of many other haplotypes are suggestive of a clonal expansion. Phylogenetic analysis shows that *P. vivax* population within pregnant women clustered with other Brazilian samples in the region.

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Keywords: Malaria in pregnancy; Placental malaria; *P. vivax*; Recurrence; Genetics

*Corresponding author. Department of Parasitology, Institute of Biomedical Sciences - ICB, University of São Paulo – USP, Av. Prof. Lineu Prestes, 1374, São Paulo, 05508-000, Brazil.
**Corresponding author. Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine - LSHTM, Keppel Street, WC1E 7HT, London, United Kingdom.
E-mail addresses: marinho@usp.br (C.R.F. Marinho), Susana.Campino@lshtm.ac.uk (S. Campino).
[e]These authors contributed equally to this work.

Research in context

Evidence before this study

We searched PubMed for articles published up to May 20, 2022, without date or language restrictions, using the search terms: "malaria in pregnancy" [All fields] OR "placental malaria" [All fields] OR ("malaria" AND pregnant women" [All fields]) AND "genetic diversity" [All fields] OR "genotyping" [All fields] OR "microsatellite markers" [All fields]. Our search identified many studies involving genetics and molecular markers in *Plasmodium falciparum*, but only six combined *Plasmodium vivax* with genotyping results in the pregnant population. Importantly, none of these studies wholly followed the pregnancy to fully characterize the outcome of malaria infection, either maternal clinical profile or placental changes.

Added value of this study

Our results reflect the possible impact of the parasite in various facets of malaria in pregnancy, which represent a public health problem. On the one hand, the possible implications of specific parasite haplotypes in placental pathology, and on the other hand, the type of treatment available for pregnant women may not be enough to reduce the impact of the disease. The genomic analysis of *P. vivax* allowed to clarify some biological aspects of the parasite associated with gestational malaria, such as its high genetic diversity throughout all infections during pregnancy and its location in the geographic cluster in South America.

Implications of all the available evidence

Our observations complement current studies on recurrences of *P. vivax* infections in pregnancy, opening the way for further investigations into the pathophysiology and genetics of parasites in infections in pregnant women. Thus, our results may contribute to designing new strategies for treating the disease in pregnant women in Brazil, mitigating the devastating effects of this disease in such a vulnerable population.

Introduction

Malaria in pregnancy, caused by *Plasmodium* parasites, is a serious public health problem impacting on maternal and foetal health.[1] They may result in maternal anaemia, intrauterine growth restriction (IUGR), low birth weight (LBW), and reduced foetal viability.[2] Both IUGR and LBW are strongly associated with neonatal mortality and morbidity, as well as inhibited growth and cognitive development in children with chronic diseases in adulthood.[3]

P. vivax is the most geographically distributed of all human malaria parasite species and entails a complex and distinguished biology that compromises the control and eradication of the disease. *P. vivax* has the ability to establish dormant uninucleated forms in hepatocytes upon sporozoite invasion, the hypnozoites.[4,5] Hypnozoite activation can occur in weeks, months or years after the first infection, thereby contributing to ongoing trasmission.[6] Furthermore, the only drug regimen currently available in Brazil that prevents *P. vivax* hypnozoites activation is a 7–14 days course of Primaquine, producing severe haemolysis in people who are glucose-6-phosphate dehydrogenase (G6PD) deficient. Likewise, it is not recommended for pregnant women and infants less than 6 months of age,[4] two population groups that commonly face multiple and consecutive relapses.[7,8]

P. vivax burden in pregnancy is less described than *P. falciparum*, but recent studies indicate that both species represent a threat to the mother and foetus.[2,9,10] Together with the biology of *Plasmodium* parasites, their genetic structure has been shown to impact in the development of the disease. Several studies have shown that *P. falciparum* genotypes can be correlated to differential disease outcomes, and specific haplotypes have been associated with LBW.[11–13] Knowledge of the genomic structure of *P. vivax* has increased in recent years, revealing higher genetic diversity than *P. falciparum*.[8,14–16] Also, it was suggested that *vivax*-infected pregnant women are found to be commonly infected by parasites with a specific haplotype and they have greater susceptibility to infection with increasing multiplicity of *P. vivax*.[8,17]

In recent years, the number of reliable methods for genotyping *P. vivax* populations has increased. Studies on the genetic diversity of this parasite have efficiently used microsatellite (MS) markers.[8,18] The wide use of these markers is mainly due to their abundance, the high degree of mutations, and their neutral or nearly neutral evolution.[18] Furthermore, MS markers are an important tool in studies that seek to distinguish recrudescence/relapse from new infections.[18,19]

Due to the high number of recurrences, we believe that through the genetic study of the parasite it is possible to distinguish these recurrences and verify the association of specific haplotypes with adverse pregnancy outcomes. Here, we study *P. vivax* genetic diversity on samples isolated from pregnant women collected throughout the pregnancy, through the application of candidate-gene target sequencing, microsatellite genotyping and whole-genome sequencing. This combined approach allows for the investigation of different recurrences involving parasites that are genetically heterologous or homologous, and an assessment of associations with placental pathology and adverse pregnancy outcomes. In addition, in geographical regions where *P. vivax* populations have a high

degree of genetic diversity,[18] it is possible to assess whether different haplotypes of the parasite are related to a greater severity of disease. The resulting clarification of *P. vivax* biological aspects associated with gestational malaria may contribute to help the design of more adequate tools for the control and elimination of malaria, especially in the pregnant women population.

Methods

Samples and phenotypes

We conducted a prospective follow-up study (cohort) for a period of 23 months in the Juruá region—Acre (Brazil), which enrolled 600 pregnant women malaria-infected and non-infected that upon informed consent accepted to integrate within the study. The enrolled women were followed until delivery, with at least three domiciliary visits to monitor their clinical state and collect a peripheral blood sample, and placenta at delivery. In each malaria episode during pregnancy, an additional blood sample was collected. All the samples collected throughout the pregnancy were screened for the presence of malaria parasites, through microscopic analysis and confirmed by a specific real-time PCR technique (PET-PCR) that detect the *Plasmodium* genus, quantify abundance and discriminate species. Further details on the technique have been described elsewhere.[20,21] For this study, we selected samples positive for *P. vivax*. All *vivax*-infected pregnant women were treated with a regimen of Chloroquine for three days, a total of 25 mg/kg, according to the Brazilian Ministry of Health guidelines. At the end of the process of exclusion, a total of 397 pregnant women remained for evaluation. However, only 85 had their samples genotyped, being distributed throughout the study region (Fig. 1A and B). All details of the exclusion and sampling process are detailed in the supplementary material (Appendix p 2 and Supplementary Table S1)

Histopathology evaluation

The histopathologic examination involved using placental tissue slides. The Hematoxylin-Eosin-staining allowed the evaluation of syncytial knots, fibrin deposition, fibrinoid necrosis, and placental barrier thickness.[22] The leukocyte (CD45) and mononuclear inflammatory cells infiltrate (CD68) were assessed by immunohistochemistry using the tissue microarray (TMA) technique. The images of placenta were analysed by Image J software.

Pvmsp1 gene sequencing

A very polymorphic region of 800bp of the *Pvmsp1* gene was sequenced using Sanger sequencing and following a previously described protocol.[23] Briefly, the cycling parameters for PCR were as follows: an initial denaturation step at 94 °C for 3 min preceded the 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 35 s and extension at 72 °C for 45 s. After a final annealing step followed by 10 min of extension, the reaction was stopped. PCR products were purified using the QIAquick PCR Purification Kit according to manufacturer's instructions (Qiagen) and stored at 10 °C until analysis. A total of 10 µl of purified PCR samples were sent to the Sanger Sequencing Service at University College London (UCL), following the institution's protocol - capillary sequencing was performed using the Big Dye™ Terminator v3.1 and analysis using the ABI

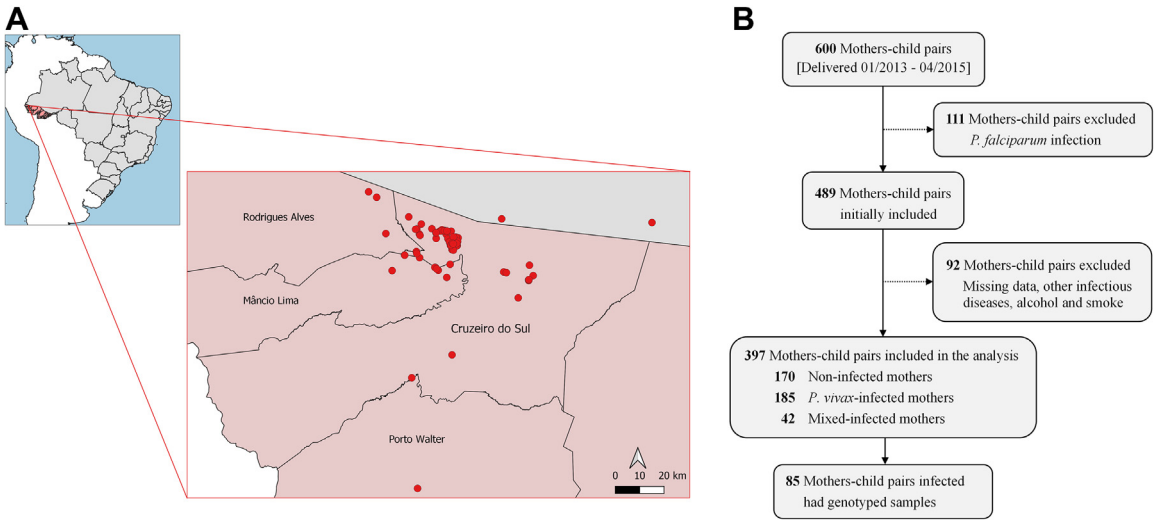

Fig. 1: **A) Map with the spatial distribution of pregnant women with genotyped samples. B) Flow chart detailing exclusion criteria applied prior to data analysis.**

3730xl DNA Analyzers for capillary electrophoresis and fluorescent dye terminator detection. A total of 175 samples (85 pregnant women) had enough DNA quantity and quality for sequencing. Results were analysed using BLAST (NCBI) to confirm gene sequences, and Clustal Omega (EBI) and Aliview[24] for sequence alignment and visualisation.

Microsatellite genotyping

The following MS markers: msp1F3, pv3.27, MS16, MS2, MS8 and MS10[18,25] were used. Each MS contains a forward primer labelled with different fluorescent reporter dyes (6-FAM, VIC, NED) (Supplementary Table S3). A PCR was performed individually for each MS marker with the following condition: 5.0 µl (5×) of New England BioLabs Q5 reaction buffer, 0.5 µl (10 nm) of Roche Diagnostics dNTPs, 0.125 µl (100 nm) of each MS forward primer, 0.125 µl (100 nm) of each microsatellite reverse primer, 0.2 µl of New England BioLabs high fidelity hot start DNA polymerase, 17.5 µl of H2O, and 1.5 µl of template DNA. Cycling parameters were the same for all primer pairs: 1 cycle at 98 °C for 30 s; 40 cycles at 98 °C for 10 s, 55 °C for 25 s, and 72 °C for 25, and a final cycle of 5 min at 72 °C.

To confirm successful amplification of the *P. vivax* DNA had taken place, a 2% agarose gel electrophoresis stained with SYBR Safe was used. The PCR products of each microsatellite markers were diluted in accordance with strength of the bands' fluorescence seen on the gel image. The samples were diluted to prevent over fluorescence by any one marker. Length variation of labelled PCR products was performed using an Applied Biosystems 3500 Genetic Analyser. The fragment size data was standardised using the LIZ600 internal DNA sizing standard, and the data was analysed on Applied Biosystem's GeneMapper software, version 4.0.

Whole genome sequencing

Four of the samples from two pregnant women (two time points from each pregnant woman—ID 30 and 77 in the S1 Dataset), assigned the same haplotype from the primary Sanger sequencing and microsatellite markers analysis, were randomly selected to be whole genome sequenced. The 150bp paired-end DNA libraries for WGS Illumina MiSeq analysis were prepared using the QIAseq FX DNA Library Kit (Qiagen) in accordance with manufacturer's instructions. Library concentration was estimated by qPCR using the NEBNext Library Quant Kit for Illumina (New England BioLabs), with The Agilent Technologies 2100 Expert Bioanalyser with 7500 DNA reagents (Agilent Technologies) and DNA chips (Agilent Technologies) being used to estimate individual library DNA fragment sizes. The DNA libraries were pooled to create a final library concentration of 4 nM, spiked with 1% of Phi X (Illumina) control library. Cluster generation and 150bp read paired-end sequencing were conducted according to manufacturers (Illumina) instructions on the Illumina MiSeq system with the corresponding v2 (500-cycles) reagent kit.

Whole genome sequence data for samples from Colombia, Peru, Mexico and Brazil were publicly available from MalariaGEN (https://www.malariagen.net/parasite/p-vivax-genome-variation) and our own sequence data.[26,27]

Raw whole genome sequence data was mapped to a *P. vivax* reference genome (*P. vivax* P01_v1, http://genedb.org) using the bwa-mem read mapping tool under the parameters for perfect read matching with allowance for pre-identified SNPs. (Sub-)telomeric, repetitive and non-unique regions (e.g., *vir* genes) were excluded from the analysis. Using the resulting alignments and coverage, genetic variants (e.g., SNPs, small insertions and deletions, and copy number variations) were identified by GATK (version 4.1.4.1—https://gatk.broadinstitute.org/hc/en-us) and samtools software (version 1.9—http://www.htslib.org/). These variants used for further population genetic analysis. Details of the bioinformatics pipeline have been described elsewhere.[26,28]

To assess parasite population diversity a pairwise distance matrix calculated based on matching sequence SNPs. The matrix was used to create principal component analysis (PCA) plots and a neighbour joining (NJ) tree (made using "ape" package in R (version 5.4-1)). All raw sequence data from the samples sequenced in this study are available from the European Nucleotide Archive (see https://www.ebi.ac.uk/ena/browser/view/PRJEB36199).

Population genetics and statistical analysis of association

The R statistical package was used to analyse the genomic variation data. Allele frequencies were calculated for parasite populations across groups based on geographical regions and clinical status of pregnant patients. Expected heterozygosity (H_E) was calculated as a measurement of genetic diversity at each SNP using the formula $H_E = (n/(n-1))(2pq)$, where n is the number of samples and p and q (=1 − p) the frequency of each allele. Linkage disequilibrium was calculated to identify potential associations between marker loci. Specifically, the R^2 (Pearson correlation coefficient) measure was implemented using the "LDcorSV" library within the R statistical package.

Descriptive statistics were carried out using Stata, version 14.2 or GraphPad Prism, version 6.0 software. Differences between groups were evaluated using Mann–Whitney test and Kruskal–Wallis tests, followed by the Dunn post hoc multiple comparison test as appropriate. Differences between categorical data and

proportions were analysed using Pearson's chi-squared test or Fisher's exact test. To assess the association between haplotypes and adverse pregnancy outcomes, adjusted odds ratios (aOR) with 95% confidence intervals (CI) were estimated using multivariate logistic regression. Statistical significance was set at a p-value of 0.05 and all statistical tests were 2-sided.

Ethics statement

Ethical clearance was provided by the committees for research of the University of São Paulo (Plataforma Brasil, CAAE: 03930812.8.0000.5467), according to resolution number 466/12 of Brazilian National Health Committee. Informed written consent was obtained from all participants. The study was conducted in accordance with the Good Clinical Practice Guidelines and the Declaration of Helsinki. This study is following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement guidelines (Supplementary Table S2).

Role of the funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Study population and baseline characteristics

A total of 397 pregnant women were eligible for further analysis after application of the exclusion criteria (Fig. 1B, Appendix p 2 and Supplementary Table S1). Of these, 170 were classified as non-infected (NI)—as they did not present infection during the current pregnancy-,185 as *P. vivax (Pv)*-infected and 42 mixed infected during gestation (Fig. 1B). To avoid bias in epidemiological and clinical analyses, pregnant women who had mixed infection during pregnancy (*P. vivax* + *P. falciparum*), and their respective samples, were used only in genetic analyses. The overall socio–demographic profile and clinical characteristics of the stratified participants is summarised (Table 1). We observed that *Pv*-infected pregnant women are marginally younger and, despite the higher percentage of rural residence in relation to NI pregnant women, it is worth noting that more than 70% (134/185) live in urban areas. *Pv* positive women reported more previous malaria episodes (163/185, 88.6%) than their NI counterparts (92/170, 54.1%) regardless the number of recurrences during current pregnancy.

Regarding the clinical characteristics, *Pv*-infected pregnant women presented reduced maternal weight gain, lower haematocrit, and haemoglobin, as well as reduced placental weight when compared to NI-pregnant women. Moreover, the stratification of the infected pregnant women by number of recurrences revealed that there is no significant difference in clinical characteristics between those *Pv*-infected with only 1-episode and those with 2 or more episodes in pregnancy (Table 1).

Placental histologic alterations resulting from *Pv*-infection are independent of the number of recurrences

Key placental histologic features of malaria in pregnancy[22] were assessed to address whether or not the number of *Pv*-infections leads to histologic and cytological alterations. We only had access to 297 placentas (155 from non-infected pregnant women and 142 from pregnant women with *Pv* infection). The analysis revealed that marked differences between placentas collected from *Pv* women and non-infected pregnant women (Table 2). We observed a significant increase in the number of syncytial nuclear aggregates (SNA) and fibrin deposition even with only 1 episode during pregnancy (median, SNA – 1-*Pv* 15.5 *vs.* NI 13.0, $p = 0.02$; fibrin – 1-*Pv* 2.0 *vs.* NI 1.9, $p = 0.0003$). In the same extent, we detected a significant increase in leukocyte and monocyte infiltrate in placentas from the *Pv*-1 episode group (median, leucocytes – 1-*Pv* 23 *vs.* NI 15.0, $p < 0.0001$; monocytes – 1-*Pv* 8.0 *vs.* NI 4.0, $p < 0.0001$) (Table 2). Additionally, we observed that these marked placental changes are also present in the group of pregnant women with more than 2 episodes of *Pv* during pregnancy, mainly fibrin deposition and fibrinoid necrosis (median, fibrin – 2-*Pv* 2.4 *vs.* NI 1.9, $p = 0.0001$; fibrinoid necrosis – 2-*Pv* 7.9 *vs.* NI 6.8, $p = 0.03$) (Table 2).

Haplotype diversity

Of the 177 pregnant women who met the inclusion criteria for the genetic study (details in Appendix p 2 and Supplementary Table S1) and who had blood samples collected across their pregnancy (330 samples), we obtained nucleotide sequencing and MS marker data for 85 woman (175 samples). From these, 40 women (47.1%) had only one recurrence in pregnancy with a median of 56 days from the first infection (interquartile range [IQR] 41–100 days); 27 women (31.8%) had 2 recurrences after the first infection (median 118 days; IQR 93–156 days); and 17 women (20%) had 3 or more recurrences of infections during pregnancy (median 167 days; IQR 126–205 days). It is important to note that all pregnant women were treated only with chloroquine following the guidance of the Brazilian Ministry of Health, therefore not using primaquine.

A total of 143 samples had nucleotide sequencing data for the *Pvmsp1* gene, which revealed the presence

Characteristics	Non-infected (N = 170)	*P. vivax* (N = 185)	p value[a]	*P. vivax* 1-episode (N = 79)	p value[b]	*P. vivax* 2+ episodes (N = 106)	p value[c]
Age, years	23.0 (19.0–28.0)	21.0 (17.0–26.0)	0.004	23.0 (17.0–28.0)	0.12	20.0 (17.0–25.0)	0.0004
Gravidity	2.0 (1.0–2.0)	2.0 (1.0–2.0)	0.47	2.0 (1.0–2.0)	0.06	2.0 (1.0–2.0)	0.43
Occupation			0.001		0.009		0.006
Farmer	3 (1.8)	11 (6.0)		5 (6.3)		6 (5.7)	
Housewife	77 (45.3)	99 (53.5)		43 (54.4)		56 (52.8)	
Student	36 (21.2)	46 (24.9)		18 (22.8)		28 (26.4)	
Other	54 (31.7)	29 (15.6)		13 (16.5)		16 (15.1)	
Bed net[d]	93 (79.5)	113 (68.9)	0.05	51 (71.8)	0.23	62 (66.7)	0.04
Rural residence	8 (4.7)	51 (27.6)	<0.0001	19 (24.1)	<0.0001	32 (30.2)	<0.0001
Malaria history[e]	92 (54.1)	163 (88.6)	<0.0001	69 (88.5)	<0.0001	94 (88.7)	<0.0001
Clinical outcomes							
Gestational age at delivery, weeks[f]	40.0 (39.0–40.0)	39.0 (38.0–40.0)	0.12	39.0 (38.0–40.0)	0.04	39.0 (38.0–40.0)	0.19
Maternal weight gain, Kg[g]	13.5 (10.0–16.8)	11.0 (8.0–14.0)	<0.0001	10.5 (7.0–14.0)	0.0003	11.0 (8.0–14.5)	0.0002
Haematocrit during pregnancy, %[h]	36.6 (34.5–38.6)	34.8 (32.4–37.4)	<0.0001	34.9 (32.0–37.5)	0.001	34.6 (32.4–37.3)	<0.0001
Haemoglobin during pregnancy, g/dL[i]	12.2 (11.6–12.8)	11.7 (10.8–12.3)	<0.0001	11.8 (10.6–12.6)	0.002	11.6 (10.8–12.2)	<0.0001
Placental weight, g[j]	577.4 (508.3–658.9)	542.3 (483.3–612.1)	0.01	544.0 (501.3–608.6)	0.05	542.3 (468.5–615.8)	0.009

Abbreviations: N, total number of individuals; Infec, infection; Kg, kilograms; g, grams. Results are presented as median and interquartile range or total number of events (n) and percentage (%). Statistical tests were applied according to the type of variable and groups: Mann–Whitney test to compare Non-infected and *P. vivax* groups, Kruskal–Wallis test with Dunn post-hoc test to compare Non-infected × 1-*P. vivax* × 2-*P. vivax* groups, and Chi-square test to compare the variables with percentage; statistically significant difference was observed between the 1-*P. vivax* and 2-*P. vivax* groups only in the age variable - p = 0.04. [a]Comparisons between Non-Infected and *P. vivax* group. [b]Comparisons between Non-Infected and *P. vivax* 1-episode groups. [c]Comparisons between Non-Infected and *P. vivax* 2 or more episodes. [d]The bed net variable was calculated based on the pregnant women who owned and regularly used a bed net. [e]Malaria history was recorded in 184 *P. vivax*-pregnant women (78 *P. vivax* 1-episode). [f]Gestational age was recorded in 168 non-infected pregnant women and 172 *P. vivax* (74 *P. vivax* 1-episode and 98 *P. vivax* 2 or more episodes). [g]Maternal weight gain was recorded in 163 non-infected pregnant women and 139 *P. vivax* (55 *P. vivax* 1-episode and 84 *P. vivax* 2 or more episodes). It was determined as the final weight minus the initial weight during pregnancy. [h]Haematocrit during pregnancy was recorded in 152 non-infected pregnant women and 154 *P. vivax* (65 *P. vivax* 1-episode and 89 *P. vivax* 2 or more episodes). [i]Haemoglobin during pregnancy was recorded in 157 non-infected pregnant women and 154 *P. vivax* (64 *P. vivax* 1-episode and 90 *P. vivax* 2 or more episodes). [j]Placental weight was recorded in 155 non-infected pregnant women and 142 *P. vivax* (56 *P. vivax* 1-episode and 86 *P. vivax* 2 or more episodes).

Table 1: **Socio-demographic profile and clinical characteristics of the women who participated in the study according to infection status.**

of 12 haplotypes of which one was the most frequent (54.6%). The other haplotypes range in frequency between 0.7% and 13.3% (Supplementary Table S4). For 50 pregnant women, it was possible to obtain sequence data for at least one more time point during pregnancy, and 78% present the same haplotype in the 2 time points. Of the samples analysed, 171 (97.7%) had full allelic data for at least 3 of the MS loci (MSP1f3, Pv3.27 and MS16—primary panel). These 3 markers formed the basis of the original haplotype (H) designation system. Any sample with more than one allele for a single marker was considered a polyclonal infection. A total of 27 unique haplotypes were identified with the initial 3 markers, more than double compared to using msp1 sequence data alone. From these markers, 20% (44/220) of total isolates were typed as having haplotype 1 (H1) (Supplementary Table S5), rising to approximately 26% (23/89) when polyclonal infections were discarded from the analysis (Supplementary Table S6); Haplotypes 15 (H15) and 16 (H16) are the next most frequent (9.5 and 15.9%, respectively) and were present in many polyclonal infections, but when only analysing non-mixed infections, the frequency drops to 2.2% and 9%, respectively. The remaining 24 haplotypes occur at a far lower frequency, only occurring in a maximum of 3 patients (8%) each, some reoccurred in patients, others were unique cases.

Of 85 patients analysed with the original MS loci panel, 28.2% contained a recurring single haplotype. The recurring haplotype was either a monoclonal infection at all analysed time points during pregnancy or a polyclonal infection followed by a monoclonal infection containing a haplotype seen in the patient's previous infections.

These samples were analysed with a secondary panel (MS2, MS8 and MS10), along with several polyclonal infections that contained H1, H15 or H16 (S1 Dataset). Nineteen of these patients originally thought to have recurrent infections with the same *P. vivax* clone, were found to be distinguishable infections with different haplotypes or had a polyclonal infection. The remaining five patients had a clonal parasite reoccurring throughout their infections.

Genotyping data from the MS markers was used to calculate the number of distinct alleles and the heterozygosity (H_E) (Supplementary Table S7). The high number of distinguishable allelic forms observed for each locus and the high value of heterozygosity indicated that overall, the *P. vivax* isolates circulating in the pregnant women had a high degree of genetic diversity.

Characteristics	Non-infected (N = 155)	*P. vivax* (N = 142)	p value[a]	*P. vivax* 1 episode (N = 56)	p value[b]	*P. vivax* 2+ episodes (N = 86)	p value[c]
Syncytial nuclear aggregates	13.0 (10.0–17.0)	15.0 (11.0–21.0)	0.01	15.5 (11.0–20.5)	0.02	15.0 (10.0–21.0)	0.01
Fibrin deposition score	1.9 (1.9–2.8)	2.1 (1.9–2.8)	<0.0001	2.0 (1.9–2.8)	0.0003	2.4 (1.9–2.8)	0.0001
Fibrinoid necrosis[d]	6.8 (3.9–10.2)	7.5 (5.1–10.6)	0.07	7.0 (5.1–10.8)	0.18	7.9 (5.1–10.4)	0.03
Placental barrier thickness[e]	3.1 (2.8–3.5)	3.1 (2.8–3.5)	0.98	3.2 (2.8–3.5)	0.32	3.1 (2.8–3.4)	0.35
Leukocytes infiltrate[f]	15.0 (9.0–21.0)	21.5 (14.0–29.0)	<0.0001	23.0 (15.0–30.0)	<0.0001	21.0 (14.0–28.0)	0.0003
Mononuclear cells infiltrate[g]	4.0 (2.0–6.0)	8.0 (5.0–12.0)	<0.0001	8.0 (6.0–14.0)	<0.0001	8.0 (5.0–11.0)	<0.0001

Abbreviations: N, total number of individuals. Results are presents in median and interquartile range. Statistical tests were applied according to the type of variable and groups: Mann–Whitney test to compare Non-infected and *P. vivax* groups, and Kruskal–Wallis test with Dunn post-hoc test to compare Non-infected × 1-*P. vivax* × 2-*P. vivax* groups; no statistically significant difference was observed between the 1-*P. vivax* and 2-*P. vivax* groups. [a]Differences between Non-Infected and *P. vivax* group. [b]Differences between Non-Infected and *P. vivax* 1 episode group. [c]Comparisons between Non-Infected and *P. vivax* 2 or more episodes. [d]Fibrinoid necrosis was recorded in placentas from 124 *P. vivax*-pregnant women (51 *P. vivax* 1 episode and 73 *P. vivax* 2 or more episodes). [e]Placental barrier thickness was recorded in placentas from 128 *P. vivax*-pregnant women (51 *P. vivax* 1 episode and 77 *P. vivax* 2 or more episodes). [f]Leukocyte infiltrate (CD45+) was recorded in placentas from 153 non-infected and 120 *P. vivax*-pregnant women (50 *P. vivax* 1 episode and 70 *P. vivax* 2 or more episodes). [g]Mononuclear cells infiltrate (CD68+) was recorded in placentas from 154 non-infected and 141 *P. vivax*-pregnant women (85 *P. vivax* 2 or more episodes).

Table 2: **Placental parameters of non-infected and infected pregnant women.**

This observation holds even when stratified by the number of recurrences of infection during pregnancy (Supplementary Table S8).

To detect any non-random associations between alleles, linkage disequilibrium (LD) was assessed using the R^2 measure (correlation coefficient). All R^2 values were below 0.1, which indicates no association between the alleles at each marker (Supplementary Table S9). This was expected as all MS loci are present on different chromosomes but reassures that none of the markers are introducing bias to the analysis, by influencing the allele present in the remaining loci.

Monoclonal and polyclonal infections

Of the 175 isolates analysed, 48.6% (85) demonstrated indistinguishable polyclonal *P. vivax* infections (had more than one allele present at, at least one marker of the primary panel: MSP1f3, Pv3.27 and MS16). The remaining 90 infections were assigned one of the 27 haplotypes corresponding to their allelic profile.

Samples that begun with a polyclonal infection were not found to increase the likelihood of recurrent mixed infections. However, samples were more likely to be polyclonal the more infections a patient had, suggesting the pregnant women are potentially relapsing due to the inactivity of chloroquine against hypnozoites and/or being re-infected with new haplotypes. The polyclonal infections preceded or followed by single haplotype infections contained the original haplotype 64.3% (18/28) of the time. Therefore, these recurrent infections are probably relapsing from the initial polyclonal infection (S1 Dataset).

Distribution of haplotypes across different clinical characteristics and placental parameters

Based on Tables 1 and 2, we selected the main variables to identify the association with the most frequent haplotypes (H1, H15 and H16). For this analysis, only pregnant women who had *P. vivax* infection during pregnancy were used (n = 63). Therefore, a multivariate logistic regression analysis was performed to find any association between the most frequent haplotypes and the clinical and placental variables of pregnancy (Supplementary Table S10). Multivariate logistic regression revealed that none of the haplotypes were associated with these variables in the unadjusted analysis. However, when they were adjusted for the place of residence, number of antenatal visit and for the other variables in the block, we observed a lower chance of inflammatory infiltrate, mainly by mononuclear cells, when the pregnant woman was infected with H1 (adjusted odds ratio [aOR] 0.81, CI 95% 0.67–0.99, p = 0.038). These results are inconclusive due to the small sample size, and a larger number of pregnant women would be essential for a robust analysis.

Genomic diversity of samples with haplotype 1

Two randomly selected patients (two time points from each pregnant woman—ID 30 and 77 in the S1 Dataset), each had two consecutive H1 infections, were also whole genome sequenced. To analyse the genetic diversity of these samples, WGS was performed, and 87,433 SNPs were identified across the 4 samples, averaging around 21,000 SNPs per sample (sample 1: 26,893, sample 2: 21620, sample 3: 23,787 and sample 4: 16,045) compared to the reference strain. These samples contained high quantities of human DNA in comparison to parasite DNA, as the DNA was extracted directly from unprocessed blood. This resulted in a lower number of parasite SNPs to analyse from WGS.

A neighbour joining (NJ) tree (Fig. 2) using data from Brazil, Colombia, Mexico and Peru shows that the samples from this Brazilian pregnant population cluster together with the non-pregnant *P. vivax* infected

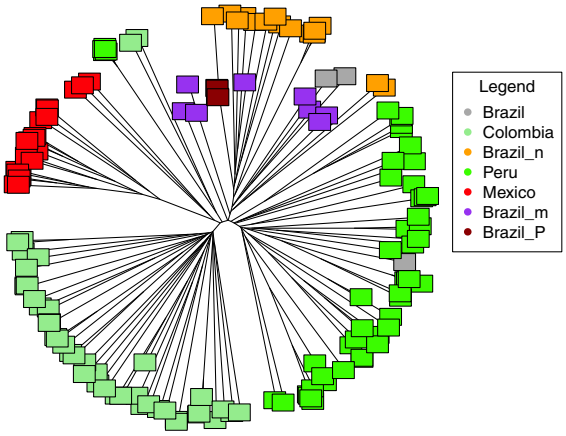

Fig. 2: **Neighbour-Joining Tree of *Plasmodium vivax* samples from Brazil and other regions in South America.** Samples from Colombia, Peru, Mexico, and Brazil were publicly available from MalariaGEN (https://www.malariagen.net/parasite/p-vivax-genome-variation); Brazil_n are samples collected by Oliveira et al.[30] also in the state of Acre; Brazil_m are samples from non-pregnant women collected in the same region and at similar times as the samples in this study (Brasil_P), in which we published in our previous studies[26,29]; Brazil_P are the samples of the two pregnant women in this study.

population from Brazil.[26,29] This demonstrates high levels of relatedness. All these samples from Brazil were collected in the state of Acre.[26,29,30] The rest of the samples in the NJ tree show geographical clustering of parasites. The 4 samples from the pregnant women are clustered together on the same branch indicating high similarity in the number of SNPs shared between these samples. These 4 samples appear to have emerged from the same clone, and a single sample from a non-pregnant patient also present on the branch suggests it is the same haplotype.

Discussion

To assess the burden of vivax relapses during pregnancy, it is necessary to distinguish the recurrences, which can be achieved through the target sequence of candidate genes, genotyping or sequencing of the entire genome of the parasites.[31] In this study, we used these approaches to characterise the genetic diversity of *P. vivax* populations in samples obtained from pregnant women in an endemic region in Brazil, in addition to looking for the association of frequent haplotypes with adverse pregnancy outcomes.

Currently, the only drug used to prevent *P. vivax* relapses is primaquine. However, this drug is not recommended for pregnant women or children, the population groups most affected by malaria. In fact, when compared to other groups these two groups face more cases of *P. vivax* recurrences, possibly relapses.[7] The genetic analysis of *P. vivax* makes it possible to clarify several biological aspects of the parasite, such as, for example, a better understanding of recurrences and possible distinction between relapse/recrudescence and new infections. Thus, the results described here reflect the many facets that malaria in pregnancy represents as a public health problem.[32] When evaluating the maternal clinical variables, it is observed that, in general, the infection has a strong impact on pregnancy, with a reduction in maternal weight gain as well as in haematocrit and haemoglobin levels. It is already well described that not only *P. falciparum* infection, but *P. vivax* infection in pregnancy is also associated with a high risk of maternal morbidity and mortality.[10,33,34] In addition, the pathology caused by the disease can influence the development of the foetus,[10,35] reinforcing the idea that the type of treatment available to pregnant women, in the case of *P. vivax*, may not be sufficient to reduce the impact of the disease.

Our data also indicated an aggravation when the pregnant woman had two or more recurrences during pregnancy, mainly in the variable placental weight reduction. The placental inflammatory process that acts on *P. vivax* infection may contribute to poor outcomes during pregnancy.[10,22] This is supported by the observation of significant histopathological changes in placentas when the mothers were infected by *P. vivax*. A localised inflammation can generate a frame of hypoxia/ischemia that would alter the transport of both nutrients and respiratory gases to the foetus, which can impact on foetal development due to the lack of adequate supply of nutrients and oxygen.[36] Also, the oxidative stress caused by hypoxia causes several structural and functional alterations in intrauterine development.[37] In the analysis of MS markers, we found that haplotype 1 (H1), the most frequent in populations, was associated with a lower inflammatory infiltrate. However, it is not possible to draw strong conclusions about the association of this haplotype with the improvement of the placental inflammatory process due to the small sample size, and further studies are needed.

Since the distinction between recrudescence, reinfection and relapse based on clinical observations alone is difficult and inconclusive, genomic studies of the parasite are essential. When genetically evaluating this dataset, we believe recrudescence is unlikely as all women were treated with chloroquine and recurrent infections were detected only 28 days after treatment, which is not typical of a recrudescence due to treatment failure. On the other hand, it is likely that the recurrent episodes analysed in this study were relapses originating from hypnozoites, as pregnant women cannot be treated with primaquine to eliminate this hepatic form of the parasite. The presence of the same parasite haplotype in recurrent infections might be indicative of a relapse, especially if the haplotype is rare or unique in the population. However, if a haplotype is frequent in the

population, such as H1, H15, and H16 seen here, it is more difficult to determine whether recurrent infections are due to a relapse or a new infection. Reinfections from mosquito bites are also likely to occur in particular in this *P. vivax* endemic region, which is considered one of the most endemic regions in Brazil.[33,38]

The high genetic diversity and the large number of polyclonal infections observed may be due to several factors, such as reduced levels of immunity to infections, increased susceptibility to mosquito bites, and physiological changes in pregnancy, as reported in other studies.[8,18] In addition, it was observed that most pregnant women had a previous history of malaria, and it is possible that these women may harbour hypnozoites upon preconception, and relapses may occur due to their immunocompromised state during pregnancy. The latent hypnozoites may result in heterologous relapse where the relapse appears to be a different haplotype to the original haplotype, confounding the results.[39]

The high number of distinguishable allelic forms observed for each locus and the high value of heterozygosity indicate that, in general, *P. vivax* circulating in the population of pregnant women showed a high degree of genetic diversity, which is in line with other studies carried out in Brazil in non-pregnant women.[14,30] The observed high frequency of specific alleles and haplotypes suggests a clonal expansion of the *P. vivax* population in this population in Acre. It is possible that this is a remnant of a historic clonal outbreak, and over time, low-frequency haplotypes are re-emerging, explaining the identification of a high number of alleles at lower frequencies and high genetic diversity. In a complementary way, population bottlenecks can also explain the observed clonal expansion, similar to the events observed in other South American countries.[40,41]

Phylogenetic analysis show that *P. vivax* population within pregnant women clustered with other Brazilian samples in the region.[29,30] The close distance between the Brazilian and Peruvian *P. vivax* samples is expected, as Acre borders with Peru.

Some limitations of this study must be noted. There are two caveats connected with sampling that need to be considered before drawing firm conclusions. First, for a genetic study, a larger number of pregnant women and samples might be needed. Second, although we were able to analyse a total of 175 isolates in 85 pregnant women, the association of haplotypes with adverse gestational events, which we present in the S10 Table, was impaired. This is because 23 pregnant women also had *P. falciparum* infection during pregnancy, being excluded from the clinical association analyses, which further reduced our sample size. Although this study does not provide a clear association between the genetic component of *P. vivax* infection and pregnancy outcomes, these results open the possibility for future investigations.

Overall, our work provides insights into the genomic diversity of *P. vivax* in pregnant women, which represents a serious public health problem. In addition, it contextualizes the population of these parasites with the population found in non-pregnant women in Brazil and in other South American countries. As relapses can be very common during pregnancy, it is crucial to find drugs to eliminate hypnozoites that will neither affect the mother nor the baby. In particular, it highlights that the type of treatment available for pregnant women is currently not adequate to reduce the impact of the disease. It is also important to implement better control strategies to avoid new infections from mosquito bites during pregnancy, such as expanding their distribution and encouraging the use of bed nets and insecticides. Finally, this work is extremely important since molecular surveillance of the genetic variability of *P. vivax* in the context of malaria in pregnancy is scarce and can assist in the design of more adequate tools for the control and elimination of malaria.

Contributors

JGD and CRFM designed the study. JGD, HAP, MC, EPMS, SE, TGC, CRFM and SC were involved in the data acquisition and scientific input. JGD, HAP, MC, TGC, CRFM and SC contributed to the analysis and/or interpretation of the data. JGD and SC had access and verified the data. JGD and SC wrote the manuscript. CRFM was the main funder of this work, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All the authors reviewed and approved the final version of this manuscript.

Data sharing statement

This data also includes samples from the MalariaGEN *P. vivax* Genome Variation project (publicly available data: https://www.malariagen.net/parasite/p-vivax-genome-variation), as described elsewhere.[27] Additional data is available online as Supplementary Material. All raw sequence data from the samples sequenced in this study are available from the European Nucleotide Archive (see https://www.ebi.ac.uk/ena/browser/view/PRJEB36199).

Editor note

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Declaration of interests

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lana.2022.100407.

References

1 WHO. *Word malaria report 2021*. 2021.
2 Fried M, Duffy PE. Malaria during pregnancy. *Cold Spring Harb Perspect Med*. 2017;7(6):a025551. https://doi.org/10.1101/cshperspect.a025551.
3 United Nations Children's Fund and World Health Organization. *Low birthweight: country, regional and global estimates*. 2004.
4 WHO. *Control and elimination of Plasmodium vivax malaria*. 2015.
5 Krotoski WA, Collins WE, Bray RS, et al. Demonstration of hypnozoites in sporozoite-transmitted Plasmodium vivax infection. *Am J Trop Med Hyg*. 1982;31(6):1291–1293. https://doi.org/10.4269/ajtmh.1982.31.1291.
6 Battle KE, Karhunen MS, Bhatt S, et al. Geographical variation in Plasmodium vivax relapse. *Malar J*. 2014;13:144. https://doi.org/10.1186/1475-2875-13-144.
7 White NJ, Imwong M. Relapse. *Adv Parasitol*. 2012;80:113–150. https://doi.org/10.1016/B978-0-12-397900-1.00002-5.
8 Thanapongpichat S, McGready R, Luxemburger C, et al. Microsatellite genotyping of Plasmodium vivax infections and their relapses in pregnant and non-pregnant patients on the Thai-Myanmar border. *Malar J*. 2013;12:275. https://doi.org/10.1186/1475-2875-12-275.
9 McGready R, Lee SJ, Wiladphaingern J, et al. Adverse effects of falciparum and vivax malaria and the safety of antimalarial treatment in early pregnancy: a population-based study. *Lancet Infect Dis*. 2012;12(5):388–396. https://doi.org/10.1016/S1473-3099(11)70339-5.
10 Dombrowski JG, Barateiro A, Peixoto EPM, et al. Adverse pregnancy outcomes are associated with Plasmodium vivax malaria in a prospective cohort of women from the Brazilian Amazon. *PLoS Negl Trop Dis*. 2021;15(4):e0009390. https://doi.org/10.1371/journal.pntd.0009390.
11 Ranjit MR, Das A, Das BP, Das BN, Dash BP, Chhotray GP. Distribution of Plasmodium falciparum genotypes in clinically mild and severe malaria cases in Orissa, India. *Trans R Soc Trop Med Hyg*. 2005;99(5):389–395. https://doi.org/10.1016/j.trstmh.2004.09.010.
12 Rorick MM, Rask TS, Baskerville EB, Day KP, Pascual M. Homology blocks of Plasmodium falciparum var genes and clinically distinct forms of severe malaria in a local population. *BMC Microbiol*. 2013;13:244. https://doi.org/10.1186/1471-2180-13-244.
13 Minja DTR, Schmiegelow C, Mmbando B, et al. Plasmodium falciparum mutant haplotype infection during pregnancy associated with reduced birthweight, Tanzania. *Emerg Infect Dis*. 2013;19(9):1446–1454. https://doi.org/10.3201/eid1909.130133.
14 Batista CL, Barbosa S, Da Silva Bastos M, Viana SAS, Ferreira MU. Genetic diversity of Plasmodium vivax over time and space: a community-based study in rural Amazonia. *Parasitology*. 2015;142(2):374–384. https://doi.org/10.1017/S0031182014001176.
15 Friedrich LR, Popovici J, Kim S, et al. Complexity of infection and genetic diversity in Cambodian Plasmodium vivax. *PLoS Negl Trop Dis*. 2016;10(3):e0004526. https://doi.org/10.1371/journal.pntd.0004526.
16 Arango EM, Samuel R, Agudelo OM, et al. Genotype comparison of Plasmodium vivax and Plasmodium falciparum clones from pregnant and non-pregnant populations in North-west Colombia. *Malar J*. 2012;11:392. https://doi.org/10.1186/1475-2875-11-392.
17 Marín-Menéndez A, Bardají A, Martínez-Espinosa FE, et al. Rosetting in Plasmodium vivax: a cytoadhesion phenotype associated with anaemia. *PLoS Negl Trop Dis*. 2013;7(4):e2155. https://doi.org/10.1371/journal.pntd.0002155.
18 Menegon M, Bardají A, Martínez-Espinosa F, et al. Microsatellite genotyping of Plasmodium vivax isolates from pregnant women in four malaria endemic countries. *PLoS One*. 2016;11(3):e0152447. https://doi.org/10.1371/journal.pone.0152447.
19 Restrepo E, Imwong M, Rojas W, Carmona-Fonseca J, Maestre A. High genetic polymorphism of relapsing P. vivax isolates in northwest Colombia. *Acta Trop*. 2011;119(1):23–29. https://doi.org/10.1016/j.actatropica.2011.03.012.
20 Dombrowski JG, Souza RM de, Lima FA, et al. Association of malaria infection during pregnancy with head circumference of newborns in the Brazilian Amazon. *JAMA Netw Open*. 2019;2(5):e193300. https://doi.org/10.1001/jamanetworkopen.2019.3300.
21 Lucchi NW, Narayanan J, Karell MA, et al. Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR. *PLoS One*. 2013;8(2):e56677. https://doi.org/10.1371/journal.pone.0056677.
22 Souza RM, Ataíde R, Dombrowski JG, et al. Placental histopathological changes associated with Plasmodium vivax infection during pregnancy. *PLoS Negl Trop Dis*. 2013;7(2):e2071. https://doi.org/10.1371/journal.pntd.0002071.
23 Imwong M, Pukrittayakamee S, Grüner A, et al. Practical PCR genotyping protocols for Plasmodium vivax using Pvcs and Pvmsp1. *Malar J*. 2005;4:20. https://doi.org/10.1186/1475-2875-4-20.
24 Larsson A. AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics*. 2014;30(22):3276–3278. https://doi.org/10.1093/bioinformatics/btu531.
25 Koepfli C, Mueller I, Marfurt J, et al. Evaluation of Plasmodium vivax genotyping markers for molecular monitoring in clinical trials. *J Infect Dis*. 2009;199(7):1074–1080. https://doi.org/10.1086/597303.
26 Benavente ED, Manko E, Phelan J, et al. Distinctive genetic structure and selection patterns in Plasmodium vivax from South Asia and East Africa. *Nat Commun*. 2021;12(1):3160. https://doi.org/10.1038/s41467-021-23422-3.
27 Pearson RD, Amato R, Auburn S, et al. Genomic analysis of local variation and recent evolution in Plasmodium vivax. *Nat Genet*. 2016;48(8):959–964. https://doi.org/10.1038/ng.3599.
28 Campino S, Benavente ED, Assefa S, et al. Genomic variation in two gametocyte non-producing Plasmodium falciparum clonal lines. *Malar J*. 2016;15:229. https://doi.org/10.1186/s12936-016-1254-1.
29 Benavente ED, Campos M, Phelan J, et al. A molecular barcode to inform the geographical origin and transmission dynamics of Plasmodium vivax malaria. *PLoS Genet*. 2020;16(2):e1008576. https://doi.org/10.1371/journal.pgen.1008576.
30 de Oliveira TC, Corder RM, Early A, et al. Population genomics reveals the expansion of highly inbred Plasmodium vivax lineages in the main malaria hotspot of Brazil. *PLoS Negl Trop Dis*. 2020;14(10):e0008808. https://doi.org/10.1371/journal.pntd.0008808.
31 White MT, Karl S, Koepfli C, et al. Plasmodium vivax and Plasmodium falciparum infection dynamics: re-infections, recrudescences and relapses. *Malar J*. 2018;17(1):170. https://doi.org/10.1186/s12936-018-2318-1.
32 Dellicour S, Tatem AJ, Guerra CA, Snow RW, Ter Kuile FO. Quantifying the number of pregnancies at risk of malaria in 2007: a demographic study. *PLoS Med*. 2010;7(1):e1000221. https://doi.org/10.1371/journal.pmed.1000221.
33 Pincelli A, Neves PAR, Lourenço BH, et al. The hidden burden of Plasmodium vivax malaria in pregnancy in the Amazon: an observational study in Northwestern Brazil. *Am J Trop Med Hyg*. 2018;99(1):73–83. https://doi.org/10.4269/ajtmh.18-0135.
34 Rogerson SJ, Desai M, Mayor A, Sicuri E, Taylor SM, van Eijk AM. Burden, pathology, and costs of malaria in pregnancy: new developments for an old problem. *Lancet Infect Dis*. 2018;18(4):e107–e118. https://doi.org/10.1016/S1473-3099(18)30066-5.
35 Moore KA, Simpson JA, Wiladphaingern J, et al. Influence of the number and timing of malaria episodes during pregnancy on prematurity and small-for-gestational-age in an area of low transmission. *BMC Med*. 2017;15(1):117. https://doi.org/10.1186/s12916-017-0877-6.
36 Mir IN, Johnson-Welch SF, Nelson DB, Brown LS, Rosenfeld CR, Chalak LF. Placental pathology is associated with severity of neonatal encephalopathy and adverse developmental outcomes following hypothermia. *Am J Obstet Gynecol*. 2015;213(6):849.e1–849.e7. https://doi.org/10.1016/j.ajog.2015.09.072.
37 Kurinczuk JJ, White-Koning M, Badawi N. Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum Dev*. 2010;86(6):329–338. https://doi.org/10.1016/j.earlhumdev.2010.05.010.

38 BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Situação Epidemiológica da Malária no Brasil. Brasília/DF. https://portalarquivos.saude.gov.br/images/pdf/2019/dezembro/03/3. a - Situação Epidemiológica da Malária_Brasil_CIT_28.11.2018.pdf; 2019.
39 Imwong M, Snounou G, Pukrittayakamee S, et al. Relapses of Plasmodium vivax infection usually result from activation of heterologous hypnozoites. *J Infect Dis*. 2007;195(7):927–933. https://doi.org/10.1086/512241.
40 Delgado-Ratto C, Gamboa D, Soto-Calle VE, et al. Population genetics of Plasmodium vivax in the Peruvian Amazon. *PLoS Negl Trop Dis*. 2016;10(1):e0004376. https://doi.org/10.1371/journal.pntd.0004376.
41 Villena FE, Lizewski SE, Joya CA, Valdivia HO. Population genomics and evidence of clonal replacement of Plasmodium falciparum in the Peruvian Amazon. *Sci Rep*. 2021;11(1):21212. https://doi.org/10.1038/s41598-021-00806-5.