Plasmodium ovale spp *dhfr* mutations associated with reduced susceptibility to pyrimethamine in sub-Saharan Africa: a retrospective genetic epidemiology and functional study

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Summary

Background Mutations in the *Plasmodium falciparum dhfr* gene confer resistance to pyrimethamine, which is widely used for malaria chemoprevention in Africa. We aimed to evaluate the frequency and evolution of *dhfr* mutations in *Plasmodium ovale* spp in Africa and their functional consequences, which are incompletely characterised.

Methods We analysed *dhfr* mutations and their frequencies in *P ovale* spp isolates collected between Feb 1, 2004, and Aug 31, 2023, from the French National Malaria Reference Centre collection and from field studies in Benin, Gabon, and Kenya. Genetic patterns of positive selection were investigated. Full-length recombinant wild-type and mutant DHFR enzymes from both *P ovale curtisi* and *P ovale wallikeri* were expressed in bacteria to test whether the most common mutations reduced pyrimethamine susceptibility.

Findings We included 518 *P ovale* spp samples (314 *P ovale curtisi* and 204 *P ovale wallikeri*). In *P ovale curtisi*, Ala15Ser-Ser58Arg was the most common *dhfr* mutation (39%; 124 of 314 samples). In *P ovale wallikeri*, *dhfr* mutations were less frequent, with Phe57Leu-Ser58Arg reaching 17% (34 of 204 samples). These two mutants were the most prevalent in central and east Africa and were fixed in Kenyan isolates. We detected six and four other non-synonymous mutations, representing 8% (24 isolates) and 2% (five isolates) of the *P ovale curtisi* and *P ovale wallikeri* isolates, respectively. Whole-genome sequencing and microsatellite analyses revealed reduced genetic diversity around the mutant *pocdhfr* and *powdhfr* genes. The mutant DHFR proteins showed structural changes at the pyrimethamine binding site in-silico, confirmed by a 4-times increase in pyrimethamine half-maximal inhibitory concentration in an *Escherichia coli* growth assay for the Phe57Leu-Ser58Arg mutant and 50-times increase for the Ala15Ser-Ser58Arg mutant, compared with the wild-type counterparts.

Interpretation The widespread use of sulfadoxine–pyrimethamine for malaria chemoprevention might have exerted fortuitous selection pressure for *dhfr* mutations in *P ovale* spp. This calls for closer monitoring of *dhfr* and *dhps* mutations in *P ovale* spp.

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Introduction

Malaria is a vector-borne parasitic disease causing approximately 249 million cases and 608 000 deaths worldwide in 2022, with about 95% of cases occurring in sub-Saharan Africa.[1] *Plasmodium ovale* spp, now referred to as *P ovale wallikeri* and *P ovale curtisi*,[2] account for up to 1·69% of cases in sub-Saharan Africa and 0·77% worldwide.[3] The prevalence of these overlooked species is likely to be underestimated due to low parasite densities and difficulties in detection by microscopy and rapid diagnostic tests, as recently demonstrated in Tanzania.[4] Like *Plasmodium vivax*, both *P ovale curtisi*[5] and *P ovale wallikeri*[6] have the ability to cause relapses, characterised by recurrent asexual parasitaemia arising from dormant liver forms (hypnozoites) after an initial infection. These dormant forms are refractory to most antimalarials and can therefore limit the control of those relapsing species.[7]

Introduced in many countries in the 1960s, sulfadoxine–pyrimethamine was formally adopted as first-line treatment for malaria in nine African countries in the 1990s[8] (and as chemoprophylaxis for travellers in the 1980s[9]). Its effectiveness was then compromised by the emergence and spread of sulfadoxine–pyrimethamine-resistant *Plasmodium falciparum* parasites.[10] This led to its replacement by artemisinin-based combination therapies or mefloquine as first-line treatment in the 2000s. However, sulfadoxine–pyrimethamine still forms the basis of preventive treatment strategies in vulnerable populations. The use of sulfadoxine–pyrimethamine for intermittent preventive treatment in pregnancy (IPTp) significantly reduces low

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Research in context

Evidence before this study

The antimalarial combination sulfadoxine–pyrimethamine is widely used in sub-Saharan Africa for intermittent preventive treatment in pregnancy, in infants, and for seasonal malaria chemoprevention. Over the past 30 years, *Plasmodium falciparum* has developed resistance to these drugs due to point mutations in the dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) genes, challenging the efficacy of sulfadoxine–pyrimethamine in sub-Saharan Africa. In a recent report from 2022, non-synonymous mutations in the *dhfr* gene of *P ovale* spp have been observed in sub-Saharan Africa. The frequency in a larger sample and the evolution of these *pocdhfr* and *powdhfr* mutations in sub-Saharan Africa remain to be fully elucidated, as does their functional effect.

We searched PubMed for articles published since database inception until Dec 31, 2023, using the search terms "(Plasmodium ovale) AND (dhfr OR powdhfr OR pocdhfr)". Of seven studies, three reported *dhfr* mutations in *P ovale curtisi* or *P ovale wallikeri* isolates from sub-Saharan Africa. In total, fewer than 100 isolates for these two malaria parasite species were screened for *dhfr* mutations, with missing data for many east African countries and incomplete *dhfr* coding sequences for 147 isolates. Chen and colleagues reported in 2022 reduced genetic diversity around *pocdhfr* in 69 isolates, possibly related to selective sweep, but *powdhfr* remains to be studied. Whole-genome data for *P ovale curtisi* and *P ovale wallikeri* are also very scarce, with approximately 20 genomes for either species available in the European Nucleotide Archive and National Center for Biotechnology Information databases, limiting population genomics studies. Finally, no clinical follow-up nor in-vitro experiments have been performed to demonstrate that *pocdhfr* and *powdhfr* mutations are associated with altered susceptibility to pyrimethamine. The paucity of data prompted us to conduct this study.

Added value of this study

We analysed the spectrum and frequency of *dhfr* mutations. To our knowledge, this is to date the largest study related to *P ovale* spp genomics and pyrimethamine resistance. We evidenced a selective sweep targeting both *pocdhfr* Ala15Ser-Ser58Arg and *powdhfr* Phe57Leu-Ser58Arg and provided data that support the different evolution of the two species at this locus associated with drug resistance. In particular, we described at least two distinct emergences for the Ala15Ser-Ser58Arg allele and only one dominant haplotype for the Phe57Leu-Ser58Arg allele. In addition, PocDHFR and PowDHFR in-silico modelling and half-maximal inhibitory concentration measurements both suggested a reduced susceptibility to pyrimethamine associated with these two main *dhfr* mutants.

Implications of all the available evidence

The overlooked *P ovale curtisi* and *P ovale wallikeri* species account for a meaningful proportion of malaria infections, particularly in east Africa (eg, up to 50% of malaria cases in Tanzania). The data collected here suggest that *P ovale curtisi* and *P ovale wallikeri* populations from central and east Africa are under antimalarial drug selection pressure, presumably from sulfadoxine–pyrimethamine. There is a need to monitor mutations in *dhfr* and other drug resistance genes in these two malaria species and to document the clinical impact of these mutations.

birth weight and neonatal mortality[11] and has been recommended by WHO since 1998. Remarkably, despite widespread pyrimethamine and sulfadoxine resistance, sulfadoxine–pyrimethamine remains of paramount clinical interest, particularly for birthweight outcomes.[12] In addition, seasonal malaria chemoprevention (SMC), consisting of a full course of sulfadoxine–pyrimethamine plus amodiaquine given monthly to children aged 3–59 months, was recommended by WHO in 2012.[13] In 2021, 15 countries in the Sahel region of Africa had active SMC programmes, representing 180 million doses of treatment per year.[14]

In *P falciparum*, pyrimethamine resistance is conferred by three main amino acid changes (Asn51Ile, Cys59Arg, and Ser108Asn) in the dihydrofolate reductase enzyme (DHFR), which are now highly prevalent in sub-Saharan Africa,[15] and an additional fourth (Ile164Leu) that confers very high rates of resistance. Due to the sustained and continuous use of sulfadoxine–pyrimethamine over decades, non-*falciparum* human malaria species have been exposed to pyrimethamine selection. Remarkably, two recent studies have reported distinct sets of mutations in the *pocdhfr* and *powdhfr* genes of African isolates,[16,17] including mutations at positions known to confer pyrimethamine resistance in *P falciparum* and *P vivax*. However, the number of isolates included was small (five *P ovale curtisi* and six *P ovale wallikeri* isolates)[17] or the *dhfr* coding sequence was not fully covered.[16] Interestingly, some evidence of positive selection on *pocdhfr* was also found.[16] Altogether, these initial results prompted us to evaluate more comprehensively the molecular epidemiology of *dhfr* mutations in a larger collection of *P ovale* spp isolates from varied African endemic regions and to test their functional effect.

Here, we investigated the frequency of *dhfr* mutations in *P ovale curtisi* and *P ovale wallikeri* isolates from 28 different African countries collected between 2004 and 2023. We searched for genetic evidence of positive selection on both *pocdhfr* and *powdhfr* using flanking microsatellite and single nucleotide polymorphism (SNP) markers. Finally, we used in-silico homology modelling and a heterologous bacterial expression system to assess whether the dominant mutations in PocDHFR and PowDHFR alter pyrimethamine susceptibility.

Methods

Sample and data collection

We retrospectively included a subset (see appendix 1 p 3 for more details on the selection) of *P ovale curtisi* and *P ovale wallikeri* samples from the DNA bank of the French

See Online for appendix 1

National Malaria Reference Centre (FNMRC; responsible for the epidemiological surveillance of imported malaria in France) collected between Feb 1, 2004, and Aug 31, 2023. Samples from three field studies, selected to improve geographical coverage, in Gabon (non-randomised clinical trial, CEI-CERMEL: 007/2014; samples collected from Dec 1, 2014, to Aug 31, 2016), Kenya (prevalence survey, WRAIR-A number 2454 and KMRI number 3628; samples collected from Aug 1, 2010, to Aug 31, 2023), and Benin (prevalence survey, number 23/CREC/CEI-CREC/SA; samples collected in 2020) were also included. Sample data and processing are described in detail in appendix 1 (p 3).

Informed consent was not required as the clinical and biological data were collected from the FNMRC database in accordance with the common public health mission of all the National Reference Centres in France, in coordination with the organisation Santé Publique France for malaria surveillance and care. The study was considered as non-interventional research according to article L1221–1.1 of the Public Health Code in France and only required the patient's non-opposition (according to article L1211–2 of the Public Health Code). All data were anonymised before use. For the Benin isolates from the *Etude des déterminants individuels du paludisme asymptomatique dans la zone sanitaire ALLADA-ZE-TOFFO* (Study of individual determinants of asymptomatic malaria in the ALLADA-ZE-TOFFO health zone) ethical approval was obtained from the *Comité d'Ethique Institutionnelle* (Institutional Ethics Committee) of the Centre de Recherche Entomologique de Cotonou (reference number: 23/CREC/CEI-CREC/SA). For the Gabon field study isolates, ethical approval was obtained from the Centre de Recherches Médicales de Lambaréné (reference number: CEI-CERMEL: 007/2014). Ethical approval for the Kenya field study isolates was obtained from the Walter Reed Army Institute of Research (number 2454) and the Kenya Medical Research Institute (number 3628).

Procedures

Sanger sequencing of the pocdhfr *and* powdhfr *coding sequences*

The complete coding sequences of *pocdhfr* and *powdhfr* were amplified by single round or semi-nested PCR. PCR reaction mixtures, cycling conditions, and sequencing strategy are described in appendix 1 (pp 4, 28).

PCR microsatellite analysis

We used previously published primers to amplify four microsatellites flanking the *pocdhfr* gene[16] and developed six microsatellites flanking the *powdhfr* gene (appendix 1 p 28). The location, PCR mixes, cycling programmes, and analysis are described in appendix 1 (pp 5, 28).

Whole-genome sequencing

Leukodepletion or selective whole-genome amplification was used to obtain samples enriched for *P ovale* spp DNA (appendix 1 p 6). *P ovale* spp DNA-enriched samples were then used for library preparation and sequenced at 150 bp paired-end on a NextSeq 500 system (Illumina, San Diego, CA, USA). The full procedure is described in appendix 1 (p 7).

Variant calling and analysis

Raw reads were aligned to the reference genomes (PocGH01 or PowCR01) using the Burrows-Wheeler Aligner algorithm (version 0.7.17; default parameters). Aligned reads were processed using SAMtools (version 1.4) and coverage statistics and depth estimates were obtained using Qualimap (version 2.2.1). Duplicate reads were removed using Picard MarkDuplicates (version 2.26.10). Genome-wide SNPs were identified using BCFtools *mpileup* (version 1.13) according to previously published quality criteria.[17] The full analysis procedure is described in appendix 1 (pp 8–9).

Analysis of the predicted PocDHFR and PowDHFR tertiary structures

The tertiary structures of wild-type PocDHFR and PowDHFR were predicted by homology modelling using the wild-type PfDHFR structure obtained by x-ray diffraction at a resolution of 2·6 Ångstrom (Protein Data Bank ID: 3UM8) as a template.[18] Mutations were introduced using the *swapaa* function in UCSF Chimera (version 1.14). The models were aligned with PfDHFR co-crystallised with pyrimethamine to identify the pocket where the drug should dock to PocDHFR and PowDHFR. The procedure is fully described in appendix 1 (p 10). Molecular drawings were generated using UCSF Chimera.[19]

Expression of PocDHFR and PowDHFR in bacterial system and drug assays

The protocol for expression of the wild-type and mutant versions of both PocDHFR and PowDHFR domains, preparation of the bacterial suspension for the drug assay, and drug assays were similar to the overall strategy described by Chusacultanachai and colleagues[20] but with several modifications. The full procedure is detailed in appendix 1 (p 11).

Statistical analysis

All statistical analyses and graphs were performed using R software (version 4.2.1). Quantitative results were expressed as median (IQR). The Mann-Whitney *U* test was used to test whether the distribution of ranks for one variable was significantly different between the two groups. Proportions were compared by using the $\chi 2$ or Fisher's exact test, depending on the sample size. A threshold of 0·05 was chosen for significant results.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

	West Africa	Central Africa	East Africa	All isolates
***P ovale curtisi dhfr* haplotype**				
Total	101	183	27	314*†‡
Wild type	94 (93%)	70 (38%)	1 (4%)	166 (53%)*
Ala15Ser-Ser58Arg	2 (2%)	96 (52%)	25 (93%)	124 (39%)†
Ala15Ser-Ser113Thr	0	8 (4%)	0	8 (3%)
Ala15Ser-Ser58Arg-Ser113Thr	0	2 (1%)	1 (4%)	3 (1%)
Ser113Thr-Ile169Thr	0	1 (1%)	0	1 (0%)
Lys48Arg	3 (3%)	5 (3%)	0	9 (3%)‡
Ala97Ser	0	1 (1%)	0	1 (0%)
Pro98His	2 (2%)	0	0	2 (1%)
***P ovale wallikeri dhfr* haplotype**				
Total	85	97	22	204
Wild type	85 (100%)	77 (79%)	3 (14%)	165 (81%)
Phe57Leu-Ser58Arg	0	15 (15%)	19 (86%)	34 (17%)
Asn105Ile	0	1 (1%)	0	1 (0%)
Thr62Arg-Ser113Asn	0	2 (2%)	0	2 (1%)
Cys49Arg-The62Arg-Ser113Asn	0	1 (1%)	0	1 (0%)
Ile169Leu	0	1 (1%)	0	1 (0%)

Data are N or n (%). We used the definition from the UN for the African regions. *One isolate (OC30) originates from Thailand. †One isolate (OC139) has an unknown country of origin. ‡One isolate (OC37) has an unknown country of origin.

Table: **Frequency of *dhfr* mutations in *Plasmodium ovale* spp isolates**

Results

A total of 518 *Plasmodium ovale* spp samples (314 *P ovale curtisi* and 204 *P ovale wallikeri*) collected between Feb 1, 2004, and Aug 31, 2023, were retrospectively selected from the FNMRC DNA bank (n=465) and field studies in Benin (n=4), Gabon (n=13), and Kenya (n=36; appendix 2). Isolates were predominantly from central Africa (n=280), west Africa (n=186), and east Africa (n=49). The three remaining isolates were from Thailand (n=1) or an unknown country (n=2).

See Online for appendix 2

The complete *dhfr* coding sequence of all isolates was analysed by the Sanger method. 312 *P ovale curtisi* isolates had the His98Pro amino acid change compared with the PocGH01 reference strain and Pro98 was found in all 204 *P ovale wallikeri* isolates. We therefore considered Pro98 to be wild type in both species. The *pocdhfr* Ala15Ser-Ser58Arg double mutation was found with a frequency of 39% (n=124 of 312; appendix 1 p 29). We identified six other *pocdhfr* mutant haplotypes at much lower frequencies: Lys48Arg (3%, n=9), Ala15Ser-Ser113Thr (3%, n=8), Ala15Ser-Ser58Arg-Ser113Thr (1%, n=3), Pro98His (1%, n=2), Ser113Thr-Ile169Thr (0%, n=1), and Ala97Ser (0%, n=1; table, appendix 1 p 29). The mutants were predominantly found in central and east Africa (table, figure 1), with Kenya (100%, n=22 of 22), Cameroon (83%, n=75 of 90), and the Central African Republic (68%, n=21 of 31) having the highest proportions of mutants (appendix 1 p 29). The frequency of the Ala15Ser-Ser58Arg mutation increased in Cameroon between 2004–07 and 2008–23 ($p<0·0001$; Fisher's exact test; note, however, the low number of isolates available [n=9] before 2008; appendix 1 p 12) and in central Africa between 2012–15 and 2020–23 ($p=0·011$; χ^2 test; appendix 1 p 12).

In *P ovale wallikeri*, five *powdhfr* mutant haplotypes were identified: Phe57Leu-Ser58Arg (17%, n=34 of 204), Thr62Arg-Ser113Asn (1%, n=2), Cys49Arg-Thr62Arg-Ser113Asn (0%, n=1), Asn105Ile (0%, n=1), and Ile169-Leu (0%, n=1; appendix 1 p 30). All the mutant isolates (n=39) were exclusively from central and east Africa (table, figure 1). 20 (21%) of 97 isolates from central Africa and 19 (86%) of 22 isolates from east Africa carried a mutant *powdhfr* gene (table). Kenya, the Democratic Republic of the Congo, and the Republic of the Congo had the highest proportions of mutant *powdhfr*, with 100% (n=16 of 16), 67% (n=4 of 6), and 41% (n=7 of 17), respectively (appendix 1 p 30).

We tested for selective sweep(s) associated with the most common *dhfr* mutant haplotypes—ie, *pocdhfr* Ala15Ser-Ser58Arg and *powdhfr* Phe57Leu-Ser58Arg. To this end, we performed whole-genome sequencing for 65 *P ovale curtisi* and 28 *P ovale wallikeri* isolates from 24 different African countries (whole-genome sequencing metrics detailed in appendix 1 pp 31–33). Two *P ovale curtisi* isolates had a within-infection fixation index (F_{WS}) value below 0·95 (isolate OC150: F_{WS}=0·91; isolate OC156: F_{WS}=0·92) and were considered polyclonal. Using single nucleotide polymorphisms located in the 100 kb *pocdhfr*-flanking regions, a discriminant analysis of principal components (DAPC) showed that the *P ovale curtisi* Ala15Ser-Ser58Arg and wild-type isolates grouped separately (appendix 1 p 13). Examination of the ten discriminant positions identified by DAPC (appendix 1 p 14) showed that 22 of the 28 isolates carrying Ala15Ser-Ser58Arg (all from central Africa) were associated with two main, related discriminant haplotypes (I-A and I-B; figure 2A) that differed at the two discriminant SNPs located upstream of *pocdhfr*. Six other *P ovale curtisi* Ala15Ser-Ser58Arg isolates were associated with discriminant haplotypes that were either different or possibly related to I-A (OC230) or I-B. The extended haplotype homozygosity (EHH) test and furcation plot, performed on a subset of 37 isolates (22 and 15 isolates carrying *pocdhfr* wild-type and Ala15Ser-Ser58Arg, respectively), confirmed reduced genetic diversity around the mutant *pocdhfr* Ala15Ser-Ser58Arg compared with the wild type, suggesting positive selection (figure 2B, appendix 1 p 15). A genome-wide association study between Ala15Ser-Ser58Arg and wild-type isolates revealed that the SNPs most associated with the double mutants were located near *pocdhfr*, suggesting a hitchhiking effect (appendix 1 p 16). 12 of the top 25 associated SNPs were located outside chromosome 5 (where *pocdhfr* is located) and were mainly involved in protein biosynthesis and transcription (appendix 1 p 34). The *P ovale curtisi* Ala15Ser-Ser58Arg isolates carrying haplotypes I-A or I-B did not cluster together in the phylogenetic tree based on genome-wide SNPs (appendix 1 p 17), indicating that these two major mutant haplotypes were segregating in different genomic backgrounds.

The four *P ovale wallikeri* isolates carrying *powdhfr* Phe57Leu-Ser58Arg shared a common haplotype defined by six SNPs in a short region between –3·3 and +11·9 kb around *powdhfr*. This haplotype was not found in any of the

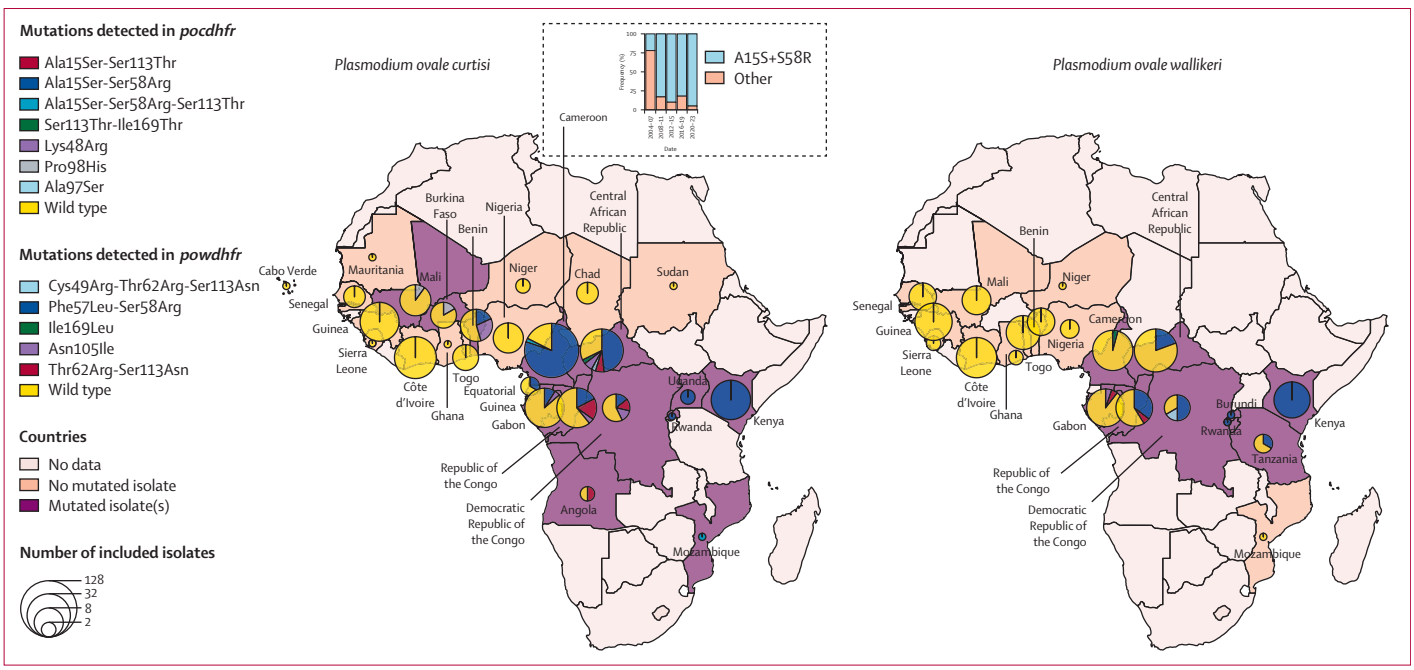

Figure 1: **Prevalence of *dhfr* status in Africa for *P ovale curtisi* and *P ovale wallikeri***

Countries with mutant isolates are shown in purple, countries without mutant isolates are shown in orange, and countries without data are shown in beige. The proportion of mutations detected in each country is shown as a pie chart, with different colours used for each mutation. The proportion of parasites carrying the *pocdhfr* Ala15Ser-Ser58Arg in Cameroon is represented above the map for five periods: 2004–07 (n=2 of 9), 2008–11 (n=5 of 6), 2012–15 (n=17 of 19), 2016–19 (n=28 of 34), and 2020–23 (n=21 of 22).

24 wild-type isolates (figure 2C). Interestingly, in the phylogenetic tree based on genome-wide SNPs, isolates carrying wild-type *powdhfr* from west Africa (except OW110) grouped together (appendix 1 p17).

To explore the genetic diversity of *dhfr* haplotypes in a larger number of isolates, we genotyped 232 *P ovale curtisi* isolates with four microsatellites flanking *pocdhfr*[16] and 101 *P ovale wallikeri* isolates with six novel microsatellites flanking *powdhfr* (appendix 1 pp 35–36). Whether the analysis was restricted to a country or a larger region, the expected heterozygosity (H_E) was lower in the Ala15Ser-Ser58Arg than in wild-type *P ovale curtisi* isolates (figure 3A, appendix 1 pp 18, 37). The majority of Ala15Ser-Ser58Arg mutants from central Africa shared the same flanking haplotype M1 (273-101-418-131 bp) or its related haplotype M7 (268-101-418-131 bp; figure 3B, appendix 1 p 19) corresponding to the SNP-based discriminant haplotypes I-A and I-B, respectively. The downstream-haplotype 101-418-131 bp (ie, the one shared by the M1 and M7 related haplotypes) contributed to at least 46% of the Ala15Ser-Ser58Arg mutants in central Africa across all study periods (appendix 1 p 21). Remarkably, the majority of isolates from east Africa shared another different haplotype, M30 (268-91-426-123 bp) or its related haplotypes M31 to M36, suggesting at least one additional origin of the Ala15Ser-Ser58Arg mutation (figure 3B). Of the two Ala15Ser-Ser58Arg-Ser113Thr triple-mutants from Cameroon, one clustered with I-A and the other with I-B, suggesting that the two main Ala15Ser-Ser58Arg mutants are their direct ancestors. In *P ovale wallikeri*, H_E decreased near *powdhfr* for Phe57Leu-Ser58Arg isolates at microsatellites located from −4·8 to +17·6 kb of *powdhfr* compared with wild-type isolates (figure 3C, appendix 1 pp 18, 37), confirming a selective sweep involving the *powdhfr* Phe57Leu-Ser58Arg mutation. Most *powdhfr* Phe57Leu-Ser58Arg isolates (originating only from central and east Africa) clustered together, suggesting a common origin of the mutation (figure 3D, appendix 1 p 22). Based on the decreasing east-to-central frequency gradient, this haplotype might have originated in east Africa and then spread to central Africa.

We then used homology modelling to generate in-silico DHFR structures for *P ovale curtisi* and *P ovale wallikeri* using the *P falciparum* DHFR structure as a template (appendix 1 pp 23–24). Except for the rare Ala97Ser and Pro98His mutations in PocDHFR, all the other natural PocDHFR and PowDHFR mutations detected in this study were located near or in the pocket where pyrimethamine was docked (appendix 1 p 25). The Lys48Arg, Cys49Arg, Ser58Arg, and Thr62Arg mutations probably reduced the size of the channel at the entrance of the binding pocket, whereas the Ala15Ser, Phe57Leu, Asn105Ile, Ser113Thr/Asn, and Ile169Thr/Leu mutations either reduced the pocket volume or changed its architecture (appendix 1 p 25). Therefore, these mutations might alter the interaction between pyrimethamine and the PocDHFR or PowDHFR enzymes and reduce susceptibility to the drug.

To experimentally test this hypothesis for the dominant PocDHFR Ala15Ser-Ser58Arg and PowDHFR Phe57Leu-Ser58Arg mutations, we measured the in-vitro growth in the presence of pyrimethamine of *Escherichia coli* bacteria

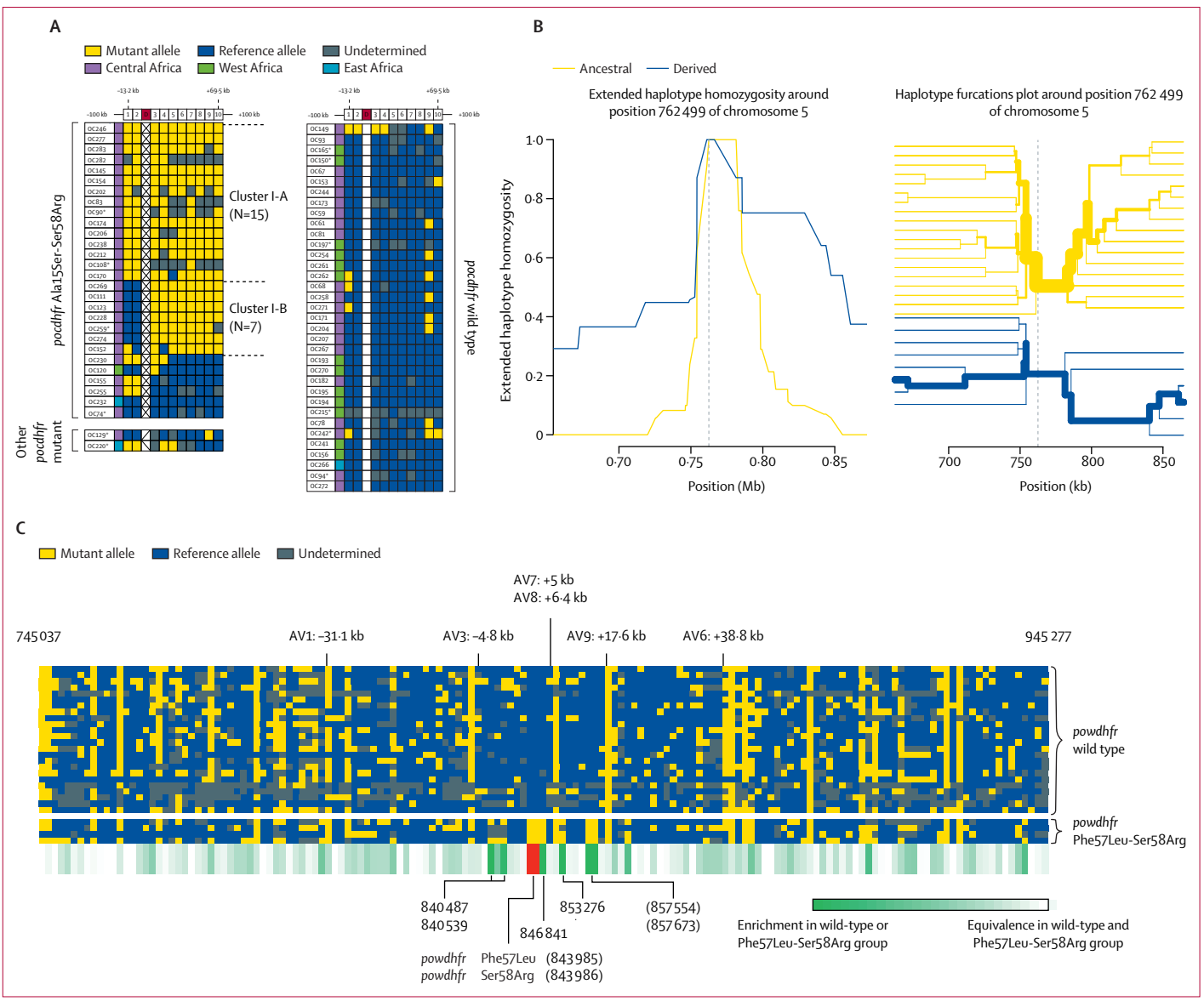

Figure 2: **Analysis of *dhfr* flanking regions sequenced by whole-genome sequencing**

(A) Genotypes at the 10 discriminating single nucleotide polymorphisms (SNPs) highlighted in the discriminant analysis of principal components analysis (appendix 1 pp 13–14) around the *pocdhfr* gene (n=65 isolates). Mutated positions are shown in yellow, non-mutated positions in blue, and undetermined positions in grey. The geographical origin of the isolates is indicated next to the isolate identified using a coloured box. The *pocdhfr* genotype is also indicated: (X) for Ala15Ser-Ser58Arg, (/) for Ala15Ser-Ser113Thr, and (\) for Ala15Ser-Ser58Arg-Ser113Thr. For isolates marked with an asterisk, the *pocdhfr* gene was not covered by next-generation sequencing and its genotype was obtained by Sanger sequencing. Cluster I was defined as the main cluster of Ala15Ser-Ser58Arg isolates. This cluster was subdivided into clusters I-A and I-B according to the genotype of the two discriminating positions located upstream of *pocdhfr*. (B) Extended haplotype homozygosity (left) and haplotype furcation (right) plots around position 762 499 of chromosome 5 in *pocdhfr* wild-type (ancestral, n=22) and Ala15Ser-Ser58Arg (derived, n=15) genes. Position 762 499 of chromosome 5 corresponds to the Gcg→Tcg substitution resulting in the Ala15Ser amino acid change. The extended haplotype homozygosity (EHH) plot shows the loss of haplotype homozygosity with increasing distance from the core SNP, represented here as the derived and ancestral alleles at codon 15 in the *pocdhfr* gene. The x-axis is the chromosomal position of the SNPs spanning chromosome 5 and the y-axis is the probability that two randomly chosen chromosomes are identical at all SNPs for the entire interval from the core region to distance x. EHH=0 indicates that all extended haplotypes are different, whereas EHH=1 indicates that all extended haplotypes are the same. The yellow colour refers to wild-type isolates and the blue colour to Ala15Ser-Ser58Arg isolates. The EHH and the furcation plots for the entire chromosome 5 are represented in appendix 1 p 15. (C) Chromosome painting of the flanking regions around *powdhfr*. Mutated positions are shown in yellow, non-mutated positions in blue, and undetermined positions in grey. SNPs enriched in either Phe57Leu-Ser58Arg or wild-type isolates are shown in green and the Phe57Leu and Ser58Arg SNPs are shown in red. Discriminating positions between mutant and wild-type isolates are listed as their chromosomal position. The positions of the six microsatellites flanking *powdhfr* (named AV1, AV3, AV7, AV8, AV9, and AV6) are also shown.

expressing either the wild type or the double-mutant malarial DHFRs and in which the bacterial DHFR was chemically inactivated. The half-maximal inhibitory concentration (IC_{50})—ie, the concentration of pyrimethamine that inhibited 50% of *E coli* growth—was 50-times higher for the Ala15Ser-Ser58Arg than for the wild-type PocDHFR (median 211 µM [IQR 191–213] *vs* 4·2 µM [4·0–8·2]; n=5 replicates; p=0·0079, Mann-Whitney *U* test; figure 4,

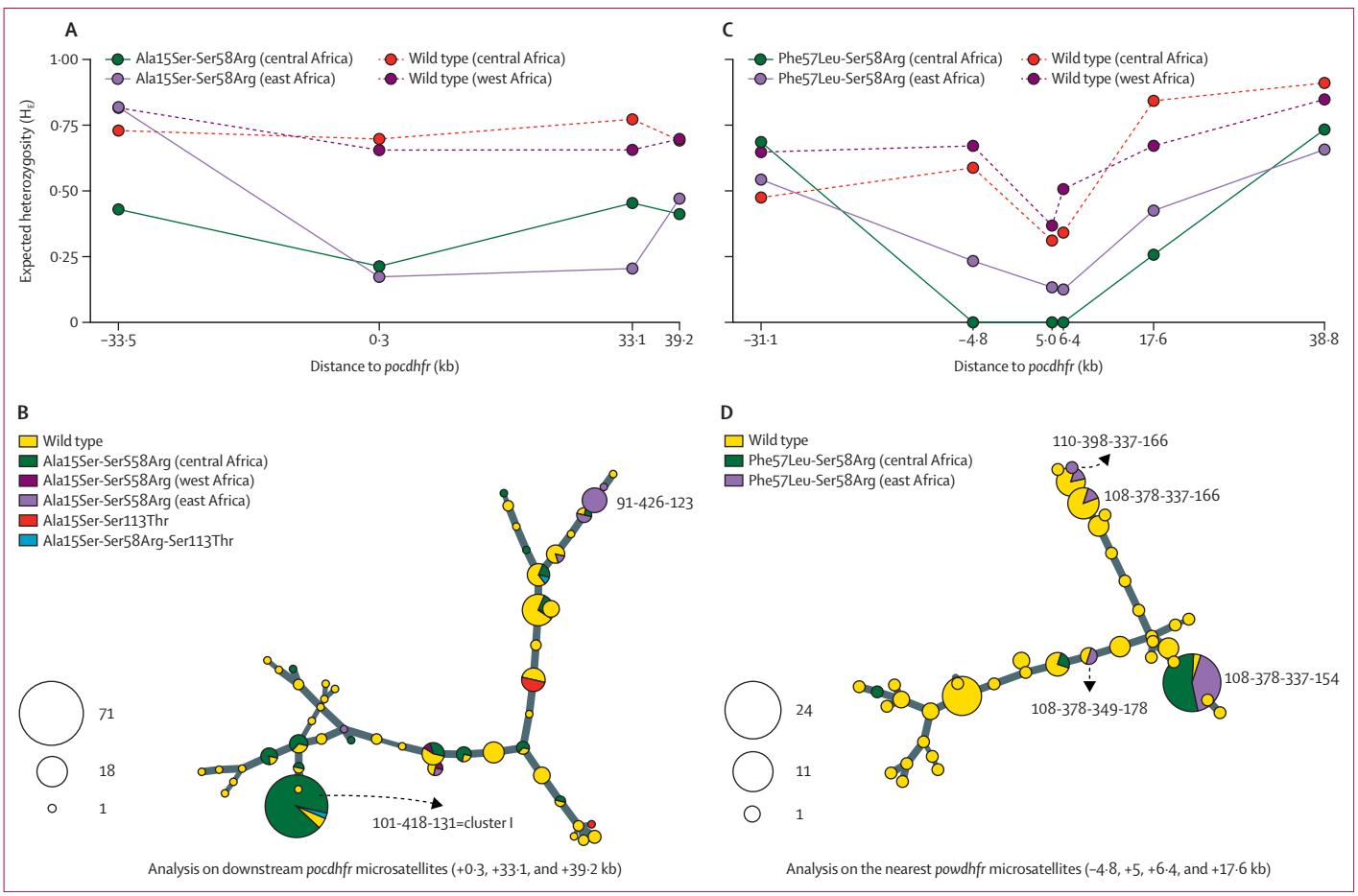

Figure 3: **Analysis of *dhfr*-flanking microsatellites**

Expected heterozygosity (H_E) for four microsatellites flanking *pocdhfr* Ala15Ser-Ser58Arg and wild-type isolates (A) or six microsatellites flanking *powdhfr* Phe57Leu-Ser58Arg and wild-type isolates (C) according to their geographical origin. Mutant isolates from central and east Africa are coloured green and light purple, respectively. Wild-type isolates from west and central Africa are coloured dark purple and red, respectively. (B) Minimum spanning network obtained with the downstream *pocdhfr* microsatellites (STR564-6, STR564-8, and STR564-9). *pocdhfr* Ala15Ser-Ser58Arg isolates from central, west, and east Africa are coloured green, dark purple, and light purple, respectively. *pocdhfr* wild-type, Ala15Ser-Ser113Thr, and Ala15Ser-Ser58Arg-Ser113Thr isolates are coloured yellow, red, and blue, respectively. Haplotype composition (allele lengths in base pairs) is described for the two main *pocdhfr* Ala15Ser-Ser58Arg haplotypes from central and east Africa. (D) Minimum spanning network for the nearest *powdhfr* microsatellites (AV3, AV7, AV8, and AV9). *powdhfr* Phe57Leu-Ser58Arg isolates from central and east Africa are coloured green and light purple, respectively. Wild-type *powdhfr* isolates are coloured yellow. Haplotype composition (allele lengths in base pairs) is described for the main *powdhfr* Phe57Leu-Ser58Arg haplotypes from central and east Africa.

appendix 1 p 26) and 4-times higher for the Phe57Leu-Ser58Arg than for the wild-type PowDHFR (11·0 μM [8·3–16·8] *vs* 2·9 [2·3–4·7]; n=5 replicates; p=0·012, Mann-Whitney *U* test; figure 4, appendix 1 p 26). These results suggest that pyrimethamine inhibited the double-mutant DHFRs less efficiently than the wild-type counterparts.

Finally, since pyrimethamine is always partnered with sulfadoxine as an antimalarial, we also investigated from the whole-genome sequencing data the sequences of *pocdhps* and *powdhps*, which are orthologous to known molecular markers associated with sulfadoxine resistance in *P falciparum*. None of the 11 mutations we detected corresponded to those associated with sulfadoxine resistance in *P falciparum* and *P vivax* (appendix 1 p 38), and they were all located quite far from the sulfadoxine binding site and mostly in long loops from the PocDHPS or PowDHPS enzymes (appendix 1 p 27).

Discussion

Using the complete *dhfr* coding sequence from 518 isolates of *P ovale curtisi* and *P ovale wallikeri*, we mapped the frequency of *dhfr* mutations in west and central Africa and partially in east Africa. The two sets of *pocdhfr* and *powdhfr* mutations—some of which were previously described at a similar prevalence in the retrospective study by Chen and colleagues[16]—are species-specific, showing that the two species have evolved differently at this drug resistance locus. Most of the mutations we detected are located at homologous positions in *P falciparum*[21] and *P vivax*[22,23] *dhfr* known to confer pyrimethamine resistance.

We provide several lines of evidence that the *pocdhfr* and *powdhfr* genes are under positive selection in central and east Africa: (1) the frequency of mutants is high, particularly for *pocdhfr* Ala15Ser-Ser58Arg, whose frequency is fixed in the small sample set from Kenya and has increased over

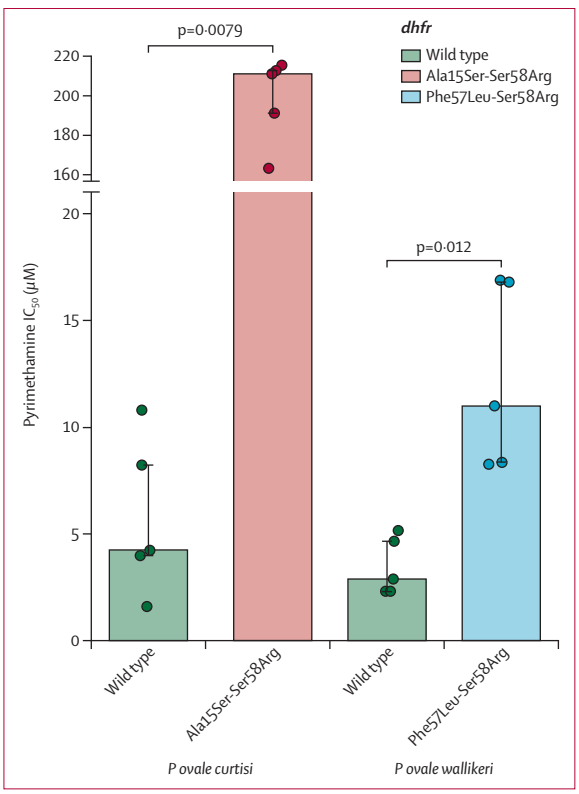

Figure 4: **Effect of *dhfr* mutations on bacterial growth**
Pyrimethamine IC_{50} measured in an *Escherichia coli* growth assay transformed with wild-type or Ala15Ser-Ser58Arg GST-PocDHFR plasmid (five replicates) or wild-type or Phe57Leu-Ser58Arg GST-PowDHFR plasmid (five replicates). IC_{50}=half-maximal inhibitory concentration. GST=glutathione S-transferase.

time in central Africa during a period of sustained sulfadoxine–pyrimethamine use; (2) the genetic diversity flanking *pocdhfr* Ala15Ser-Ser58Arg and that flanking *powdhfr* Phe57Leu-Ser58Arg are reduced in both central and east African isolates, suggesting a selective sweep as previously described for *pocdhfr* in central Africa;[16] (3) the two triple-mutants *pocdhfr* Ala15Ser-Ser58Arg-Ser113Thr from Cameroon had flanking *dhfr* haplotypes closely related to that of the Ala15Ser-Ser58Arg double-mutant, suggesting that the double mutant later acquired the Ser113Thr mutation; we speculate that *P ovale curtisi* might accumulate *dhfr* mutations that confer a selective advantage, as observed in *P falciparum* and *P vivax*;[21,22,24] (4) the pyrimethamine binding site is predicted to be altered in PocDHFR Ala15Ser-Ser58Arg and PowDHFR Phe57Leu-Ser58Arg structures generated in-silico; and (5) bacteria expressing the double-mutant PocDHFR or PowDHFR malarial enzymes grow significantly better in the presence of pyrimethamine than those expressing the wild-type malarial enzymes, a phenotypic change that is particularly large for the PocDHFR Ala15Ser-Ser58Arg. For PowDHFR Phe57Leu-Ser58Arg, the increase in IC_{50} was quite similar to that previously measured for the same *P vivax* DHFR double-mutant expressed in a *Saccharomyces cerevisiae* model (7-times increase).[22] Regarding the *pocdhfr* Ala15Ser-Ser58Arg, we do not have a reference IC_{50} value: the *pfdhfr* Ala16Val-Cys59Arg mutation (at positions similar to Ala15Ser-Ser58Arg) was a non-functional allele in the yeast model and has not been studied in any other *Plasmodium* species nor using purified enzymes.

Notably, none of the single mutants Ala15Ser, Phe57Leu, and Ser58Arg were detected in our African samples. Chen and colleagues described the Ser58Arg mutation alone, but their forward PCR primer did not cover the Ala15Ser mutation.[16] Therefore, it is currently uncertain whether the dominant *pocdhfr* Ala15Ser-Ser58Arg and *powdhfr* Phe57Leu-Ser58Arg haplotypes are of African origin or have been imported to the continent by migration. The source could be the southeast Asian region where the two species have been reported,[2] similarly to the introduction of *pfdhfr* Asn51Ile-Cys59Arg-Ser108Asn into sub-Saharan Africa.[25] However, there are several arguments in favour of an African origin: firstly, *P ovale* spp have a low prevalence in southeast Asia and the western Pacific region;[3] secondly, at least two independent origins of the *pocdhfr* Ala15Ser-Ser58Arg mutation are evidenced in isolates from central and east Africa; and thirdly, local African emergence of single and double *dhfr* mutants has also been reported in *P falciparum*.[26]

It is interesting to note that *pocdhfr* and *powdhfr* mutants were rare in the west African isolates studied here. Chen and colleagues found in this same region a comparatively higher (but still quite low) frequency of mutants,[16] suggesting that there could be geographical heterogeneity in the region. The low mutant frequencies in west Africa could be explained by the fact that *P ovale* spp parasites have fewer migratory events within the sub-Saharan Africa region than *P falciparum*, perhaps because *P ovale* spp infections are much less frequent. The rate of spread also depends largely on the intensity of drug use and of the selective advantage conferred by *pocdhfr* and *powdhfr* mutations. The sustained use of sulfadoxine–pyrimethamine is very likely to continue and one hypothesis could therefore be that the level of pyrimethamine resistance conferred by some of the *P ovale* spp *dhfr* mutants might be moderate, potentially slowing their spread. The absence of seemingly relevant partner mutations in *pocdhps* and *powdhps*—suggesting only partial clinical resistance to sulfadoxine–pyrimethamine—and the relatively small size of the genomic regions affected by the *dhfr*-related genetic hitchhiking (at least for *P ovale wallikeri*, synonymous with low strength of selection[27]) in our sampling support this hypothesis.

Finally, using genome-wide data for *P ovale curtisi* and *P ovale wallikeri*, we provide a first insight into the population genomic structure of these two species. We highlighted the segregation of *P ovale wallikeri* genomes according to their region of origin, suggesting genetic differentiation between west Africa and central or east Africa. Regarding *P ovale curtisi*, despite the high rate of monoclonal infections and low parasite prevalence that would both favour inbreeding,[28] the segregation of the dominant *pocdhfr* Ala15Ser-Ser58Arg

haplotypes (I-A and I-B) in multiple *P ovale curtisi* genome-wide backgrounds suggests the existence of sexual recombination in *P ovale curtisi*. Further studies with larger datasets and more sophisticated analyses are needed to confirm these initial findings.

This study has several limitations. Firstly, we did not include isolates from several countries in sub-Saharan Africa (especially east and southern Africa) or Asia. Secondly, the *E coli* model we used is not sufficient to prove the resistance to pyrimethamine in the parasite. An orthologous exchange strategy in *P falciparum* or *P knowlesi*[29] as the recipient parasite could be used to confirm the *E coli* findings. Finally, the clinical impact of the mutations on the efficacy of pyrimethamine-containing treatments to prevent or cure *P ovale curtisi* and *P ovale wallikeri* infections was not assessed.

In conclusion, we report here a moderate to high prevalence of *dhfr* mutations in *P ovale curtisi* and *P ovale wallikeri* in some areas of central and east Africa. Selective sweeps targeting the *pocdhfr* Ala15Ser-Ser58Arg and *powdhfr* Phe57Leu-Ser58Arg alleles were evidenced, probably caused by the sustained selective pressure of pyrimethamine (a hypothesis previously proposed by Chen and colleagues[16]), which has been part of multiple curative and preventive strategies for decades: first as sulfadoxine–pyrimethamine curative therapy, and then as chemopreventive therapy with the deployment of IPTp, intermittent preventive treatment in infants, and SMC. Based on the experience with *P falciparum*, the role of *dhfr* mutations has become critically important, as *pfdhfr* mutations have been described as a precursor to the acquisition of *pfdhps* mutations[24] and the combination of *pfdhfr* and *pfdhps* mutations has led to a reduction in the efficacy of IPTp in certain regions of sub-Saharan Africa.[30] This calls for closer monitoring of *dhfr* and *dhps* mutations in *P ovale curtisi* and *P ovale wallikeri*, as well as other candidate drug resistance markers.

Contributors

VJ, RC, JC, and SH designed the study. VJ, MG, BP, GC, HA, and SH supervised the sample collection. VJ and RC analysed whole-genome data. VJ and YR generated Sanger and microsatellite data. RC performed structural modelling. VJ, JB, FGTT, YR, SA, and JC performed in-vitro experiments in the *E coli* system. VJ, RC, JB, FGTT, YR, SA, and JC interpreted the data. VJ, RC, JB, and JC drafted the manuscript. RC and JC verified the data reported in this study. All authors made important intellectual contributions during the preparation of the manuscript and take responsibility for the entire work and ensure that questions regarding the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

Next-generation sequence files are available from the European Nucleotide Archive under the accession numbers ERS10659512 to ERS10659516 and ERS15941390 to ERS15941449 for *P ovale curtisi* and ERS10659518 to ERS10659521, ERS14392651 to ERS14392657, and ERS17576819 to ERS17576836 for *P ovale wallikeri*.

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