Changes in the frequencies of *Plasmodium falciparum* *dhps* and *dhfr* drug-resistant mutations in children from Western Kenya from 2005 to 2018: the rise of *Pfdhps S436H*

M. Andreína Pacheco1, Kristan A. Schneider2, Qiuying Cheng3, Elly O. Munde4,5, Caroline Ndege4,6, Clinton Onyango4,6, Evans Raballah4,7, Samuel B. Anyona4,8, Collins Ouma4,6, Douglas J. Perkins3,4*, and Ananias A. Escalante1*

**Abstract**

**Background:** Sulfadoxine-pyrimethamine (SP) is the only anti-malarial drug formulation approved for intermittent preventive treatment in pregnancy (IPTp). However, mutations in the *Plasmodium falciparum dhfr* (*Pfdhfr*) and *dhps* (*Pfdhps*) genes confer resistance to pyrimethamine and sulfadoxine, respectively. Here, the frequencies of SP resistance-associated mutations from 2005 to 2018 were compared in samples from Kenyan children with malaria residing in a holoendemic transmission region.

**Methods:** Partial sequences of the *Pfdhfr* and *Pfdhps* genes were amplified and sequenced from samples collected in 2005 (n = 81), 2010 (n = 95), 2017 (n = 43), and 2018 (n = 55). The frequency of known mutations conferring resistance to pyrimethamine and sulfadoxine were estimated and compared. Since artemisinin-based combination therapy (ACT) is the current first-line treatment for malaria, the presence of mutations in the propeller domain of *P. falciparum* *kelch13* gene (*Pfk13*) linked to ACT-delayed parasite clearance was studied in the 2017/18 samples.

**Results:** Among other changes, the point mutation of *Pfdhps S436H* increased in frequency from undetectable in 2005 to 28% in 2017/18. Triple *Pfdhfr* mutant allele (*CIRN*) increased in frequency from 84% in 2005 to 95% in 2017/18, while the frequency of *Pfdhfr* double mutant alleles declined (allele *CICN* from 29% in 2005 to 6% in 2017/18, and *CNRN* from 9% in 2005 to undetectable in 2010 and 2017/18). Thus, a multilocus *Pfdhfr/Pfdhps* genotype with six mutations (*HGEAA/CIRN*), including *Pfdhps S436H*, increased in frequency from 2010 to 2017/18. Although none of the mutations associated with ACT-delayed parasite clearance was observed, the *Pfk13* mutation *A578S*, the most widespread *Pfk13* SNP found in Africa, was detected in low frequency (2.04%).

**Conclusions:** There were changes in SP resistance mutant allele frequencies, including an increase in the *Pfdhps S436H*. Although these patterns seem consistent with directional selection due to drug pressure, there is a lack of information to determine the actual cause of such changes. These results suggest incorporating molecular
surveillance of \textit{Pfdhfr/Pfdhps} mutations in the context of SP efficacy studies for intermittent preventive treatment in pregnancy (IPTp).

**Keywords:** Drug resistance genes, Dhfr, Dhps, \textit{k13} gene, SP resistance, \textit{Plasmodium falciparum}

**Background**

Despite a worldwide decline, malaria remains a significant and resilient global health problem. Approximately 228 million cases and 405,000 associated deaths were reported globally in 2018; of those, more than 90% of the malaria morbidity and mortality occurred in Africa [1]. \textit{Plasmodium falciparum} is the most prevalent malaria parasite in the African continent, accounting for 99.7% of the estimated cases in sub-Saharan Africa. Pregnant women and children under 5 years of age are the most vulnerable groups and account for 67% of all malaria deaths worldwide. The interventions available to mitigate the adverse effects of malaria during pregnancy include intermittent preventive treatment in pregnancy (IPTp), insecticide-treated bed-nets (ITNs), and case management [2, 3]. Currently, sulfadoxine-pyrimethamine (SP) is the only anti-malarial drug formulation approved for use in IPTp [3]. The SP drug inhibits the enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). These enzymes are involved in the folate pathway of nucleic acid synthesis [4, 5]. However, mutations in the parasite genes \textit{dhfr} (\textit{Pfdhfr}) and \textit{dhps} (\textit{Pfdhps}) confer different degrees of resistance to pyrimethamine and sulfadoxine, respectively [4–8]. Specifically, there are four-point mutations in \textit{Pfdhfr} (N51I, C59R, S108N, and I164L) and five in \textit{Pfdhps} (S436A/F, A437G, K540E, A581G, and A613S/T) [6, 7, 9–13].

Due to the increasing SP resistance and pervasive chloroquine resistance, the World Health Organization (WHO) recommended artemisinin-based combination therapy (ACT) as first-line treatment for uncomplicated malaria in most endemic countries [3, 14]. However, ACT is still not approved for the prevention of malaria in pregnant women due to the absence of adequate safety data [3, 15]. Thus, SP remains the only drug used for IPTp and is being considered for intermittent preventive treatment in infants (IPTi) [15–17].

Several studies in Kenya have shown an association between the \textit{Pfdhfr} triple mutant (N51I, C59R, S108N) combined with the \textit{Pfdhps} double mutant (A437G, K540E), and resistance to SP in vivo [13, 18]. Even after SP was no longer the first-line drug in Kenya as of 2004, the \textit{Pfdhfr/Pfdhps} quintuple mutant genotype (N51I, C59R, S108N/A437G, K540E) continued to be prevalent [13]. Given that SP remains in use for IPTp, is considered as a possible ACT partner drug, and is a candidate for IPTi, the frequencies of SP resistance-associated mutations were investigated in samples collected from pediatric malaria patients in Siaya (Western Kenya) during three periods: 2005, 2010, and 2017/18. Among other well-known mutations associated with SP resistance, the change in frequency of a novel mutation identified in \textit{Pfdhps} (S436H) [19] was also estimated. In addition, to obtain a more comprehensive picture of the mutations related with anti-malarial drug resistance, the presence of mutations linked to the delayed parasite clearance phenotype against artemisinin-based combinations were assessed by studying the polymorphism in the propeller domain of the \textit{P. falciparum} \textit{k13} gene (\textit{Pfk13}) in a group of samples collected in 2017/18 [1, 20].

**Methods**

**Study sites, sample collection, and DNA isolation**

Samples were initially collected as part of an immunooepidemiologic study approved by the Ethics Committee of the Kenya Medical Research Institute, the University of New Mexico Institutional Review Board, the Los Alamos National Laboratory (LANL) Institutional Review Board, and the Maseno University Ethics Review Committee. The study was conducted at Siaya County Referral Hospital (SCRH), a holoendemic \textit{P. falciparum} transmission region in Western Kenya. Details of the study design and study area have been previously published [21]. Individuals inhabiting the study area are predominantly from the Luo ethnic group (>96%). Children (primarily aged <12 months), who presented at the paediatric ward for their first ‘hospital contact’ (for any reason), were identified and screened for malaria parasites. Children were enrolled in the cohort studies unless they met any of the following exclusion criteria: positive blood smears with non-\textit{P. falciparum} species, previous hospitalization (for any reason), documented or reported use of anti-malarial therapy 2 weeks prior to enrollment, and/or cerebral malaria diagnosis (though rare in this study area). Informed written consent was obtained from the parents/legal guardians of all participating children. All children were treated with standard anti-malarials approved at the time following the local guidelines (Coartem™: artemether and lumefantrine). About 2 mL of venous blood was obtained from each study participant at enrollment or visit and used for genotyping analysis.

To explore changes in mutations linked to drug resistance, \textit{P. falciparum} genomic DNA was extracted from 200 µl of 81 blood samples collected in 2005 and 95 blood
samples collected in 2010 using QIAamp DNA Micro Kit (Qiagen, GmbH, Hilden, Germany). For samples collected in 2017/18, genomic DNA was extracted using Direct-zol DNA/RNA miniprep kit (Zymo Research, Tustin, CA, USA) from 200 µL aliquots of each of the 98 blood samples that were mixed with Tri reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Genotyping analysis of *P. falciparum* drug resistance genes and *Pfk13* gene

Drug resistance genes and *kelch13* gene (*Pfk13*) of *P. falciparum* were amplified by polymerase chain reaction (PCR). DNA samples were genotyped for mutations at: (1) *P. falciparum* hydroyxymethylidihydropterin pyrophosphokinase-dihydropteroate synthase gene (*Pfdhps*) codons 436, 437, 540, 581, and 613; (2) *P. falciparum* dihydrofolate reductase-thymidylate synthase gene (*Pfdhfr-ts*) codons 51, 59, 108, and 164; and (3) the propeller domain coding region (720 bp) of *Pfk13* (with an open reading frame of 2,181 bp in length). *Pfk13* mutation analysis was only performed for samples collected in the period of 2017/18. A fragment of 750 out of 2418 bp for *Pfdhps* and a fragment of 1688 out of 1827 bp for *Pfdhfr* were amplified. Sequences of PCR primers used in this study were: (1) for *Pfdhps*, 5'-GAT ATA TGT ATT AAA AGA TAG AAT TTC-3' (forward) and 5'-CTT GTC TTT CCT CAT GTA ATT C-3' (reverse); (2) for *Pfdhfr*, 5'-GCM ATA TGT GCA TGT TGT AAR G-3' (forward) and 5'-GCC ATA TCC ATT KAA ATT TTW TC-3' (reverse); and (3) for *Pfk13*, 5'-GAT AAA CAA GGA AGA ATA TTC T-3' (forward) and 5'-CGG AAT CTA ATC ATA TGT TAT GTT CA-3' (reverse) [22].

PCR amplifications were carried out in 50 µl reactions using 2 µl of total genomic DNA, 1X PCR buffer, 2.5 mM MgCl2, 0.25 mM of each deoxynucleoside triphosphate, 0.4 µM of each primer, and 0.03 U/µl AmpliTaq polymerase (Applied Biosystems, Thermo Fisher Scientific). The PCR conditions for *Pfdhps* were a partial denaturation at 95 °C for 7 min, and 40 cycles with 30 s at 95 °C, 50 s at 50 °C and 1 min extension at 68 °C, and a final extension step of 5 min at 68 °C. For *Pfdhfr*, the conditions were a partial denaturation at 95 °C for 7 min, and 40 cycles with 1 min at 95 °C, 1 min at 54 °C and 2 min extension at 72 °C, and a final extension of 10 min at 72 °C. For *Pfk13* gene, the PCR conditions were: a partial denaturation at 94 °C for 4 min, and 36 cycles with 1 min at 94 °C, 1 min at 53 °C and 2 min extension at 72 °C, and a final extension of 10 min at 72 °C. Negative control (nuclease-free DIH2O as a template) and positive control (*Pf DNA*) were included in each batch of PCR. PCR products from each reaction (50ul) were resolved using 1% agarose electrophoresis, excised from the gel, and purified using the QIAquick® Gel extraction kit (Qiagen, GmbH, Hilden, Germany). Purified PCR products were directly sequenced for both strands using an Applied Biosystems 3730 capillary sequencer. All *Pfk13* sequences obtained in this study were deposited in GenBank under the accession numbers MT130102 to MT130200.

Evaluation of *Pfdhps* and *Pfdhfr* allele frequencies

After thorough inspections of each electropherogram, mutations associated with drug resistance genes (*Pfdhps* and *Pfdhfr*) or artemisinin delayed parasite clearance resistance *Pfk13* gene were identified and recorded. First, frequency of each (a) point mutations, (b) allele in *Pfdhps* and *Pfdhfr* genes, as well as (c) the combination of *Pfdhps* and *Pfdhfr* multilocus genotypes were estimated, dividing the total of point mutations/allele/combination of multilocus genotypes by the total of the samples (N) per year that successfully amplified, which corresponds to the frequency of patients with parasites that have a specific codon or allele. However, given the mixed infections found in these samples, the frequencies do not add 1. Additionally, via inspecting multiple peaks at the mutations of *Pfdhps* and *Pfdhfr* that were associated with drug resistance, polyclonal *P. falciparum* infections were identified, and their corresponding frequencies were estimated using the samples collected in the three periods (2005, 2010, and 2017/18). Statistical comparisons in the prevalence of all SNP mutations in *dhps* and *dhfr* genes in samples collected between the 2005 and 2017/18 surveys were performed using Fisher’s exact tests. Statistical significance was defined by a two-sided p value < 0.05.

Results

*Pfdhps* and *Pfdhfr* allele frequencies and *P. falciparum* polyclonal infections

Figure 1 shows the frequencies of genotypes at each codon for *Pfdhps* and *Pfdhfr* genes. *Pfdhps* mutations at codons 437 and 540, i.e., A437G and K540E, were detected in more than 95% of all sampled years (frequency > 0.95). However, mutation S436A was found at a low frequency (0.02, two patients) only in 2010, while the mutation S436H [19] seemed to significantly increase in frequency from 0.12 in 2010 to 0.28 in 2017/18 (p value < 0.05, Fig. 1). The A581G mutation was also present at a low frequency in 2010 (0.01, one patient) and 2017/18 (0.03, three patients; Fig. 1), and mutation A613T was not detected in any of these groups of samples. In the case of *Pfdhfr*, only the N51I, C59R, and S108N mutations were found at high frequency (> 0.85) in all sampled years. The presence of I164L mutation was only detected in three patients (frequency of 0.04, Fig. 1) in 2017/18.

The frequencies of *Pfdhfr* and *Pfdhps* alleles, estimated by dividing the number of each *Pfdhps* and *Pfdhfr* alleles...
by the total of the samples (N) per year that successfully amplified, are shown in Fig. 2. *Pfdhps* SGEAA (Figs. 2a) and triple mutant for *Pfdhfr* CIRNI (Fig. 2b) are the most frequent alleles across the sampled periods in this study population. The *Pfdhps* HGEAA allele, having a novel mutation S436H, appears to significantly increase in frequency between 2010 and 2017/18 (*p* value < 0.05, Fig. 2a). Mutations associated with SP resistance in *Pfdhps* and *Pfdhfr* genes revealed 13 *Pfdhps/Pfdhfr* multilocus genotypes, with frequency changing through time during the sampled periods (Fig. 2c). SGEAA/CIRNI and SGEAA/CICNI were the most frequent multilocus genotypes for all sampled years, however, SGEAA/CICNI seems to be significantly decreasing over time (*p* value < 0.05, Fig. 2c). Interestingly, the multilocus genotype HGEAA/CIRNI was significantly increasing in frequency between 2010 and 2017/18 (*p* value < 0.05, Fig. 2c).

Polyclonal infections detected by *Pfdhps* and *Pfdhfr* SNPs were found via examination of electropherograms. The frequency of the polyclonal *Pfdhps* infections significant increased to 0.2 in 2017/18 from 0.09 (in 2005) and 0.06 (in 2010) (*p* value < 0.05). It is worth noticing that 84% of these polyclonal infections have the novel *Pfdhps* mutation S436H (CAT substituted the codon TCT) in 2017/18. Since the *Pfdhps* mutation S436H is increasing in frequency and polyclonal infections are detectable by the polymorphism present at the sampled SNPs (*Pfdhps* and *Pfdhfr* in this case), it is not surprising that most of the *Pfdhps* S436H alleles are part of polyclonal infections. In contrast, the frequency of the polyclonal infections in *Pfdhfr* gene significantly decreased gradually from 0.23 in 2005 to 0.12 in 2010 and 0.05 in 2017/18 (*p* value < 0.05); this shows the fixation of the CIRNI allele.

### Pfk13 population analyses

Upon inspecting sequences of the *Pfk13* propeller domain in 98 samples collected in 2017/18, none of the mutations associated with the delayed parasite clearance phenotype were found [14, 22, 23]. However, in the *Pfk13* propeller region, a nonsynonymous substitution at codon AGCT 578STCT (2.04%, 2 patients), a nonsynonymous substitution at codon VGTT 637IATT (2.04%, two patient), a synonymous substitution at the same codon VGTT 637VGTA (1.02%, one patient), and a nonsynonymous substitution at codon E642DGAT (2.04%, two patients) were detected in the paediatric malaria patients from Siaya (Western Kenya).

### Discussion

Molecular surveillance is considered a valuable tool to monitor the prevalence of mutations that may affect the efficacy of anti-malarial drugs [24–27]. In the context of IPTp, following the dynamic of mutations conferring resistance to SP is critical because it is the only drug approved for use in pregnant women [3, 15].

This study found that the quintuple *Pfdhps/Pfdhfr* mutant (the *Pfdhps* double mutant A437G, K540E allele together with a *Pfdhfr* triple mutant N51I, C59R, S108N allele) associated with clinical SP treatment failure [12], remained high (0.86) in Siaya (Western Kenya) in 2010 and 2017/18 (Fig. 2c), more than a decade after the
withdrawal of SP in Kenya (Fig. 3). The increase in \( Pfdhfr \) triple mutants is linked to a decline in the double mutant \( Pfdhfr \) alleles in the population, evidenced by the absence of the C59R, S108N allele in the 2010 and 2017/18 samples (Fig. 2). These patterns are consistent with the prediction made using 1992–1999 samples that allowed estimates of the relative fitness of these resistant alleles assuming drug pressure [28]. The triple \( Pfdhfr \) mutant that conferred higher resistance was found to have higher fitness than the two double mutant alleles [28]. Furthermore, the double \( Pfdhfr \) mutant alleles N51I, S108N showed a higher fitness than double mutant alleles C59R, S108N [28]. Thus, the less fit \( Pfdhfr \) allele under drug pressure (i.e., the allele of C59R, S108N) is absent in the more recent samples.

Although the frequency of \( Pfdhps \) allele A437G, K540E was similar during the sampled years (0.91 in 2005 to 0.88 in 2017/18), there is an increase in the frequency of a triple mutant allele S436H, A437G, K540E (0.28), which has the mutation at codon 436. This mutation has been previously reported in low frequency in pregnant women from Nyanza Province (located in Western Kenya, covering the area of nowadays six counties, including Siaya county) between 2002 and 2009: 2.3% in 2002–2008 and 3.8% in 2008–2009 [19]. Although the results presented here are consistent with a scenario that positive directional selection is playing a role in the frequency increase of this new allele (S436H, A437G, K540E) in Western Kenya, the phenotypic effect of the \( Pfdhps \) S436H mutation to clinical drug resistance has not been determined. Thus, whether the results observed here relate to actual anti-malarial drug pressure or other processes is difficult to ascertain.

SP was the second-line anti-malarial drug until 1998 when it became the first-line malaria treatment [29]. Due to the increased frequency of chloroquine treatment
failures, there was a growing SP drug pressure that may have led to the observed high prevalence of \textit{Pfdhfr} mutations in 1998 [29]. Then, the increased use of SP selected for highly resistant mutations in \textit{Pfdhps} [29]. By 2004, just after 5 years of SP usage in Kenya, the widespread treatment failures prompted a change in the malaria treatment drug policy to ACT in Kenya, like other African countries [30]. However, ACT did not have widespread distribution at many of the health facilities in Kenya until mid-2006. Considering the timeline described previously, the observed trends in \textit{Pfdhfr} and \textit{Pfdhps} mutations were unanticipated because SP has not been a first-line anti-malarial treatment in Kenya for almost 15 years.

Unlike mutations linked to chloroquine resistance, SP resistant mutations have shown to be resilient in Africa even after the drug is no longer the first-line anti-malarial treatment [13, 30, 31]. A possibility in Africa is that mutations conferring resistance to SP may not have a relative fitness cost because of the lack of wild-type alleles in the population that can outcompete the resistant ones in the absence of drug pressure, as has been suggested in South America [32, 33]. However, the significant increase in the frequencies of the \textit{Pfdhfr} triple mutant and the \textit{Pfdhps} allele with the S436H mutation is consistent with selective drug pressure. This drug pressure can be explained, at least in part, by the fact that 56% of pregnant women in Kenya took at least two SP doses in the context of IPTp in 2018, as reported by the Maternal & Child Survival Program from USAID [34].

A factor to consider is the ongoing treatment of HIV/AIDS patients with cotrimoxazole, a bacterial \textit{dhfr/dhps} inhibitor used to treat respiratory tract infections and to prevent opportunistic infections. The use of cotrimoxazole in the population may have played a role in the increased frequency of mutations linked to SP resistance in malarial parasites [35] and should be considered now. There were reports showing cross-resistance of \textit{P.falciparum} in vitro to cotrimoxazole with pyrimethamine and sulfadoxine [35, 36]. However, a recent study showed that cotrimoxazole remains effective in controlling malaria infection despite the high prevalence of SP-resistant parasites, and its use does not select for mutations associated with SP resistance [37]. Thus, at this point, the use of cotrimoxazole is not a plausible selective force that can explain the pattern observed in \textit{Pfdhfr} and \textit{Pfdhps} mutations.

In the case of \textit{Pfdhps} A581G and A613T mutations associated with high-level SP resistance, they have been
observed in Africa [28, 38–41], South America, and Southeast Asia [9, 10, 42, 43]. However, these mutations were detected in low frequency in the samples included in this study, and the results presented here are consistent with previous reports from Western Kenya [13, 28, 30, 40].

ACT is the first-line anti-malarial treatment in holoendemic *P. falciparum* malaria-endemic nations, including Kenya. As a result, there is ongoing molecular surveillance aimed to detect *Pfk13* mutations linked to delayed parasite clearance [1, 20]. Up to now, there is still no report on the presence of a delayed parasite clearance phenotype for ACT in Kenya. Mutation A578S, found in this study, is the most predominant mutation in sub-Saharan Africa [23, 44–48] and has been reported in both pre- and post-ACT parasites, with frequencies between 1.2 and 10% in samples from different malaria ecological zones in Kenya [48, 49]. For example, A578S mutation has a frequency of 2.8% in samples from Kisumu (Western Kenya) [45]. These results support the notion that A578S is the most widespread *Pfk13* SNP observed in Africa, including countries such as Mali, Angola, Democratic Republic of Congo, Uganda, Gabon, Ghana, and Kenya [20, 22, 23, 45, 47, 48, 50–52]. However, in all the studies, this mutation was detected at low frequencies. This finding is consistent with the proposed model that many of these *Pfk13* mutations are slightly deleterious and maintained as the *P. falciparum* population expanded, making selection less efficient [22]. Although the functional impact of A578S in terms of ACT efficacy remains unclear, recent studies have hinted at a potential effect. In particular, A578S is very close to the C580Y mutation, and molecular modelling and mutational sensitivity prediction performed by Mohon et al. [52] suggested that the A578S SNP could disrupt the function of the propeller domain. Nevertheless, experimental evidence is still lacking [20, 53].

**Conclusion**

Although the evolutionary processes driving the observed pattern remain elusive because of a lack of specific phenotypic information on the S436H mutation, its increase in frequency seems consistent with drug pressure. SP is no longer the first-line anti-malarial drug in Kenya, but it is still widely used as part of IPTp [15]. More studies are warranted to discern whether *Pfdhps/Pfdhfr* multilocus mutant (S436H, A437G, K540E/ N51I, C59R, S108N) adversely impact the efficacy of SP in IPTp in the context of drug efficacy evaluations. Such information is critical, considering that SP in IPTp remains an essential tool for reducing disease burden in sub-Saharan Africa [15]. On a separate note, there is no evidence indicating that *Pfk13* mutations linked with the delayed phenotype were present in Kenya when these samples were collected. However, sustaining the molecular surveillance is important since there are reports in China of an imported malaria case from Rwanda with the mutation R561H linked to artemisinin resistance [54].

**Abbreviations**

ACT: Artemisinin-based combination therapy; CBC: Complete blood counts; DHFR: Dihydrofolate reductase; DHPS: Dihydropteroate synthase; IPTi: Intermittent preventive treatment in infants; IPTp: Intermittent preventive treatment in pregnancy; ITNs: Insecticide-treated bed-nets; SP: Sulfadoxine-pyrimethamine; WHO: World Health Organization.

**Acknowledgements**

We thank Scott Bingham from the DNA laboratory at the School of Life Sciences (Arizona State University) for their technical support. This study was supported in part by grants from the US National Institute of Health, R01AI130473.

**Authors’ contributions**

MAP, AAE, and DJP conceived the study. DJP supervised the sample collection, trained field personal, and supported the fieldwork. QC, EOM, CN, CO, ER, SBA, CO conducted the field and lab work in Kenya, including the sample collection and administrated the informed consent forms. MAP genotyped the samples. MAP, KS, and AAE analyzed the data. MAP and AAE wrote a first draft of the manuscript. All authors read and approved the final manuscript.

**Funding**

This study was supported by grant R01AI130473 from the US National Institutes of Health to D. J. Perkins.

**Availability of data and materials**

Sequences were deposited in the GenBank with the accession numbers MT130102 to MT130200.

**Ethics approval and consent to participate**

The study protocol was approved by the Ethics Committee of the Kenya Medical Research Institute, the University of New Mexico Institutional Review Board, the Los Alamos National Laboratory (LANL) Institutional Review Board and the Maseno University Ethics Review Committee.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

1 Biology Department/Institute of Genomics and Evolutionary Medicine (iGEM), Temple University, Philadelphia, PA, USA. 2 Department of Applied Computer and Biosciences, University of Applied Sciences Mittweida, Technikumplatz, Mittweida, Germany. 3 Center for Global Health, University of New Mexico Health Sciences Center, Albuquerque, NM, USA. 4 University of New Mexico-Kenya Global Health Programs, Kisumu, Siaya, Kenya. 5 Department of Clinical Medicine, School of Health Sciences, Kenyatta University, Kerugoya, Kenya. 6 Department of Biomedical Sciences and Technology, Masinde Muliro University, Maseno, Kenya. 7 Department of Medical Laboratory Sciences, School of Public Health, Biomedical Sciences and Technology, Masinde Muliro University of Science and Technology, Kakamega, Kenya. 8 Department of Medical Biochemistry, School of Medicine, Maseno University, Maseno, Kenya.

Received: 13 August 2020 Accepted: 18 October 2020

**Published online: 22 October 2020**

**References**

1. World Health Organization. World Malaria Report 2019. Geneva: World Health Organization; 2019.
2. World Health Organization. Technical expert group meeting on preventive chemotherapy: report of the technical consultation on intermittent preventive treatment in infants (IPTi). Geneva: World Health Organization; 2009.

3. World Health Organization. Guidelines for the treatment of malaria. 3rd ed. Geneva: World Health Organization; 2015.

4. Talisuna AO, Bolland P, D’Alessandro U. History, dynamics, and public health importance of malaria parasite resistance. Clin Microbiol Rev. 2004;17:235–54.

5. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev. 2005;57:117–45.

6. Peterson DS, Wallace D, Wellems TE. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci USA. 1988;85:914–8.

7. Triglia T, Menting JG, Wilson C, Cowman AF. Mutations in dihydrofolate reductase-thymidylate synthase confer resistance to pyrimethamine in Plasmodium falciparum. Proc Natl Acad Sci USA. 1997;94:13494–9.

8. Lozovsky ER, Chookajorn T, Brown KM, Imwong M, Shaw PJ, Kamchom-Angkaw EK, et al. Stepwise acquisition of pyrimethamine resistance in the malaria parasite. Proc Natl Acad Sci USA. 2000;96:12025–30.

9. Foote SJ, Gaitakis D, Cowman AF. Amino acids in the dihydrofolate reductase-thymidylate synthase gene of Plasmodium falciparum confer resistance to pyrimethamine and chlorproguanil-dapone treatment of Plasmodium falciparum malaria. J Infect Dis. 2002;185:380–8.

10. Juma DW, Omondi AA, Ingasia L, Oput B, Cheruiyot A, Yeda R, et al. Trends in drug resistance codons in Plasmodium falciparum dihydrofolate reductase and dihydrofolate reductase synthase field isolates: correlation between pyrimethamine and chlorproguanil activity in vitro and point mutations in the dihydrofolate reductase domain. Antimicrob Agents Chemother. 1999;42:164–9.

11. Kublin JS, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martin LM, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapone treatment of Plasmodium falciparum malaria. J Infect Dis. 2000;182:155–65.

12. Juma DW, Omondi AA, Ingasia L, Oput B, Cheruiyot A, Yeda R, et al. Trends in drug resistance codons in Plasmodium falciparum dihydrofolate reductase and dihydrofolate reductase synthase field isolates: correlation between pyrimethamine and chlorproguanil activity in vitro and point mutations in the dihydrofolate reductase domain. Antimicrob Agents Chemother. 1999;42:164–9.

13. Juma DW, Omondi AA, Ingasia L, Oput B, Cheruiyot A, Yeda R, et al. Trends in drug resistance codons in Plasmodium falciparum dihydrofolate reductase and dihydrofolate reductase synthase field isolates: correlation between pyrimethamine and chlorproguanil activity in vitro and point mutations in the dihydrofolate reductase domain. Antimicrob Agents Chemother. 1999;42:164–9.

14. World Health Organization. Artemisinin and artemisinin-based combinations in the management of acute uncomplicated falciparum malaria: WHO policy recommendations. Geneva: World Health Organization; 2009.

15. Roh ME, Kulle FO, Rolerle F, Glymour MM, Shibuski S, Gosling R, et al. Overall, anti-malarial, and non-malarial effect of intermittent preventive treatment during pregnancy with sulfadoxine-pyrimethamine on birth weight: a mediation analysis. Lancet Glob Health. 2020;8:e942–53.

16. Ong’echa JM, Keller CC, Were T, Duma C, Otieno RO, Landis-Lewis Z, et al. Parastigmia, anemia, and malaria in infants and young children in a rural holoendemic Plasmodium falciparum transmission area. Am J Trop Med Hyg. 2006;74:376–85.

17. Gosling RD, Carneiro J, Chandramohan D. Intermittent preventive treatment of malaria in infants: how does it work and where will it work? Trop Med Int Health. 2009;14:1003–10.

18. Khalil I, Ronn AM, Alifrangis M, Gabar HA, Satti GM, Bygbjerg IC. Dihydrofolate reductase and dihydropteroate synthase genotypes associated with in vitro resistance of Plasmodium falciparum to pyrimethamine, trimethoprim, sulfadoxine, and sulfamethoxazole. Am J Trop Med Hyg. 2003;68:586–9.

19. Spalding MD, Eyase FL, Akala HM, Bedno SA, Prigge ST, Coldren RL, et al. Increased prevalence of the pfdhfr/pfdhps quintuple mutant and rapid emergence of pfdhps resistance mutations at codons 581 and 613 in Kenya. Malar J. 2010;9:393.

20. Heuchert A, Abduselam N, Zeynudin A, Eshetu T, Löscher T, Wieser A, et al. Artemisinin resistance and Artemisinin-resistant-based interventions in Kenya. Malar J. 2007;6:72.

21. McCollum AM, Schneider KA, Mwangi M, Chededer A, Gantenbein J, Nair S, et al. The prevalence and antifolate drug resistance profiles of Plasmodium falciparum in study participants randomized to discontinue or continue cotrimoxazole prophylaxis. J Infect Dis. 2009;199:739–49.

22. Pacheco MA, Kadakia ER, Chaudhary Z, Perkins DJ, Kelley J, Ravishankar S, et al. Evolution and genetic diversity of the k13 gene associated with artemisinin delayed parasite clearance in Plasmodium falciparum. Antimicrob Agents Chemother. 2019;63:e02550-e2618.

23. Conrad MD, Bigio V, Kapito J, Mulindo M, Kamya MR, Havlir DV, et al. Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in Plasmodium falciparum isolated from Ugandan children. PLoS ONE. 2014;9:e105690.

24. Escalante AA, Feireira MU, Vinzet JM, Volkman SK, Cui L, Gamboa D, et al. Malaria molecular epidemiology: lessons from the international centers of excellence for Malaria Research Network. Am J Trop Med Hyg. 2015;93:79–86.

25. Dalmat R, Naughton B, Kwan-Getts TS, Sylker J, Stuckey EM. Use cases for genetic epidemiology in malaria elimination. Malar J. 2019;18:163.

26. Escalante AA, Pacheco MA. Malaria molecular epidemiology: An evolutionary genetics perspective. Microbiol Spectr. 2019;7:1128.

27. Shretta R, Oumwongo J, Rupada B, Snow RW. Using evidence to change anti-malarial drug policy in Kenya. Trop Med Int Health. 2000;5:755–64.

28. Arrojo SM, Roper C. Mapping “partially resistant”, “fully resistant” and “super resistant” malaria. Trop Med Int Health. 2009;14:1003–10.

29. McCollum AM, Schneider KA, Chededer A, Gantenbein J, Nair S, et al. The prevalence and antifolate drug resistance profiles of Plasmodium falciparum in study participants randomized to discontinue or continue cotrimoxazole prophylaxis. J Infect Dis. 2009;199:739–49.

30. Pacheco MA, Foerero-Peña DA, Schneider KA, Chededer A, Gantenbein J, Nair S, et al. The prevalence and antifolate drug resistance profiles of Plasmodium falciparum in study participants randomized to discontinue or continue cotrimoxazole prophylaxis. J Infect Dis. 2009;199:739–49.

31. Escalante AA, Feireira MU, Vinzet JM, Volkman SK, Cui L, Gamboa D, et al. Malaria molecular epidemiology: lessons from the international centers of excellence for Malaria Research Network. Am J Trop Med Hyg. 2015;93:79–86.

32. Dalmat R, Naughton B, Kwan-Getts TS, Sylker J, Stuckey EM. Use cases for genetic epidemiology in malaria elimination. Malar J. 2019;18:163.

33. Pacheco MA, Forero-Peña DA, Schneider KA, Chededer A, Gantenbein J, Nair S, et al. The prevalence and antifolate drug resistance profiles of Plasmodium falciparum in study participants randomized to discontinue or continue cotrimoxazole prophylaxis. J Infect Dis. 2009;199:739–49.

34. McCollum AM, Poe AC, Hamel M, Huber C, Zhou Z, Shi Y, et al. Antifolate resistance in Plasmodium falciparum: multiple origins and identification of novel dhfr alleles. J Infect Dis. 2006;194:190–9.
43. Vinayak S, Alam MT, Mixson-Hayden T, McCollum AM, Sem R, Shah NK, et al. Origin and evolution of sulfadoxine resistant Plasmodium falciparum. PLoS Pathog. 2010;6:e1000830.
44. Isozumi R, Uemura H, Kimata I, Ichiinose Y, Logedi J, Omar AH, et al. Novel mutations in K13 propeller gene of artemisinin-resistant Plasmodium falciparum. Emerg Infect Dis. 2015;21:490–2.
45. Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson K, et al. K13-propeller polymorphisms in Plasmodium falciparum parasites from sub-Saharan Africa. J Infect Dis. 2015;211:1352–5.
46. Ouattara A, Kone A, Adams M, Fofana B, Maiwa AW, Hampton S, et al. Polymorphisms in the K13-propeller gene in artemisinin-susceptible Plasmodium falciparum parasites from Bougoula-Hameau and Bandiagara. Mali Am J Trop Med Hyg. 2015;92:1202–6.
47. Taylor SM, Parobek CM, DeConti DK, Kayentao K, Coulibaly SO, Greenwood BM, et al. Absence of putative artemisinin resistance mutations among Plasmodium falciparum in Sub-Saharan Africa: a molecular epidemiologic study. J Infect Dis. 2015;211:680–8.
48. de Laurent ZR, Chebon LJ, Ingasia LA, Akala HM, Andagalu B, Ochola-Oyier LI, et al. Polymorphisms in the K13K13 gene in Plasmodium falciparum from different malaria transmission areas of Kenya. Am J Trop Med Hyg. 2018;98:1360–6.
49. Wamae K, Okanda D, Ndewiga L, Oost V, Kimenyi KM, Abdal AI, et al. No evidence of P. falciparum K13 artemisinin conferring mutations over a 24-year analysis in Coastal Kenya, but a near complete reversion to chloroquine wild type parasites. Antimicrob Agents Chemother. 2019;63:e01067-e1119.
50. Muwanguzi J, Henriques G, Sawa P, Bousema T, Sutherland CJ, Beshir KB. Lack of K13 mutations in Plasmodium falciparum persisting after artemisinin combination therapy treatment of Kenyan children. Malar J. 2016;15:36.
51. Ocan M, Bwanga F, Okeng A, Katabazi F, Kigozi E, Kyobe S, et al. Prevalence of K13-propeller gene polymorphisms among Plasmodium falciparum parasites isolated from adult symptomatic patients in northern Uganda. BMC Infect Dis. 2016;16:428.
52. Lu F, Culleton R, Zhang M, Ramaprasad A, von Seidlein L, Zhou H, et al. Emergence of indigenous artemisinin-resistant Plasmodium falciparum in Africa. N Engl J Med. 2017;376:991–3.
53. Mohon A, Alam M, Bayi F, Folefoc A, Shahan J, Dhaque R, et al. Mutations in 386 Plasmodium falciparum K13 propeller gene from Bangladesh (2009–2013). Malar J. 2014;13:431.
54. Wang X, Ruan W, Zhou S, Huang F, Lu Q, Feng X, et al. Molecular surveillance of Plkrt and k13 propeller polymorphisms of imported Plasmodium falciparum cases to Zhejiang Province, China between 2016 and 2018. Malar J. 2020;19:59.

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