

Molecular Markers of Antimalarial Resistance in Children with Uncomplicated Plasmodium falciparum at Two Sites in Liberia in 2022-2023

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Abstract

Background: Between 2022-2023, as part of a therapeutic efficacy study in Liberia, children were recruited from two rural health facilities in Saclepea and Sinje. Participants were treated with artemisinin-based combination therapy and monitored for 28 days. Following treatment, samples were analyzed to identify molecular markers of antimalarial drug resistance.

Methods: Blood from participants was collected and dried on filter paper. All baseline (D0) and Day of Failure (DoF) samples with recurrent parasitemia were sent to the Centers for Disease Control and Prevention in Atlanta. A total of 91 samples (all treatment failure pairs and 34% of unpaired D0) were first screened for *P. falciparum* monoinfection using a multiplex real-time PCR assay, and polymorphisms associated with antimalarial drug resistance in the *Pfk13*, *Pfmdr1*, *Pfcr1*, *Pfdhfr*, *Pfdhps* and *Pfcytb* genes were determined using the NGS-based Malaria Resistance Surveillance protocol.

Results: A total of 90 samples were confirmed to be *P. falciparum* mono infections. There was no evidence of any known *Pfk13* mutations associated with artemisinin partial resistance. There were low rates of both *Pfmdr1* N86Y (0.83-5.5%) and *Pfcr1*K76T (12-22%) mutations. Moderate rates of the *Pfmdr1* Y184F (41-51%) were observed. The triplet canonical mutations conferring chloroquine resistance in *Pfcr1* were detected in 12 and 17% of samples from Saclepea and Sinje, respectively. Considering the World Health Organization thresholds for sulfadoxine-pyrimethamine (SP) resistance, the rates of the *Pfdhfr* mutations were above, but rates of the *Pfdhps* mutation were below the thresholds.

Conclusions: This study shows high efficacy of artemisinin derivatives in Liberia. There is no indication of SP resistance in either site; however, circulating parasite population may be predisposed to reduced lumefantrine susceptibility.

Trial Registration: ClinicalTrials.gov ID NCT06300970

INTRODUCTION

Despite sustained investments in control and prevention, malaria continues to cause substantial morbidity and mortality worldwide. In 2024, the mosquito-borne illness caused by Plasmodium parasites, was responsible for more than 282 million cases and 610,000 deaths worldwide [1]. *Plasmodium falciparum* (*P. falciparum*), the predominant species in Liberia, West Africa, is the primary cause of nearly every malaria case and death [2].

Malaria control relies on a multifaceted set of interventions implemented in endemic regions, including the widespread use of long-lasting insecticidal nets, indoor residual spraying and strategic case management. Central to case management is the use of efficacious oral artemisinin-based combination therapies (ACTs).

The malaria burden in Liberia is quite significant accounting for 34% of outpatient visits, 47% of inpatient cases, and 23% of hospital deaths [3]. The Liberian National Malaria Control Program (NMCP) currently

recommends artemether-lumefantrine (AL) as the first-line treatment for uncomplicated malaria. Until relatively recently, artesunate-amodiaquine (ASAQ), a drug commonly used throughout West and Central Africa [4, 5], was the preferred therapy. However, results from the most recent therapeutic efficacy study (December 2017 – May 2018) demonstrated a decline in ASAQ efficacy, while no such decline was demonstrated for AL. [6]

As recommended by the World Health Organization (WHO), the efficacy of antimalaria drugs must be regularly monitored to ensure continued effectiveness. Therapeutic efficacy studies (TES) are considered the gold standard for evaluating clinical and parasitological responses to treatment of uncomplicated malaria. Complementing TES, molecular surveillance of *P. falciparum* for validated and candidate resistance-associated genetic markers provides valuable information on the emergence and spread of drug resistance. Numerous studies have shown that parasite resistance is often associated with genetic mutations and single nucleotide polymorphisms (SNPs), individually or in specific combinations. [7–10] For example, SNPs at codons 86, 184, and 1246 in the *P. falciparum* multidrug resistance 1 gene (*Pfmdr1*) have been implicated in parasite resistance to multiple anti-malarial drugs, with individual polymorphisms leading to adverse outcomes such as recurrent infections [11–13]. Mutations at *Pfmdr1* 86Y and 1246Y have been linked to decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to lumefantrine, mefloquine and artemisinin [7, 12], while mutations at 184F have been associated with reduced susceptibility to lumefantrine [13, 14]. Moreover, mutations at codons 72 to 76 of the *P. falciparum* chloroquine resistance transporter gene (*Pfcr1*) have been associated with resistance to chloroquine and amodiaquine [15–17].

To address these challenges and complex potential outcomes, genomic surveillance using next-generation sequencing (NGS) has emerged as a powerful tool for detecting SNPs associated with antimalarial drug resistance [18]. Characterizing the distribution and frequency of these SNPs in *P. falciparum* populations is critical for informing treatment policies and guiding malaria control strategies in high-burden settings such as Liberia.

Building on a TES conducted in Liberia from August 2022 to July 2023 [6], this study analyzed *P. falciparum* samples for WHO validated and candidate molecular markers associated with resistance to a battery of antimalarial drugs, providing insights into the local prevalence of resistance and informing ongoing surveillance and treatment strategies.

MATERIALS AND METHODS

Study design and enrollment. Using samples from the 2022–2023 Liberia TES [4], we performed a retrospective analysis of molecular markers associated with antimalaria drug resistance. The TES employed an open-label parallel-arm design in which patients received either AL or ASAQ. Treatments were administered at two sentinel sites: Saclepea (Saclepea Comprehensive Health Center in Nimba County) and Sinje (Sinje Health Center in Grand Cape Mount County).

Participants were followed for 28 days and evaluated clinically and parasitologically in accordance with the 2009 WHO *in vivo* TES protocol [19]. At each site, 88 participants were enrolled per treatment arm, resulting in 176 participants per site and a total TES enrollment of 352 children across both sites.

Eligibility criteria included febrile illness, defined as an axillary temperature $\geq 37.5^{\circ}\text{C}$ and/or history of fever within 24 hours prior to enrollment and confirmed uncomplicated *P. falciparum* mono-infection with a parasite density of 2,000-200,000 asexual parasites/ μL (Table 1). Written informed consent from a parent or guardian was also a requirement.

TABLE 1 TES Inclusion and Exclusion Criteria

Inclusion criteria	Exclusion criteria
Age 6–59 months of age	Presence of severe malaria or danger signs, including prostration, alteration in level of consciousness, respiratory distress, convulsions, or jaundice
Weight > 5.0 kg	Severe malnutrition according to WHO child growth standards (weight for age < 3 standard deviations)
Axillary temp $\geq 37.5^{\circ}\text{C}$ or history of fever in the past 24 hours	Known hypersensitivity to AL or ASAQ
Hemoglobin ≥ 8 grams/deciliter at enrollment	Use of antimalarials or other drugs with antimalarial activity in the last two weeks of recruitment screening
Slide-confirmed mono-infection with <i>Plasmodium falciparum</i> and asexual parasitemia between 2,000 and 200,000 parasites/ μL	General clinical condition necessitates hospitalization
Live within the catchment area of the study site (10 km radius)	Diagnosis of severe illness (e.g. Sickle cell)
Ability to swallow oral medication	Evidence of concomitant infections at the time of presentation
Ability and desire to comply with the protocol for the duration of study	Plan to travel or leave the area within the next 3 months
Able and willing to comply with the study visit schedule on days 2, 3, 7, 14, 21, and 28	Previously enrolled in this study

Sample collection, storage, and quality assessment. Following enrollment, capillary blood samples were collected from children aged 6 months to 5 years for baseline testing [6]. Participants received treatment with either (AL) or (ASAQ) and were followed to assess their clinical and parasitological outcomes. Blood samples were collected on Whatman 903 filter paper (Cytiva, Massachusetts, USA) at enrollment (D0) and during scheduled follow-up visits on days 2, 3, 7, 14, 21, and 28. Detailed recruitment and follow-up procedures are described elsewhere [6].

Dried blood spots (DBS) were air-dried and stored individually in low air-permeable, resealable clear plastic bags containing a minimum of one color-indicating desiccant packet. Samples were stored at

room temperature for approximately five months prior to shipment to the U.S. Centers for Disease Control and Prevention (CDC) for molecular analysis. All day zero (D0) samples from enrollment and day-of-failure (DoF) samples from individuals with recurrent parasitemia were included in the molecular analyses.

Upon receipt at the Atlanta CDC malaria laboratory, DBS samples underwent quality assessment prior to downstream molecular processing. Quality criteria included: (1) compliance with CDC personally identifiable information (PII) protocols for labeling, ensuring that sample labels were devoid of any personal identifying information; (2) verification of appropriate collection and storage materials used (i.e. 903 Whatman filter paper and resealable, low air-permeable plastic specimen bags); (3) presence of a minimum of one unsaturated color-indicating desiccant packet; (4) absence of visible mold or degradation and (5) sufficient blood volume and appropriate spot coloration.

DNA extraction. Genomic DNA was extracted from the DBS using four 3-mm punchouts per sample with the QIAamp DNA Mini Kit (QIAGEN; Cat. No. 51304) following the manufacturer's instructions (QIAGEN, Hilden, Germany). DNA was eluted in two 100 µL aliquots using the provided elution buffer and stored at -20°C until further molecular analyses were performed.

Plasmodium species determination and estimation using PET-PCR. Plasmodium species identification and parasitemia estimation were performed on extracted DNA using Photo-induced Electron Transfer PCR (PET-PCR) assay [20]. The assay was used to detect *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*.

Negative controls included DNA extracted from DBS samples without *Plasmodium* parasites, while no template controls (NTC) were derived from molecular grade water. Positive controls included DNA extracted from *P. falciparum* 3D7 reference strain, and DNA extracted from archived clinical samples infected with *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*.

Sequencing of Molecular Markers of Resistance. Targeted amplification was performed on samples from participants with treatment failure and on a subset of samples from participants with adequate clinical and parasitological response (ACPRs), i.e. no malaria parasites were detected after Day 28. Full-length anti-malaria resistance-associated genes – *Pfk13*, *Pfmdr1*, *Pfcrt*, *Pfdhfr*, *Pfdhps*, and *Pfcytb* – were amplified using the Malaria Resistance Surveillance (MaRS) Next-Generation Sequencing (NGS) protocol (<https://cdcgov.github.io/MaRS>), as previously described [18].

Amplification success was assessed by gel electrophoresis and PCR reactions were repeated for samples that failed initial amplification. For each sample, amplicons from the six target genes were pooled and purified using AMPure XP beads (Beckman Coulter). Purified amplicons were then processed with the Illumina DNA Prep kit (Illumina; Cat No. 20060059), which includes tagmentation, library purification and indexing which ultimately generates libraries.

Prepared libraries were sequenced on an Illumina MiSeq platform using a v2 500-reagent kit (Illumina Cat# MS-102-2003) with paired-end sequencing.

Sequencing analysis and mutation frequency calculations. Sequencing data were processed using the Nf-NeST pipeline (<https://github.com/CDCgov/Nf-NeST>) to trim raw reads, align sequences and identify reportable single nucleotide polymorphisms (SNPs) for each target gene. Only reads with a minimum sequencing quality (Q) score of 35 were retained for alignment. SNPs were excluded from downstream analysis if coverage at a given codon site was fewer than five reads. Additionally, SNPs identified by fewer than two of the four variant callers implemented in the Nf-NeST toolkit (SAMtools, VarDict, GATK, and FreeBayes) were excluded from reporting.

For each study site, weighted variant allele frequencies (VAFs) were calculated for all polymorphic loci. The weighted VAF was computed as

$$\text{VAF} = \sum_{i=1}^N \text{VAF}_i w_i / \sum w_i$$

where v_i represents the variant allele frequency for sample i , w_i represents the weight assigned sample i , and N represents the total number of individual and pooled DNA samples per site. Because all samples in the study were individually sequenced, equal weighting was applied ($w_i = 1$ for all samples).

As previously described [6], all DoF samples selected for analysis were determined to be reinfections and therefore were treated as independent observations, contributing additional data points to the total number of observations used in VAF calculations.

Haplotype inference was performed only when the major allele accounted for more than 95% of sequencing reads at all loci within a given gene. Samples that did not meet this criterion were considered to contain multiple parasite strains and were excluded from haplotype construction.

D0 and DoF samples were further screened for the presence of seven *Pfcr*t mutations (M74I, N75E, K76T, A220S, Q271E, I356T, and R371I) with a VAF > 0%, stratified by treatment arm.

RESULTS

Selection for Molecular Analysis

A total of 328 DBS specimens were received by the CDC malaria laboratory in Atlanta. Upon quality inspection, 71 specimens from Sinje were excluded from further processing because of visible mold growth. Two of the rejected specimens belonged to complementary paired samples (D0 and DoF from same participant); therefore, the corresponding mold-free paired samples were also excluded. The remaining 255 specimens (77%) that met quality criteria included 10 complementary paired samples (5 D0 and 5 DoF) and 245 D0 samples from participants without recurrent parasitemia.

DNA extraction was performed from 91 samples, comprising all 10 paired samples and 81 randomly selected unpaired D0 samples from the set of 245 samples mentioned above. Of these, 90 samples were

confirmed as *P. falciparum* mono-infections, while one DoF had no detectable *Plasmodium* DNA by PET-PCR and was excluded from further analysis.

Parasitemia Estimation and PCR Amplification Process

Cycle Threshold (CT) values obtained from PET-PCR were compared with microscopy-based parasitemia estimates from the TES (**Supplemental Fig. 1**). Samples from Sinje demonstrated a stronger correlation between molecular and microscopy-based parasitemia estimates than samples from Saclepea.

Based on gel electrophoresis, all six full-length genes were successfully amplified from 79 samples. Among the remaining 11 samples with detectable *P. falciparum* DNA, six genes were successfully amplified for eight samples, four genes were amplified for two samples, and three genes were amplified for one sample (**Supplemental Table S1**).

Sequencing Coverage and Detection of Resistance-Associated Mutations

Variant allele frequencies and sequencing coverage for each polymorphic site across the *Pfcr*t, *Pfcy*tb, *Pfdh*fr, *Pfdh*ps, *Pfk*13, and *Pfmd*r1 genes are reported in **Supplemental Table S2** and visualized in Fig. 1.

Across all loci, mean sequencing depth exceeded 141 reads per polymorphic site, with average coverage of 497 reads for *Pfcr*t; 3,763 for *Pfcy*tb; 1,251 for *Pfdh*fr; 729 for *Pfdh*ps; 1,483 for *Pfk*13, and 141 for *Pfmd*r1. Mutations with a VAF greater than 0% were detected in *Pfcr*t (M74I, N75E, K76T, A220S, Q271E, I356T, R371I), *Pfdh*fr (N51I, C59R, S108N), *Pfdh*ps (I431V, S436A, A437G, K540E, A581G, A613S), *Pfmd*r1 (N86Y, Y184F, D1246Y). Mutation frequencies at each study site are summarized in Table 2, and inferred haplotypes are presented in Table 3.

Table 2
Number of samples sequenced and
observed allele frequencies from six
genes in two sites in Liberia, 2022–2023

SNP	Saclepea		Sinje	
	N	%VAF	N	%VAF
<i>Pfprt</i>				
M74I	40	12%	47	22%
N75E	40	12%	47	22%
K76T	40	12%	47	22%
A220S	40	13%	47	20%
Q271E	41	15%	47	21%
I356T	40	12%	47	18%
R371I	40	13%	46	23%
<i>Pfcytb</i>				
No mutations observed				
<i>Pfdhfr</i>				
N51I	41	68%	48	100%
C59R	41	86%	48	100%
S108N	41	93%	48	100%
<i>Pfdhps</i>				
I431V	40	0%	47	14%
S436A	40	36%	47	33%
A437G	40	79%	47	98%
K540E	40	13%	47	16%
A581G	40	0%	47	9.20%
A613S	40	9.60%	47	14%
<i>Pfk13</i>				
No mutations observed				
<i>Pfmdr1</i>				
N86Y	38	5.50%	48	0.83%

SNP	Saclepea		Sinje	
	N	%VAF	N	%VAF
<i>Pfcr</i>				
Y184F	38	51%	48	41%
D1246Y	38	0%	48	0.71%

Table 3
Distribution of haplotypes observed for six genes in two sites in Liberia, 2022–2023

Haplotype	Saclepea	Sinje
	N (% Total)	N (% Total)
Pfprt haplotypes defined at codons: 72 73 74 75 76; Wildtype: <i>CVMNK</i>		
Number of samples	40	42
<i>CVMNK</i>	35 (88%)	35 (83%)
CVIET	5 (12%)	7 (17%)
Pfcytb haplotypes defined at codons: 258 268; Wildtype: <i>IY</i>		
Number of samples	41	48
<i>IY</i>	41 (100%)	48 (100%)
Pfdhfr haplotypes defined at Pfdhfr codons 51 59 108 and Pfdhps codons 431 436 437 540 581 613; Wildtype: <i>NCS/ISAKAA</i>		
Number of samples	31	36
ICN/IAAKAA	3 (9.7%)	0
IRN/IAAKAA	2 (6.5%)	1 (2.8%)
NRN/ISGKAA	6 (19%)	0
IRN/ISGKAA	12 (39%)	19 (53%)
NRN/IAGKAA	2 (6.5%)	0
IRN/IAGKAA	0	4 (11%)
IRN/VAGKAA	0	1 (2.8%)
IRN/ISGEAA	3 (9.7%)	6 (17%)
IRN/IAGKAS	3 (9.7%)	1 (2.8%)
IRN/VAGKGS	0	4 (11%)
Pfk13 haplotypes defined at codons: 441 446 449 458 469 476 481 493 515 527 537 538 539 543 553 561 578 580 622 675; Wildtype: <i>PFGNCMAYRPNGRIPRACRA</i>		
Number of samples	39	48

Haplotype	Saclepea	Sinje
	N (% Total)	N (% Total)
PFGNCMAYRPNGRIPRACRA	39 (100%)	48 (100%)
Pfmdr1 haplotypes defined at codons: 86 184 1246; Wildtype: NYD		
Number of samples	36	37
NYD	18 (50%)	23 (62%)
NFD	16 (44%)	14 (38%)
YFD	2 (5.6%)	0

No validated or candidate mutations associated with artemisinin partial resistance were detected in *Pfk13* among the 87 successfully sequenced samples. Similarly, no mutations associated with atovaquone resistance were identified in *Pfcytb* among the 89 successfully sequenced samples.

Prevalence of Pfdhfr and Pfdhps Mutations

Three *Pfdhfr* (N51I, C59R, S108N) and five *Pfdhps* (I431V, S436A, A437G, K540E, A613S) mutations associated with resistance to sulfadoxine-pyrimethamine were detected. A total of 89 samples (Saclepea = 41, Sinje = 48) were successfully sequenced for *Pfdhfr*. In Sinje, all three *Pfdhfr* mutations were fixed (100%); however, lower frequencies were observed in Saclepea (68% N51I, 86% C59R, 93% S108N).

For *Pfdhps*, 87 samples (Saclepea = 40; Sinje = 47) were successfully sequenced. Mutation frequencies in Saclepea and Sinje, respectively, were as follows: I431V (0%, 14%), S436A (36%, 33%), A437G (79%, 98%), K540E (13%, 16%), A581G (0%, 9.2%), A613S (9.6%, 14%). The K540E mutation, a component of the quintuple *Pfdhfr*/*Pfdhps* haplotype, was detected at a range of 13–16% at both sites, while the A581G mutation, associated with the sextuple haplotype, was detected only in Sinje.

Haplotype inference was possible for 67 samples (Saclepea = 31, Sinje = 36). The quintuple *Pfdhfr*/*Pfdhps* haplotype IRN/ISGEAA was detected in 3 samples from Saclepea (9.7%) and 6 samples from Sinje (17%). The sextuple haplotype IRN/VAGKGS was detected in four samples (11%), all from Sinje.

Prevalence of Pfmdr1 Mutations

Three *Pfmdr1* mutations associated with resistance to quinoline antimalarials, including lumefantrine and amodiaquine, were detected: N86Y, Y184F, and D1246Y. The wild type N86 allele predominated, with N86Y detected at frequencies of 5.5% of in Saclepea and 0.83% in Sinje. The Y184F mutation was

detected at frequencies of 51% in Saclepea and 41% in Sinje. The D1246Y mutation was detected in Sinje at a frequency of 0.71% observed in a single sample with 34% mutant reads.

Haplotypes were inferred for 73 samples (Saclepea = 36, Sinje = 37). The wild type NYD haplotype was the most prevalent (Saclepea = 62%; Sinje = 50%), followed by the single-mutant NFD haplotype (Saclepea = 38%, Sinje = 44%;). The double-mutant YFD haplotype was detected only in Sinje (5.6%).

Prevalence of *Pfcr*t Mutations

Eighty-three D0 samples (42 AL, 41 ASAQ) and four DoF samples (2 AL, 2 ASAQ) were successfully sequenced at seven *Pfcr*t codons associated with chloroquine and amodiaquine resistance. Among the D0 samples, 15 harbored at least six of the seven mutations, while 68 harbored fewer than six.

Geographically, the canonical chloroquine resistance mutations M74I, N75E, and K76T were detected at frequencies of 12% in Saclepea and 22% in Sinje. Additional *Pfcr*t mutations (A220S, Q271E, I356T, and R371I) showed similar geographic patterns, with frequencies ranging from 13–15% in Saclepea and 18–23% in Sinje. Haplotype inference for 82 samples (Saclepea, n = 40; Sinje, n = 42) identified the wild-type CVMNK haplotype as predominant. The mutant CVIET haplotype detected at frequencies of 12% in Saclepea and 17% in Sinje, including two recurrent samples from the ASAQ study arm (**Supplemental Table S3**). In contrast, two recurrent samples from the AL arm carried the wild-type CVMNK haplotype. Molecular correction analysis classified all four DoF samples as new infections rather than recrudescence. Molecular correction results are further detailed in a separate publication [6] and the supplemental files of this report.

DISCUSSION

Malaria control and prevention remain a persistent global challenge, increasingly complicated by the emergence and spread of antimalarial drug resistance. In response to rising resistance to conventional monotherapies such as chloroquine, sulfadoxine-pyrimethamine, and amodiaquine, WHO recommended the adoption of artemisinin-based combination therapies (ACTs) in 2001 for the treatment of *Plasmodium falciparum* malaria [21]. ACTs pair a rapidly acting artemisinin derivative – which effectively kills circulating parasites but has a short half-life – with a longer acting partner drug, such as lumefantrine or amodiaquine, to eliminate residual parasites and reduce the likelihood of resistance emergence. Given the continuous selective pressure exerted by widespread ACT use, continuous clinical and molecular surveillance is essential to monitor drug efficacy and detect signals of drug resistance.

This study provides a comprehensive molecular characterization of antimalarial drug resistance markers in *P. falciparum* collected during a 2022 TES in Liberia. Collectively, the findings corroborate clinical data that demonstrate high efficacy of ACTs in Liberia. Consistent with recent studies, both AL and ASAQ demonstrated PCR-corrected efficacy above 90% at both sites. These findings align with the 2017–2018 TES conducted in Liberia and support the continued use of AL as a first-line therapy [6, 22]. The

molecular data presented here further reinforces these conclusions by demonstrating the absence of validated or candidate resistance markers to the artemisinin component of ACTs.

Specifically, this investigation found no validated or candidate mutations in the *Pfk13* propeller region. This finding is consistent with previous studies from Liberia and the broader West African region and suggest that artemisinin partial resistance, as defined by delayed parasite clearance mediated by *Pfk13* mutations, has not emerged at the study sites of Saclepea and Sinje. Similarly, no mutations associated with atovaquone resistance were identified in the mitochondrial *Pfcytb* gene, including substitutions at codon 268, which are commonly linked to clinical atovaquone failure. Together these findings indicate continued susceptibility of *P. falciparum* to both artemisinin derivatives and atovaquone in Liberia.

In contrast, resistance-associated mutations in *Pfdhfr* and *Pfdhps* – targets of pyrimethamine and sulfadoxine, respectively were common. While all three key *Pfdhfr* mutations (N51I, C59R, and S108N) were observed at near fixation, the prevalence of the most critical *Pfdhps* mutations associated with SP resistance remained relatively low. Notably, the frequencies of K540E and A581G mutations were below WHO thresholds (i.e. under 50%) used to define compromised SP effectiveness [23]. These findings suggest that there is no evidence of widespread SP resistance at the study sites, supporting the continued utility of SP for intermittent preventative treatment strategies.

Mutations in the *Pfmdr1* gene, which modulate parasite susceptibility to multiple antimalarial drugs [10, 11], were also observed. The predominance of the wild-type N86 allele, alongside moderate frequencies of the Y184F mutation, is consistent with selection patterns previously reported under lumefantrine pressure. Although Y184F has been associated with reduced lumefantrine susceptibility, its presence alone is insufficient to predict clinical failure [24, 25]. The low frequency of a third mutation, D1246Y, further suggests that the *Pfmdr1* resistance profile in this population remains compatible with continued efficacy of AL.

Analysis of *Pfcr1* mutations revealed patterns consistent with both historical chloroquine pressure and contemporary amodiaquine use [26–29]. Canonical chloroquine resistance mutations (M74I, N75E, and K76T) were detected in 18% of baseline samples, representing a decline compared with earlier reports from Liberia (2017–2018) [6]. This reduction likely reflects decreased selective pressure following the withdrawal of chloroquine as a first-line therapy and suggests a gradual repopulation of chloroquine-sensitive parasites. However, among ASAQ treatment failures, complete fixation of seven *Pfcr1* mutations was observed, indicating potential selection under amodiaquine pressure. Although the sample size was too small to make statistical inference between treatment success and failure as well as the presence of these mutations, both ASAQ pairs that were successfully sequenced at all seven *Pfcr1* mutations changed frequencies from 0% to 100% whereas the two AL pairs remained wildtype at these sites.

The contrasting selection patterns observed between AL and ASAQ are biologically plausible because both chloroquine and amodiaquine are 4-aminoquinolines and share overlapping resistance mechanisms mediated by *Pfcr1* [30, 31]. In contrast, lumefantrine exerts opposing selective pressure,

favoring wild type *Pfcr*t alleles. These findings provide a possible molecular explanation for the reduced efficacy of ASAQ observed in recent TESSs. They also provide evidence of the use of both AL and ASAQ in a multiple first-line treatment strategy to limit the spread of resistant parasites and preserve ACT efficacy [32]. More broadly, this research underscores the importance of integrating molecular surveillance data with therapeutic outcomes when informing national treatment policy.

LIMITATIONS

Several limitations should be considered. First, although the initial sample size was relatively large ($n = 328$), approximately 21% of samples were excluded because of observable mold growth, with most affected samples originating from a single site, Sinje. As a result, molecular data from this site was underrepresented. These affected samples were collected during the rainy season and stored for 6–12 months at ambient temperature (25°C) prior to analysis, underscoring the importance of routine desiccant replacement when samples are stored in high-humidity environments. Secondly, the samples included in this analysis may not be representative of the broader parasite population in Liberia; and therefore, the findings should not be extrapolated beyond the study sites. This limitation is further compounded by the exclusion of an additional 164 D0 samples, as only 81 randomly selected D0 samples were evaluated. Lastly, although the reported genotypes and SNPs have been associated with antimalarial drug resistance, the ability of these mutations to predict therapeutic outcomes remains limited. Lastly, while the MaRS protocol enables identification and estimation of mutation frequencies across six target genes, whole-genome sequencing would generate a more comprehensive assessment of genetic variation by capturing mutations across the entire parasite genome.

CONCLUSIONS

This study demonstrates the value of integrating next-generation sequencing-based molecular surveillance into antimalarial therapeutic efficacy monitoring. The investigation yielded three key findings: (1) none of the sequenced samples harbored mutations in the *k13* propeller domain associated with artemisinin resistance; (2) data suggests a resurgence of chloroquine sensitivity; and (3) no evidence of meaningful SP resistance was observed.

These data points, in combination with the PCR-corrected ACPR efficacy greater than 90% as presented in the complementary study report [6], support the continued use of AL in Liberia and reinforce the need for sustained genomic surveillance to guide evidence-based malaria control strategies.

Abbreviations

ACT
artemisinin-based combination therapy
AL
artemether-lumefantrine

ASAQ
artesunate-amodiaquine
CT
Cycle Threshold
D0
day 0
DBS
dried blood spots
DoF
day of failure
MaRS
Malaria Resistance Surveillance
Nf-NeST
Nextflow Next-generation Sequencing-analysis Toolkit
NGS
Next-Generation Sequencing
NMCP
National Malaria Control Program
PET-PCR
Photo-induced electron transfer PCR
Pf
Plasmodium falciparum
Pfcrt
Plasmodium falciparum gene coding for the chloroquine resistance transporter; PF3D7_0709000
Pfcytb
Plasmodium falciparum gene coding for cytochrome b; PF3D7_MIT02300
Pfdhfr
Plasmodium falciparum gene coding for the bifunctional dihydrofolate reductase-thymidylate synthase protein; PF3D7_0417200
Pfdhps
Plasmodium falciparum gene coding for the dihydropteroate synthase protein; PF3D7_0810800
Pfk13
Plasmodium falciparum gene coding for the kelch 13 protein; PF3D7_1343700
Pfmdr1
Plasmodium falciparum gene coding for the multidrug resistance protein 1; PF3D7_0523000
SNP
single nucleotide polymorphism
TES
therapeutic efficacy study
WHO

Declarations

Ethics review.

Ethical clearance for this study was obtained from the University of Liberia Pacific Institute for Research and Evaluation's (UL-PIRE) Institutional Review Board (ULIRB) and was determined as a non-research activity within the U.S. Centers for Disease Control and Prevention (CDC) (0900f3eb81e634a9). The study was registered with the US NIH Clinical Trials website (ClinicalTrials.gov ID# NCT06300970). Parents/guardians were informed of the study procedure, its benefits, potential risks, and gave written informed consent for their children to participate in the study prior to enrollment.

Data availability.

Data has been uploaded to NCBI under BioProject accession number PRJNA428490.

IF NEEDED SECTIONS

Consent for publication

All co-authors consent for the publication of the included results.

Competing interests

All co-authors report no competing interests or conflicts of interest. The findings and conclusions presented in this report are those of the authors and do not necessarily reflect the official position of the U.S. Centers for Disease Control and Prevention.

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Authors' contributions

VSK and JS designed the study. TW provided technical leadership. MR and MB wrote the manuscript preparation. JS, MD, LM, VSK and IC facilitated the transfer of samples to CDC. MR, MB, BH, EB, CK, BM, YVW, and GBGJ evaluated the quality of the DBSs. MR, BH, EB, and CK extracted DNA and performed PET-PCR. MR and MB amplified the genes, prepared libraries, sequenced libraries, and analyzed the sequencing results. BM facilitated laboratory data collection and tracking. MR and MB summarized the sequencing results and MB, LM, IC, and MA reviewed the results.

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Disclaimer:

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention,

References

1. World Health Organization: **World malaria report 2024: addressing inequity in the global malaria response**. 2024.
2. **Liberia: statistics & facts** [<https://www.severemalaria.org/countries/liberia>]
3. National Malaria Control Programme - NMCP/Liberia, ICF: **Liberia Malaria Indicator Survey 2022**. Monrovia, Liberia, and Rockville, Maryland, USA: NMCP and ICF; 2023.
4. Ngasala B, Bushukatale S, Chiduo M, Makene T, Mkony L, Mohamed A, Molteni F, Chacky F, Njau RJA, Mwaiswelo R: **Efficacy of artesunate-amodiaquine for treatment of uncomplicated Plasmodium falciparum malaria in mainland Tanzania**. *Malar J* 2024, **23**:90.
5. West African Network for Clinical Trials of Antimalarial D: **Pyronaridine-artesunate or dihydroartemisinin-piperaquine versus current first-line therapies for repeated treatment of uncomplicated malaria: a randomised, multicentre, open-label, longitudinal, controlled, phase 3b/4 trial**. *Lancet* 2018, **391**:1378-1390.
6. Koko VS, Skrip L, Kumeh O, Washington T, Diallo MO, Kafuko J, Inyang U, Ponce de Leon GF, Zhou Z, Laird VR, et al: **Efficacy and safety of artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) for the treatment of uncomplicated Plasmodium falciparum malaria in Liberia, 2022-2023**. *Malar J* 2025, **24**:319.
7. Efeti MT, Fankem SN, Diallo MT, Lahngong MS, Acha NL, Shey RA, Demeyer K, Souopgui J, Ghogomu SM, Njemini R: **Targeted Sequencing Identifies SNPs Associated with Antimalarial Drug Resistance and the CSP Vaccine Antigen in Plasmodium falciparum from Southwest Cameroon**. *Int J Mol Sci* 2025, **26**.
8. Gashema P, Kagame J, Iradukunda PG, Siddig EE, Tessema SK, Tegegne MA, Mazaba ML, Fallah M, Ngamije D, Harelimana JD, Muvunyi CM: **Mapping Plasmodium falciparum mutations in Africa: A critical review of emerging drug resistance and implications for malaria control**. *Int J Infect Dis* 2025, **159**:108033.
9. Junaid IT, Panda A, Deshmukh A, Sardar R, Narwal M, Agrawal P, Prakash N, Akhtar A, Dey AK, Singh SS, et al: **Role of novel mutations in food vacuole transporters beyond K13-mediated artemisinin resistance in Plasmodium falciparum**. *Antimicrob Agents Chemother* 2025, **69**:e0029325.
10. Gill J, Sharma A: **Genomic analysis of single nucleotide polymorphisms in malaria parasite drug targets**. *Parasit Vectors* 2022, **15**:309.
11. Laird VR, Plucinski MM, Venkatesan M, Rondini KA, Randrianarivelosia M, Andriamananjara MN, Moonga H, Ishengoma DS, Chidimatembue A, Dimbu PR, et al: **Plasmodium falciparum multidrug**

- resistance 1 gene polymorphisms associated with outcomes after anti-malarial treatment.** *Malar J* 2025, **24**:186.
12. Okell LC, Reiter LM, Ebbe LS, Baraka V, Bisanzio D, Watson OJ, Bennett A, Verity R, Gething P, Roper C, Alifrangis M: **Emerging implications of policies on malaria treatment: genetic changes in the Pfm-dr-1 gene affecting susceptibility to artemether-lumefantrine and artesunate-amodiaquine in Africa.** *BMJ Glob Health* 2018, **3**:e000999.
 13. Malmberg M, Ferreira PE, Tarning J, Ursing J, Ngasala B, Bjorkman A, Martensson A, Gil JP: **Plasmodium falciparum drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms.** *J Infect Dis* 2013, **207**:842-847.
 14. Ndong Ngomo JM, Mawili-Mboumba DP, M'Bondoukwe NP, Ditombi BM, Koumba Lengongo JV, Batchy Ognagosso FB, Bouyou-Akotet MK: **Drug Resistance Molecular Markers of Plasmodium falciparum and Severity of Malaria in Febrile Children in the Sentinel Site for Malaria Surveillance of Melen in Gabon: Additional Data from the Plasmodium Diversity Network African Network.** *Trop Med Infect Dis* 2023, **8**.
 15. Chen N, Russell B, Staley J, Kotecka B, Nasveld P, Cheng Q: **Sequence polymorphisms in pfcr-t are strongly associated with chloroquine resistance in Plasmodium falciparum.** *J Infect Dis* 2001, **183**:1543-1545.
 16. Foguim FT, Bogreau H, Gendrot M, Mosnier J, Fonta I, Benoit N, Amalvict R, Madamet M, Wein S, Pradines B, French National Reference Centre for Imported Malaria Study G: **Prevalence of mutations in the Plasmodium falciparum chloroquine resistance transporter, PfCRT, and association with ex vivo susceptibility to common anti-malarial drugs against African Plasmodium falciparum isolates.** *Malar J* 2020, **19**:201.
 17. Organization WH: **WHO compendium of molecular markers of antimalarial drug resistance.** Geneva2023.
 18. Talundzic E, Ravishankar S, Kelley J, Patel D, Plucinski M, Schmedes S, Ljolje D, Clemons B, Madison-Antenucci S, Arguin PM, et al: **Next-Generation Sequencing and Bioinformatics Protocol for Malaria Drug Resistance Marker Surveillance.** *Antimicrob Agents Chemother* 2018, **62**.
 19. World Health Organization: **Methods for surveillance of antimalarial drug efficacy.** Geneva2009.
 20. Souza SS, L'Episcopia M, Severini C, Udhayakumar V, Lucchi NW: **Photo-Induced Electron Transfer Real-Time PCR for Detection of Plasmodium falciparum plasmepsin 2 Gene Copy Number.** *Antimicrobial Agents and Chemotherapy* 2018, **62**:e00317-00318.
 21. Bosman A, Mendis KN: **A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies.** *Am J Trop Med Hyg* 2007, **77**:193-197.
 22. Koko VS, Warsame M, Vonhm B, Jeuronlon MK, Menard D, Ma L, Taweh F, Tehmeh L, Nyansaiye P, Pratt OJ, et al: **Artesunate-amodiaquine and artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in Liberia: in vivo efficacy and frequency of molecular markers.** *Malar J* 2022, **21**:134.

23. World Health Organization: **WHO policy recommendation on intermittent preventive treatment during infancy with sulphadoxine-pyrimethamine (SP-IPTi) for Plasmodium falciparum malaria control in Africa.** Geneva2010.
24. Millogo KS, Kabore B, Sondo P, Compaore EW, Kouevi AFC, Kambou SAE, Rouamba T, Kazienga A, Ilboudo H, Tahita MC, et al: **Trend of N86Y and Y184F Mutations in Pfmdr1 Gene in Children Under Seasonal Malaria Chemoprevention Coverage in Nanoro, Burkina Faso.** *Acta Parasitol* 2024, **69**:1967-1976.
25. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnadig N, Uhlemann AC, Martin RE, Lehane AM, Fidock DA: **Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies.** *Nat Commun* 2016, **7**:11553.
26. Wellems TE, Plowe CV: **Chloroquine-resistant malaria.** *J Infect Dis* 2001, **184**:770-776.
27. Sisowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, Fidock DA, Gil JP: **In vivo selection of Plasmodium falciparum parasites carrying the chloroquine-susceptible pfcr1 K76 allele after treatment with artemether-lumefantrine in Africa.** *J Infect Dis* 2009, **199**:750-757.
28. Beshir K, Sutherland CJ, Merinopoulos I, Durrani N, Leslie T, Rowland M, Hallett RL: **Amodiaquine resistance in Plasmodium falciparum malaria in Afghanistan is associated with the pfcr1 SVMNT allele at codons 72 to 76.** *Antimicrob Agents Chemother* 2010, **54**:3714-3716.
29. Holmgren G, Gil JP, Ferreira PM, Veiga MI, Obonyo CO, Bjorkman A: **Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pfcr1 76T and pfmdr1 86Y.** *Infect Genet Evol* 2006, **6**:309-314.
30. Wicht KJ, Mok S, Fidock DA: **Molecular Mechanisms of Drug Resistance in Plasmodium falciparum Malaria.** *Annu Rev Microbiol* 2020, **74**:431-454.
31. Tanner JD, Richards SN, Corry B: **Molecular basis of the functional conflict between chloroquine and peptide transport in the Malaria parasite chloroquine resistance transporter PfCRT.** *Nat Commun* 2025, **16**:2987.
32. World Health Organization: **Multiple first-line therapies as part of the response to antimalarial drug resistance.** Geneva2024.

Figures

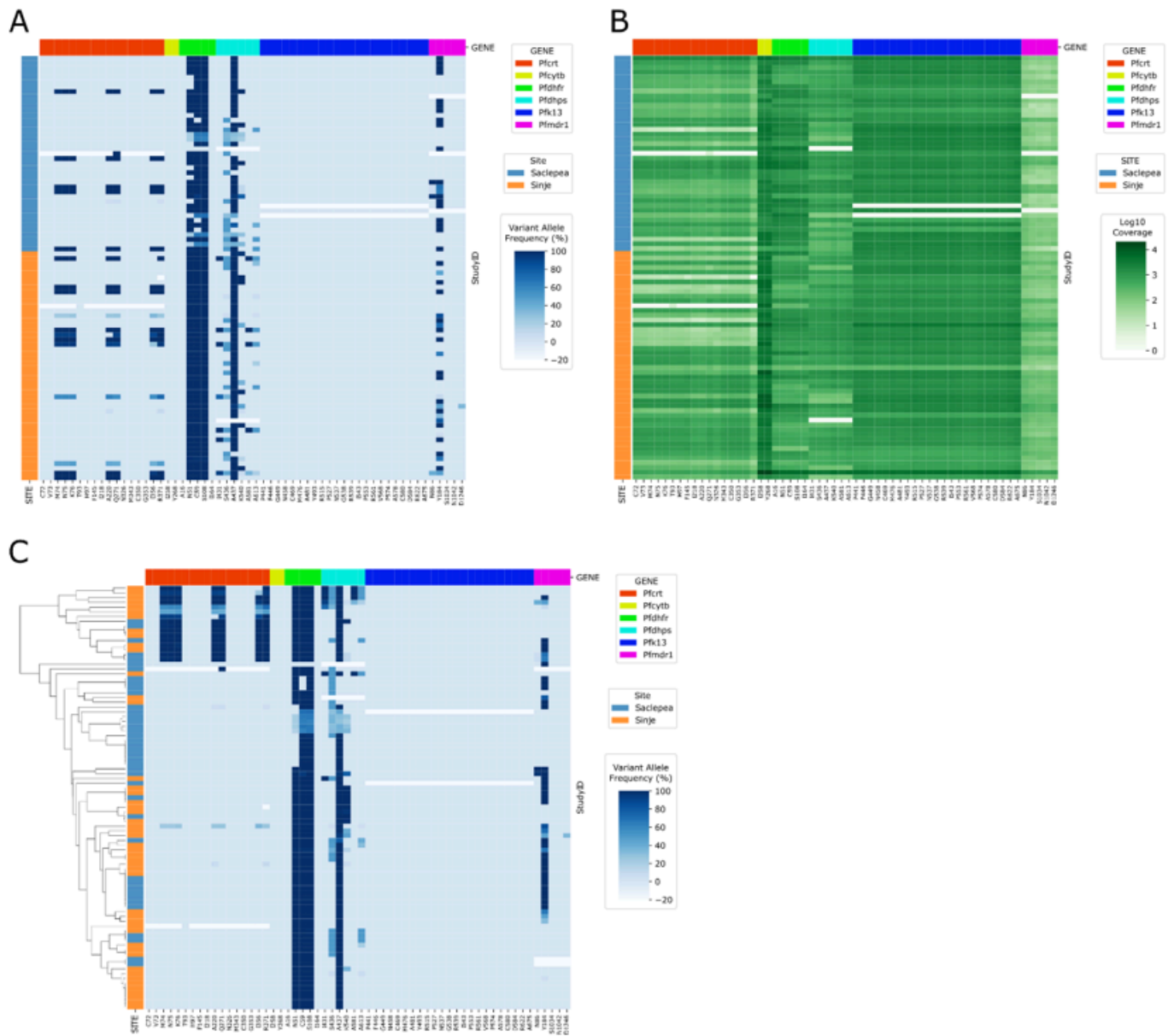


Figure 1

Observed mutations and sequencing coverage. Heatmaps represent each sequenced sample (row) at each tracked amino acid for a given gene (column) for variant allele frequencies (z-axis, **A** and **C**, 0% light blue to 100% dark blue) or log10 of the number of reads (z-axis, **B**, 10^0 light green to 10^4 dark green) at each intersecting cell. Each sample is labeled to the left of each heatmap with the site (blue = Saclepea, orange = Sinje) that sample belongs to. Each visualized amino acid is denoted at the bottom of the heatmap in numerical order within the gene, color-coded at the top of the heatmap (red = *Pfcrf*, yellow = *Pfcyfb*, green = *Pfdhfr*, cyan = *Pfdhps*, navy = *Pfk13*, pink = *Pfmdr1*), the amino acid belongs to. For all heatmaps, a white color (valued at -20 arbitrary units) indicates that no data was obtained for a given sample at those nucleotide positions. Samples are localized in the same rows in both of the unclustered heatmaps (**A** and **B**). **C**) Same heatmap as in **A** but the rows are hierarchically clustered based on row

composition, indicating the relatedness between samples in the dataset according to the frequency of mutation(s) observed for that sample.

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