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# Molecular surveillance for polymorphisms associated with artemisinin-based combination therapy resistance in *Plasmodium falciparum* isolates collected in Mozambique, 2018

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## Abstract

**Background:** Due to the threat of emerging anti-malarial resistance, the World Health Organization recommends incorporating surveillance for molecular markers of anti-malarial resistance into routine therapeutic efficacy studies (TESs). In 2018, a TES of artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) was conducted in Mozambique, and the prevalence of polymorphisms in the *pfk13*, *pfcr1*, and *pfmdr1* genes associated with drug resistance was investigated.

**Methods:** Children aged 6–59 months were enrolled in four study sites. Blood was collected and dried on filter paper from participants who developed fever within 28 days of initial malaria treatment. All samples were first screened for *Plasmodium falciparum* using a multiplex real-time PCR assay, and polymorphisms in the *pfk13*, *pfcr1*, and *pfmdr1* genes were investigated by Sanger sequencing.

**Results:** No *pfk13* mutations, associated with artemisinin partial resistance, were observed. The only *pfcr1* haplotype observed was the wild type CVMNK (codons 72–76), associated with chloroquine sensitivity. Polymorphisms in *pfmdr1* were only observed at codon 184, with the mutant 184F in 43/109 (39.4%) of the samples, wild type Y184 in 42/109 (38.5%), and mixed 184F/Y in 24/109 (22.0%). All samples possessed N86 and D1246 at these two codons.

**Conclusion:** In 2018, no markers of artemisinin resistance were documented. Molecular surveillance should continue to monitor the prevalence of these markers to inform decisions on malaria treatment in Mozambique.

**Keywords:** Antimalarial drug resistance, *Plasmodium falciparum*, *pfk13*, *Pfcr1*, *pfmdr1*, Artemisinin-based combination therapy (ACT), Polymorphisms, Mozambique

## Background

Malaria remains a leading health problem in many countries, particularly those in tropical and subtropical regions of the world [1]. In Mozambique, malaria represents a major cause of morbidity and mortality, accounting for 29% of all deaths and approximately 42% of deaths among children less than 5 years of age [2]. *Plasmodium*

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*falciparum* is the predominant malaria parasite species in the country [3].

One of the fundamental steps toward malaria control is the rapid diagnosis and correct treatment of symptomatic cases with an effective anti-malarial [4]. Anti-malarial drug resistance continues to be a major hurdle to malaria control efforts in some settings. Since replacing chloroquine (CQ) with a combination of amodiaquine (AQ) + sulfadoxine-pyrimethamine (SP) for uncomplicated malaria treatment in 2003, the Mozambique national treatment guidelines have experienced various adjustments as parasites became resistant to treatments [5]. In 2006, artemisinin-based combination therapy (ACT) was formally introduced by adopting artesunate (AS) + SP as a first-line treatment for uncomplicated *P. falciparum* malaria [6, 7]. Subsequently, the last change occurred in 2009, when the country introduced artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) as the official first-line treatments, with ASAQ as a backup in situations when AL is contraindicated [6–8]. While ASAQ is part of the national treatment algorithm, there has been limited procurement and use. In artemisinin-based combinations, the artemisinin component is short-acting and kills the majority of parasites within the first 2 days of treatment; the remaining parasites are cleared by the longer-acting partner drug [9], thus helping to abate the acquisition of parasite resistance to the treatment. However, resistance to artemisinin derivatives, defined as delayed parasite clearance (presence of > 10% parasitaemia on day 3 after the start of treatment), has been reported in Southeast Asia [10–12] and Rwanda [13]. Resistance to specific anti-malarials is associated with polymorphisms, such as a single nucleotide polymorphisms (SNPs), a combination of SNPs, or gene copy number variation in drug target genes.

To monitor the efficacy of anti-malarial treatment, the World Health Organization (WHO) recommends periodic (at least every 2 years) monitoring of the first and second-line anti-malarial treatments [14] and, in addition, molecular surveillance of resistance markers is encouraged. Artemisinin partial resistance is associated with polymorphisms in the *P. falciparum* *kelch 13* (*pfk13*) gene [10] and ten SNPs in *pfk13* gene are currently validated molecular markers for artemisinin partial resistance: F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L and C580Y [14]. One of these mutations, R561H, has been reported to be present in multiple samples from different sites in Rwanda [13, 15, 16], highlighting the importance of conducting molecular surveillance to identify emerging artemisinin and partner drug resistance genotypes. To date, there have been no reports of *pfk13* polymorphisms associated with artemisinin partial resistance in Mozambique [17, 18].

Resistance to CQ is mainly associated with SNPs in the *P. falciparum* *chloroquine resistance transporter* (*pfcr*) gene, resulting in an amino acid change from lysine (K76) to threonine (76T) at position 76; however, the *P. falciparum* *multi-drug resistance* (*pfmdr1*) gene may also play a role in CQ resistance [19, 20]. The most commonly reported *pfcr* mutations are observed in codons 72, 74–76 [21]. The wild type CVMNK haplotype is associated with CQ sensitivity, while the CVIET and SVMNT haplotypes are associated with CQ resistance, with CVIET being the more common of the latter two haplotypes in Africa [22, 23].

The *pfmdr1* gene is implicated in lower sensitivity or tolerance to several anti-malarial drugs, including CQ, AQ, and lumefantrine [22, 24]. In Africa, the most relevant polymorphisms of *pfmdr1* include N86Y, Y184F and D1246Y [8, 25]. Mutations at positions S1034C and N1042D of *pfmdr1* are rarely reported on the continent [8, 26]. The 86Y mutation has been associated with decreased CQ and AQ sensitivity, while the N86 wild type codon has been implicated in decreased sensitivity to lumefantrine. The N86, 184F, and D1246 (NFD) haplotype is associated with decreased sensitivity to AL, while the 86Y, Y184, and 1246Y (YYY) haplotype is reported to be associated with decreased sensitivity to ASAQ [25]. In Mozambique, the prevalence of *pfmdr1* mutations was low in the capital city of Maputo, although the alleles N86 and 184F showed a significantly increased prevalence after the introduction of ACT [8].

Molecular surveillance for drug resistant parasites is part of a comprehensive approach along with TESs for early detection and subsequent prevention of spread of resistant parasites by permitting timely implementation of appropriate alternative treatment policy decisions. This study's aim was to analyse the prevalence of molecular markers associated with *P. falciparum* resistance to anti-malarial drugs in the *pfk13*, *pfmdr1*, and *pfcr* genes in samples collected during a 2018 TES in four sentinel sites in Mozambique.

## Methods

### Study sites

This study was a sub-study of a TES that evaluated the efficacy and safety of AL and ASAQ in the treatment of uncomplicated *P. falciparum* malaria in children aged 6–59 months in Mozambique, based on WHO-recommended protocol [27]. Malaria transmission in the country is year-round, with seasonal peaks during and after the rainy season, which occurs between October and March. The peak of the malaria transmission extends from November into April [3]. This study was conducted between February and September 2018 in four sentinel sites: Rural Hospital of Montepuez, in Cabo Delgado

Province (Northern region), Moatize Health Center, in Tete Province (Central region), District Hospital of Mopeia, in Zambézia (Central region), and District Hospital of Massinga, in Inhambane Province (Southern region) (Fig. 1). These sites are distributed across the Northern, Central, and Southern regions of Mozambique, which represent areas with high, moderate, and low prevalence of malaria, respectively. The per protocol PCR-corrected efficacy results of this study will be reported elsewhere, but were greater than 95% for all four AL arms and greater than 98% for all three ASAQ arms (no ASAQ arm in Moatize).

**Sample collection**

Potential participants were screened for malaria parasites using microscopy at each study site. Patients were eligible for enrolment if they had uncomplicated *P. falciparum* mono-infection with an asexual blood density between 2000 and 200,000/μL, were aged 6–59 months, and had a fever at presentation (axillary temperature ≥ 37.5 °C) or history of fever in the last 24 h. A dried blood spot on Whatman 3-mm filter paper was prepared using

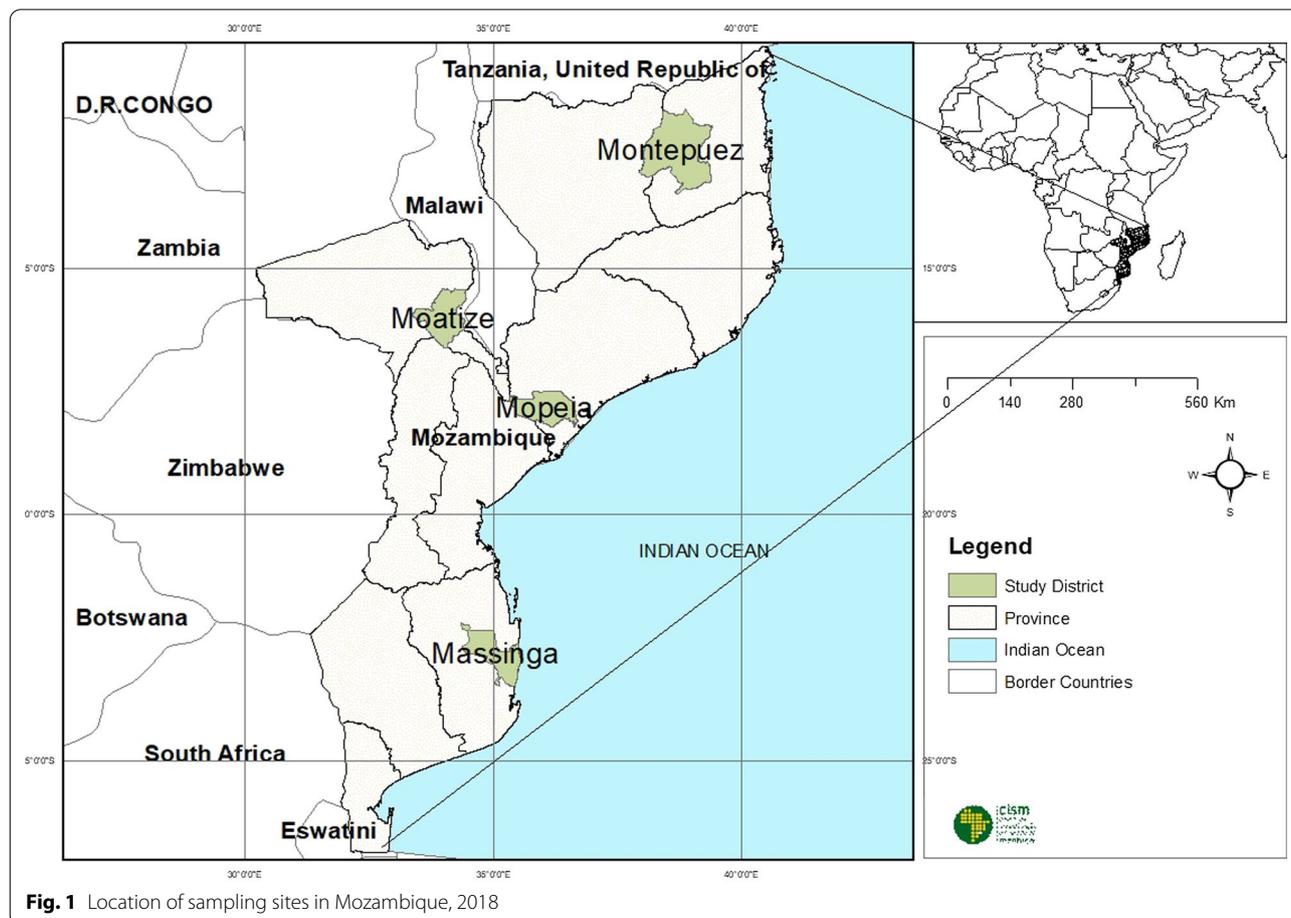
50 μL of blood collected on the day of enrolment (day 0/ pre-treatment) and on any other day the patient had a recurrent malaria infection during the follow-up period (post-treatment).

**DNA extraction**

DNA was extracted at the Manhiça Health Research Center Laboratory, Mozambique, from half of the dried blood spot using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The DNA was eluted in 150μL of elution buffer, aliquoted and transferred to the CDC Malaria Laboratory in Atlanta, GA, USA, for molecular analysis.

**Molecular genotyping of resistance markers**

Molecular analysis for drug resistance markers was performed by a laboratory technician from Mozambique with the support of staff from the CDC Malaria Laboratory in Atlanta, USA [28]. For this analysis, selected pre-treatment and all post-treatment samples were used. Samples were first screened using a multiplex real-time PCR assay (PET-PCR) for detection of *Plasmodium*



**Fig. 1** Location of sampling sites in Mozambique, 2018

genus and *P. falciparum*, as previously described [29]. Polymorphisms in the *pfk13* (propeller domain 389–649), *pfprt* (codons 72–76), and *pfmdr1* (codons 86, 184, 1034, 1042, and 1246) genes were investigated as previously described [30, 31]. Briefly, both pre-treatment and post-treatment samples were used to amplify fragments of *pfk13*, *pfprt*, and *pfmdr1* by nested PCRs. Three laboratory *P. falciparum* parasite lines, 3D7, 7G8, and Dd2, were included as controls. Direct Sanger sequencing of the purified nested PCR products was performed using a BigDye Terminator v3.1 cycle sequencing kit on an iCycler thermal cycler (Bio-Rad, CA, USA). The reaction mixtures were precipitated in 70% ethanol to clean up dye terminators, rehydrated in 10 µL HiDi formamide, and then sequenced on a 3130xl ABI genetic analyzer (ABI Prism, CA, USA). Sequence analysis was performed using Geneious R7 (Biomatters, Auckland, New Zealand). Raw sequence reads were cleaned using default settings and reads with high-quality scores (>30%) were further analysed using the 3D7 *pfk13*, *pfprt*, and *pfmdr1* genes as references.

#### Data analyses

Data were entered into a Microsoft Office Excel 2007 sheet and then exported into R 3.6.0 (R Core Team 2019) for validation, cleaning, and analysis. A statistical significance of difference in the risk of treatment failure (reinfection or recrudescence) was determined by Fisher's exact test, at a 5% significance level. All possible haplotypes from mixed infections (both wild type and mutants) were included in construction of the *pfmdr1* haplotype.

## Results

### Characteristics of study subjects

From the 641 patients enrolled in the TES, 110 (17%) pre-treatment samples were selected for the analysis. This included all the pre-treatment samples from subjects who returned with a recurrent infection ( $n=51$ ) and 10% randomly selected pre-treatment samples from patients who did not have a recurrent infection ( $n=59$ ); however, one sample was excluded due to poor quality DNA, leaving 109 pre-treatment samples. All 51 post-treatment samples from patients who had a recurrent malaria infection were included in the analysis (Fig. 2). In the AL and ASAQ arms, 7.1% (26/368) and 3.0% (8/273) of subjects, respectively, remained parasitaemic at day 3, although no subject met criteria for early treatment failure.

Table 1 summarizes the characteristics by site of 109 study participants used for molecular analysis. A total of 79 and 30 pre-treatment samples, and 48 and 3 post-treatment samples, were in the AL and ASAQ treatment arms, respectively.

### Molecular markers of drug resistance

#### *pfk13* polymorphisms

All 109 pre-treatment samples and 48/51 (94.1%) of the post-treatment samples were successfully sequenced at the *pfk13* gene. No polymorphisms associated with artemisinin partial resistance were observed in the propeller domain. One sample from Mopeia contained a synonymous mutation at codon 469 (TGC to TGT) and three samples from Mopeia contained a synonymous mutation at codon 548 (GGC to GGT). No other synonymous or nonsynonymous mutations were found.

#### *pfprt* polymorphisms

All 109 pre-treatment samples and 47/51 (92.2%) of the post-treatment samples were successfully sequenced at the *pfprt* gene. All samples showed the wild type CVMNK haplotype.

#### *pfmdr1* polymorphisms

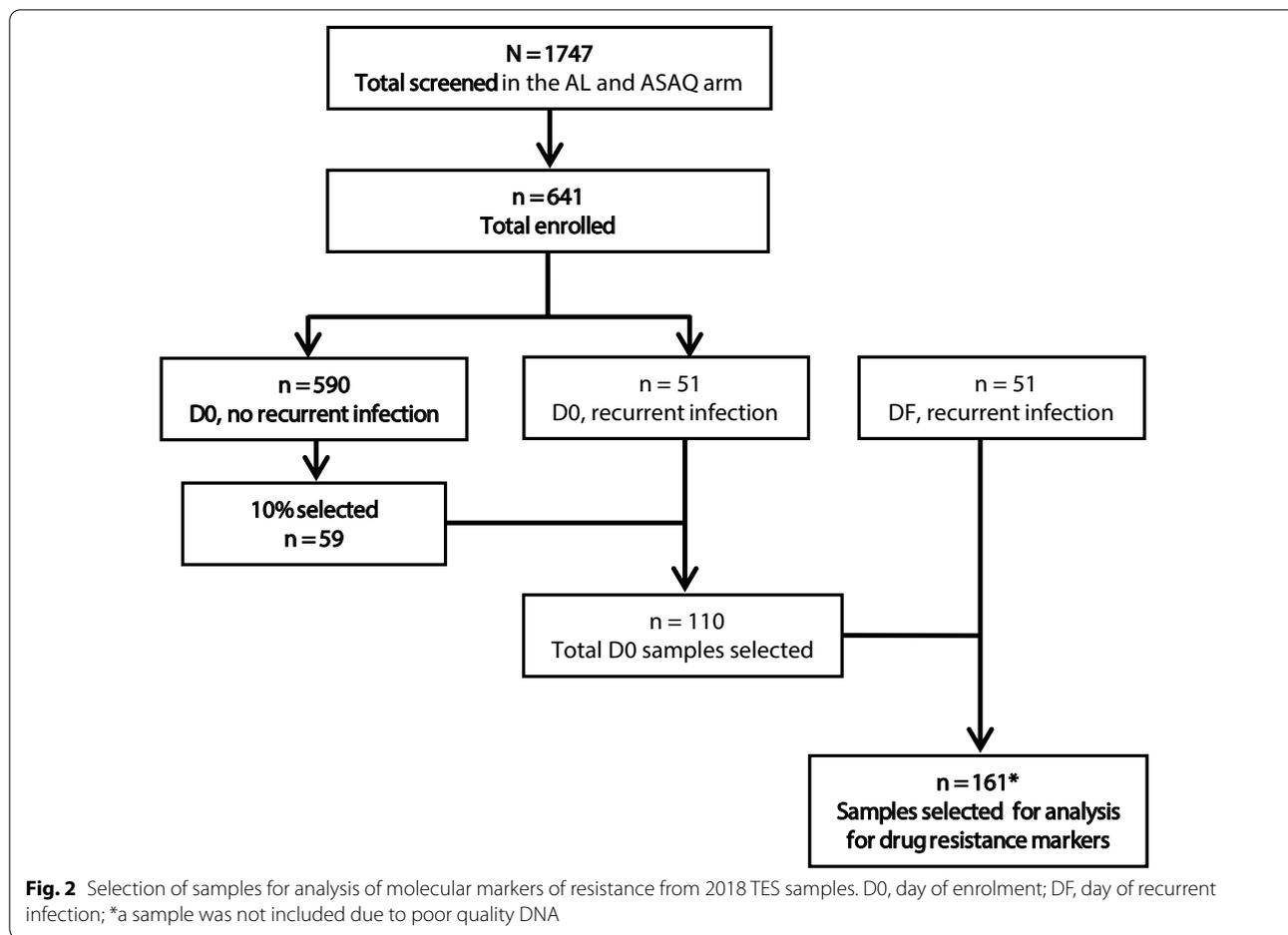
All 109 pre-treatment and 48/51 (94.1%) post-treatment samples were successfully sequenced for the *pfmdr1* gene. All pre-treatment samples possessed the N86, S1034, N1042 and D1246 alleles, with polymorphisms being observed only at codon 184: 184F in 43 (39.4%), Y184 in 42 (38.5%), and mixed Y/F in 24 (22.0%).

Among the 79 pre-treatment samples obtained from the AL arm, NFD (86, 184, 1246 codons) and NYD haplotypes were present in 49 (62.0%) and 45 (57.0%), respectively. In the ASAQ arm, NFD and NYD were present in 18 (60.0%) and 21 (70.0%) of the pre-treatment samples, respectively. Neither NFD nor NYD significantly changed ( $p>0.05$ ) in post-treatment infections after treatment with either AL or ASAQ (Table 2).

In the pre-treatment samples, NFD was present in 29 (74.4%) and 8 (66.7%) samples from Massinga and Moatize, respectively, while NYD was present in 15 (75.0%) and 26 (68.4%) samples from Montepuez and Mopeia, respectively. For the late treatment failure samples, the NFD was observed in all samples from Moatize and Montepuez and in 72.7% of the samples from Massinga and Mopeia (Table 3).

## Discussion

Mozambique has used AL and ASAQ as the two first-line anti-malarial regimens since 2009, with AL being the most widely used and ASAQ as backup for situations in which AL could not be used or is not available. This study, provides insights into the *pfk13*, *pfprt*, and *pfmdr1* genetic profiles of *P. falciparum* isolates from four sentinel sites throughout the country. In this study, no *pfk13* mutations associated with artemisinin partial resistance were observed. These findings are encouraging and suggest that artemisinin partial resistance has not yet



**Table 1** Characteristics of study participants by site, Mozambique 2018

Number of subjects	Massinga 39	Moatize 12	Montepuez 20	Mopeia 38	Total 109
Female sex n (%)	21 (53.8)	5 (41.7)	9 (45.0)	18 (47.4)	53 (48.6)
Age in months (mean ± SD)	28.5 ± 5.4	37.2 ± 16.3	21.8 ± 14.3	29.6 ± 14.4	28.6 ± 15.3
Temperature in °C (mean ± SD)	38.4 ± 0.8	38.7 ± 1.1	38 ± 0.8	37.9 ± 0.4	38.2 ± 0.8
Parasite density geometric mean (range)	28,100 (800–126,800)	52,900 (13,300–181,100)	30,500 (4800–107,500)	41,500 (4300–168,200)	35,100 (800–181,100)
Hb in g/dL (mean ± SD)	8.2 ± 1.7	9.5 ± 1.1	9.6 ± 1.9	9.4 ± 1.6	9 ± 1.7

SD, standard deviation; Hb, haemoglobin

emerged in the four study sites selected in Mozambique. A previous study in Mozambique revealed a very low prevalence (<1%) of four polymorphisms in the *pfk13* gene (L619L, F656I, V666V, and G690G)[17]. Another study revealed the presence of a V494I *K13* polymorphism, found in two samples collected after the introduction of ACT in Mozambique [32]; However, these aforementioned mutations are either synonymous or not

known to be associated with artemisinin partial resistance [17]. This current study’s findings are also consistent with most reports from Africa in which no, or a very low prevalence of, *pfk13* mutations have been reported [33, 34]. The absence of delayed parasite clearance and *pfk13* mutations known to be associated with artemisinin partial resistance is reassuring for Mozambique, at least in the short term. Nevertheless, a recently published

**Table 2** Prevalence of *pfmdr1* 184 polymorphisms in pre-treatment and post-treatment samples stratified by treatment arms

<i>pfmdr1</i> Polymorphism	AL arm			ASAQ arm		
	Pre-treatment	Post-treatment	<i>p</i> value*	Pre-treatment	Post-treatment	<i>p</i> value*
	n (%)	n (%)		n (%)	n (%)	
<i>pfmdr1</i> codon 184	n = 79	n = 46		n = 30	n = 2	
Y184	30 (38.0)	12 (26.1)	Ref	12 (40.0)	0 (0)	Ref
184Y/F	15 (10.0)	16 (34.8)	0.054	9 (30.0)	1 (50.0)	0.454
184F	34 (43.0)	18 (39.1)	0.657	9 (30.0)	1 (50.0)	0.454
<i>pfmdr1</i> haplotypes <sup>a</sup>	n = 79	n = 46		n = 30	n = 2	
NYD	45 (57.0)	28 (60.9)	Ref	21 (70.0)	1 (50.0)	Ref
NFD	49 (62.0)	34 (73.9)	0.746	18 (60.0)	2 (100)	0.597

AL, artemether-lumefantrine; ASAQ, artesunate-amodiaquine

\*Statistical significance in risk of recurrent infection (reinfection or recrudescence) was determined by Fisher's exact test; three post-treatment samples (two in the AL arm and one in the ASAQ arm) failed to amplify at one or more loci and are not included in corresponding single nucleotide polymorphism and haplotype counts

<sup>a</sup> Haplotype percentages may not sum to 100% because all possible haplotypes from mixed infections (both wild type and mutants) were included in the construction of haplotypes

**Table 3** Prevalence of *pfmdr1* polymorphisms in pre-treatment and post-treatment samples stratified by study site

<i>pfmdr1</i> <sup>a</sup>	Pre-treatment (N = 109)				Post-treatment (N = 48)			
	MEGA	MEZE	MEMP	MEIA	MEGA	MEZE	MEMP	MEIA
	n = 39	n = 12	n = 20	n = 38	n = 22	n = 1	n = 3	n = 22
Y184	10 (25.6)	4 (33.3)	9 (45.0)	19 (50.0)	6 (27.3)	0 (0)	0 (0)	6 (27.3)
184Y/F	8 (20.5)	3 (25.0)	6 (30.0)	7 (18.4)	8 (36.4)	1 (100)	0 (0)	8 (36.4)
184F	21 (53.8)	5 (41.7)	5 (25.0)	12 (31.6)	8 (36.4)	0 (0)	3 (100)	8 (36.4)
NYD	18 (46.2)	7 (58.3)	15 (75.0)	26 (68.4)	14 (63.6)	1 (100)	0 (0)	14 (63.6)
NFD	29 (74.4)	8 (66.7)	11 (55.0)	19 (50.0)	16 (72.7)	1 (100)	3 (100)	16 (72.7)

Samples are from both AL and ASAQ arms; MEGA: Massinga; MEZE: Moatize; MEMP: Montepuez; MEIA: Mopeia; three samples (two in the AL arm and one in the ASAQ arm) failed to amplify at day of failure at one or more loci and are not included in corresponding single nucleotide polymorphism and haplotype counts

<sup>a</sup> Percentages may not sum to 100% because all possible haplotypes from mixed infections (both wild type and mutants) were included in the construction of haplotypes. Tests of significance not performed due to low sample sizes in two sites

Rwandan study, using samples collected between 2012 and 2015, showed that 7.4% of the specimens carried the *pfk13* R561H mutation [15], known to be associated with artemisinin partial resistance. Another recent Rwandan study also detected the presence of two validated markers of artemisinin partial resistance, R561H and P574L, and delayed parasite clearance (parasitaemia at day 3) in more than 10% of the study participants in two sites [13]. Although this finding was not linked to clinical treatment failure of AL, it highlights the importance of conducting molecular surveillance to identify emerging patterns of parasites with artemisinin and partner drug resistance genotypes.

The *pfcr* data from this study showed that all samples sequenced contained the wild type *pfcr* haplotype (CVMNK), suggesting the return of chloroquine sensitive alleles after its use was discontinued in 2003 in Mozambique. This finding is consistent with data from previous studies carried out in Mozambique that reported

an increasing frequency of the *pfcr* CVMNK wild type, from 43.9 to 66.4% between 2009 and 2010 [22]. A Mozambique study from 2015 reported a very low prevalence of mutant alleles at codons M74I, N75E, and K76T; only 2.3% samples harbored the *pfcr* SNP 76T [17]. A separate report from 2015 reported only 0.9% samples with the *pfcr* 76T mutant allele, 3.7% samples with a mixed infection, and 95.4% samples with the wild type allele [35]. These findings confirm the likely return of CQ-susceptible *P. falciparum* and are similar to findings from studies conducted in other African countries that also observed a resurgence in the proportion of wild type *pfcr* alleles after the discontinuation of CQ for treatment [36–38]. AL has been shown to select for *pfcr* wild types [22, 39], and the widespread use of AL in most African countries may contribute to the re-emergence of these alleles associated with CQ sensitivity [39].

Polymorphisms in *pfmdr1* were only observed at codon 184, resulting in two observed haplotypes, NFD

and NYD. This is consistent with previous findings from Mozambique. In 2015, a low prevalence of 86Y (3.1%) and a higher prevalence of 184F (46.7%) were reported [17]. In addition, a high prevalence of wild type N86 (73.2%) and D1246 (96.7%) and the presence of the mutant 184F (22.7%) were reported in a 2010–2012 study [8].

The *pfmdr1* gene has been implicated in lower sensitivity or tolerance to several anti-malarial drugs, including lumefantrine, CQ, and AQ [22, 24], with the 86Y mutation being associated with decreased CQ and AQ sensitivity and the N86 wild type allele implicated in decreased sensitivity to lumefantrine [25]. The NFD haplotype increased in prevalence between the pre- and post-treatment samples in this study's AL arm, but this was not significant when compared with NYD. In Mozambique, NFD haplotype prevalence increased from approximately 22–38% between 2009 and 2010 [22]. While the sites from this study are not comparable to that report, the 61.5% pre-treatment NFD prevalence indicates that this haplotype is still circulating. Similar findings over time have been reported in other African countries in which AL was used as the first-line anti-malarial treatment [40, 41]. Some studies showed that the *pfmdr1* gene polymorphism at codons N86Y, Y184F, and D1246Y is mainly linked to AL or ASAQ drug pressure [42, 43]. Stratifying by site, NFD was identified in 100% of post-treatment samples from Moatize and Montepuez and 72.7% of samples from Massinga and Mopeia, although this was not statistically significant. Notable limitations of this study include a low sample size in some sites, due to few late recurrences, and the limited number of pre-treatment samples analysed, due to budgetary restrictions.

## Conclusion

Given that no *pfk13* or *pfprt* molecular markers of resistance were observed, the results of this study corroborate the findings of the associated TES that showed AL and ASAQ were efficacious. The high prevalence of the *pfmdr1* NFD haplotype, associated with decreased sensitivity to lumefantrine in some studies, requires further investigation to fully understand the role of this haplotype in the sensitivity of the currently used artemisinin-based combinations, AL and ASAQ. Because alleles associated with artemisinin partial resistance are emerging in the East Africa region, continued molecular surveillance for early detection of these alleles as well as relevant partner drug resistant markers remains important.

## Abbreviations

ACT: Artemisinin-based combination therapy; AL: Artemether-lumefantrine; AQ: Amodiaquine; AS: Artesunate; ASAQ: Artesunate-amodiaquine; CDC: Centers for Disease Control and Prevention; CIBERESP: Consorcio de Investigación Biomédica en Red de Epidemiología y Salud (Consortium for Biomedical Research in Epidemiology and Public Health); CISM: Centro de Investigaç

em Saúde de Manhica (Manhica Health Research Centre); CQ: Chloroquine; INS: Instituto Nacional de Saúde (Mozambican National Institute of Health); PCR: Polymerase chain reaction; *pfprt*: *Plasmodium falciparum* chloroquine resistance transporter; *pfk13*: *Plasmodium falciparum* Kelch 13; *pfmdr1*: *Plasmodium falciparum* multidrug resistance 1; SNP: Single nucleotide polymorphism; SP: Sulfadoxine-pyrimethamine; WHO: World Health Organization.

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## Authors' contributions

AC: conducted the laboratory work and wrote the first draft of the manuscript; SS, NL, IG, VU, EH: provided technical support for the laboratory work, analysed molecular data, and reviewed the manuscript; LN, AbN, CS: assisted in field-work activities and reviewed the article; ArN: provided biostatistical support; EM, PA, EC, SE: conceived and designed the study protocol and reviewed the manuscript; AM, QB, AS, BC, RZ: participated in interpretation of results and reviewed the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

The study was reviewed and approved by the National Bioethics Committee for Health of Mozambique (CNBS—IRB00002657) on December 13, 2017, (Ref. 516/CNBS/17). The Office of the Associate Director for Science in the Center for Global Health at Centers for Disease Control and Prevention (CDC) determined the work to be a non-engaged research activity without requirement for CDC IRB review (Ref. 2017-361).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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