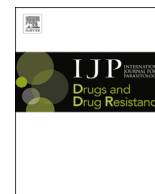




Contents lists available at ScienceDirect

# International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: [www.elsevier.com/locate/ijppaw](http://www.elsevier.com/locate/ijppaw)

## Temporal trends in prevalence of *Plasmodium falciparum* molecular markers selected for by artemether–lumefantrine treatment in pre-ACT and post-ACT parasites in western Kenya



Angela O. Achieng<sup>a</sup>, Peninah Muiruri<sup>a, b</sup>, Luicer A. Ingasia<sup>a</sup>, Benjamin H. Opot<sup>a</sup>,  
Dennis W. Juma<sup>a</sup>, Redemptah Yeda<sup>a</sup>, Bidii S. Ngalah<sup>a, c</sup>, Bernhards R. Ogutu<sup>a</sup>,  
Ben Andagala<sup>a</sup>, Hoseah M. Akala<sup>a</sup>, Edwin Kamau<sup>a, \*</sup>

<sup>a</sup> Department of Emerging Infectious Diseases, United States Army Medical Research Unit-Kenya/Kenya Medical Research Institute, Kisumu, Kenya

<sup>b</sup> Department of Biochemistry, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

<sup>c</sup> Institute of Tropical Medicine and Infectious Diseases, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

### ARTICLE INFO

#### Article history:

Received 24 March 2015

Received in revised form

27 May 2015

Accepted 28 May 2015

Available online xxx

#### Keywords:

Artemether–lumefantrine

Drug-resistance

Western Kenya

Artemisinin-based combination therapies

Africa

Chloroquine

Molecular markers

Genetics

### ABSTRACT

Artemether–lumefantrine (AL) became the first-line treatment for uncomplicated malaria in Kenya in 2006. Studies have shown AL selects for SNPs in *pfprt* and *pfmdr1* genes in recurring parasites compared to the baseline infections. The genotypes associated with AL selection are K76 in *pfprt* and N86, 184F and D1246 in *pfmdr1*. To assess the temporal change of these genotypes in western Kenya, 47 parasite isolates collected before (pre-ACT; 1995–2003) and 745 after (post-ACT; 2008–2014) introduction of AL were analyzed. In addition, the associations of parasite haplotype against the IC<sub>50</sub> of artemether and lumefantrine, and clearance rates were determined. Parasite genomic DNA collected between 1995 and 2014 was analyzed by sequencing or PCR-based single-base extension on Sequenom MassARRAY. IC<sub>50</sub>s were determined for a subset of the samples. One hundred eighteen samples from 2013 to 2014 were from an efficacy trial of which 68 had clearance half-lives. Data revealed there were significant differences between pre-ACT and post-ACT genotypes at the four codons (chi-square analysis;  $p < 0.0001$ ). The prevalence of *pfprt* K76 and N86 increased from 6.4% in 1995–1996 to 93.2% in 2014 and 0.0% in 2002–2003 to 92.4% in 2014 respectively. Analysis of parasites carrying pure alleles of K + NFD or T + YYY haplotypes revealed that 100.0% of the pre-ACT parasites carried T + YYY and 99.3% of post-ACT parasites carried K + NFD. There was significant correlation ( $p = 0.04$ ) between lumefantrine IC<sub>50</sub> and polymorphism at *pfmdr1* codon 184. There was no difference in parasite clearance half-lives based on genetic haplotype profiles. This study shows there is a significant change in parasite genotype, with key molecular determinants of AL selection almost reaching saturation. The implications of these findings are not clear since AL remains highly efficacious. However, there is need to closely monitor parasite genotypic, phenotypic and clinical dynamics in response to continued use of AL in western Kenya.

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### 1. Introduction

Recent gains made in malaria control have relied on the sustained efficacy of artemisinin-based combination therapies (ACTs) which is the recommended first-line treatment for uncomplicated *Plasmodium falciparum* malaria by the World Health Organization (WHO). ACTs consist of a short-acting artemisinin component

which rapidly reduces parasite biomass and results in consequent rapid resolution of the symptoms (Na et al., 1994; Bethel et al., 1997; White et al., 1999), and a long-acting partner drug (White et al., 1999) essential in clearing residual parasitemia. Resistance to ACTs is threatening malaria control and elimination efforts in Southeast Asia (SEA) (Noedl et al., 2008; Dondorp et al., 2009; WHO, 2014). However, ACTs remain highly efficacious in sub-Saharan Africa (SSA) with fast clearance half-lives (Ashley et al., 2014; Zwang et al., 2014). Sensitive *P. falciparum* parasites are cleared within 48 h in 95% of the patients post-ACTs treatment based on smears (White, 2008) whereas resistant parasites are

\* Corresponding author.

E-mail address: [edwin.kamau.mil@mail.mil](mailto:edwin.kamau.mil@mail.mil) (E. Kamau).

characterized by slow clearance rates (Noedl et al., 2008; Dondorp et al., 2009). The presence of parasites at 72 h is a good predictor of subsequent treatment failure (Stepniewska et al., 2010). Mutations in the *P. falciparum* K13-propeller domain have recently been shown to be important determinants of artemisinin resistance in SEA (Ariey et al., 2014; Ashley et al., 2014). However, the K13-propeller region mutations recently described in SEA have not been found in African parasites (Ashley et al., 2014; Conrad et al., 2014; Kamau et al., 2014; Taylor et al., 2014) nor has there been an association of K13 polymorphisms with parasite clearance half-lives in African parasites (Ashley et al., 2014).

The most commonly used ACT in SSA is artemether–lumefantrine (AL) (WHO, 2013) which has been used as the first-line treatment for uncomplicated malaria in Kenya since 2006 (Amin et al., 2007). In a recent study where individual patient data from 31 clinical trials was analyzed, the overall clinical efficacy for patients treated with AL was shown to be 94.8%; 93.8% efficacy in East Africa and 96.2% in West Africa (Venkatesan et al., 2014). In Kenya, AL efficacy has remained >95% (Ogotu et al., 2014). Although AL remains highly efficacious and K13 polymorphisms are not associated with reduced susceptibility to ACTs in SSA, AL is associated with selection of single nucleotide polymorphisms (SNPs) in *P. falciparum* chloroquine resistance transporter gene (*pfcr*) and *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) in parasite re-infections (Sisowath et al., 2005; Humphreys et al., 2007; Sisowath et al., 2007, 2009). The genotype associated with the parasite re-infection is the K76 in *pfcr* and N86, 184F and D1246 (NFD) in the *pfmdr1*. Further, reduced susceptibility to lumefantrine has also been linked to NFD and increase in the *pfmdr1* copy numbers (Lim et al., 2009; Some et al., 2010; Gadalla et al., 2011; Malmberg et al., 2013a,b). Recent meta-analysis of clinical trials data showed the presence of N86 allele and an increased *pfmdr1* copy number were the most significant independent risk factors for recurrence of parasites in patients treated with AL (Venkatesan et al., 2014). Monitoring the selection and emergence of these markers presents a cost-effective method for detecting evidence of declines in parasite susceptibility to AL and should be made routine (Malmberg et al., 2013a,b; Venkatesan et al., 2014).

In 1998, sulphadoxine–pyrimethamine (SP) replaced chloroquine as the first-line treatment of uncomplicated malaria in Kenya due to treatment failure and in 2006, SP was replaced with AL (Shretta et al., 2000; Amin et al., 2007; Okiro et al., 2010). In both instances, treatment failures were attributed to development of drug resistance. The genotype associated with chloroquine resistance is *pfcr* 76T (Djimé et al., 2001), modulated by *pfmdr1* 86Y, Y184 and 1246Y (YYY) (Reed et al., 2000; Babiker et al., 2001). Interestingly, chloroquine resistant parasites are AL sensitive; i.e. AL selects for chloroquine sensitive parasites. In this study, we set out to analyze the temporal trends of parasite genotypes in *pfcr* (codon 76) and *pfmdr1* (codons 86, 184 and 1246) in parasite samples from Kisumu County, western Kenya, collected before (1995–2003) and after (2008–2014) introduction of AL, referred to as pre- and post-ACT respectively. The 50% inhibition concentration (IC<sub>50</sub>) of artemether and lumefantrine in field isolates carrying combined *pfcr*/*pfmdr1* haplotype K + NFD were compared to those carrying haplotype T + YYY. Further, the association of parasite clearance rates with these haplotypes was investigated.

## 2. Materials and methods

### 2.1. Samples

Samples used in this study were obtained from Kisumu County in pre- and post-ACT periods. Forty seven pre-ACT parasite samples were collected between 1995 and 2003 whereas 745 post-ACT

parasites were collected between 2008 and 2014. Samples were collected under protocols approved by the Ethical Review Committee of the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya and Walter Reed Army Institute of Research (WRAIR) Institutional Review Board, Silver Spring, MD. The pre-ACT and post-ACT studies were conducted under approved study protocols KEMRI-SSC 1330/WRAIR 1384 (Epidemiology of malaria and drug sensitivity patterns in Kenya) and KEMRI-SSC 2518/WRAIR 1935 (*In vivo* and *in vitro* efficacy of artemisinin combination therapy in Kisumu County, western Kenya) respectively. KEMRI-SSC 2518/WRAIR 1935 efficacy study was conducted in Kombewa division, which is in Kisumu County, in 2013–2014. In both studies, patients presenting with uncomplicated malaria, aged between 6 months and 65 years were consented. Eligibility criteria included: measured temperature of  $\geq 37.5$  °C, a history of fever within 24 h prior to presentation, mono-infection with *P. falciparum* and a baseline parasitemia of 2000–200,000 asexual parasites/ $\mu$ L. Persons treated for malaria within the preceding 2 weeks were excluded from the study. Written informed consent was obtained from adult subjects ( $\geq 18$  years of age) or legal guardians for subjects <18 years of age. The presence of malaria was confirmed by microscopy and rapid diagnostic test (RDT; Parascreen<sup>®</sup>, Zephyr Biomedicals, Verna Goa, India). Whole blood was collected and aliquots preserved for analysis as specified in the study protocols. For KEMRI-SSC 1330/WRAIR 1384 protocol, subjects were treated with oral AL (Coartem) administered over three consecutive days, a standard of care for *P. falciparum* malaria in Kenya. The first dose was observed by the study team and remaining doses were self-administered at home. For KEMRI-SSC 2518/WRAIR 1935, subjects were admitted at the study facility for approximately 3 days, until two consecutive smear negative slides were obtained.

### 2.2. *Pfcr* and *Pfmdr1* genotyping

Genomic DNA was extracted from whole blood using QIAamp Blood mini kit (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. Extracted DNA was stored appropriately until analyzed. *Pfcr* K76T and *pfmdr1* N86Y, Y184F and D1246Y alleles were determined by sequencing or PCR-based single-base extension on Sequenom MassARRAY platform (Agena Biosciences, San Diego, CA, USA) following manufacturer recommendations. Previously published primers for *pfcr* K76T (Djimé et al., 2001) and *pfmdr1* SNPs (Vinayak et al., 2010) were used for PCR and sequencing analyses. The final concentration of PCR reactants were as follows: 0.5  $\mu$ M each of the primers, 1 $\times$  PCR buffer, 4 mM of magnesium chloride (MgCl<sub>2</sub>), 0.02 Units of Taq polymerase, 100  $\mu$ M (25  $\mu$ M each) of deoxynucleotides. After successfully amplifying the target regions, the isolates were purified using Exosap-it<sup>®</sup> (Affymetrix, Santa Clara, CA) as per the manufacturer's protocol. Sequencing of the target regions was done on the 3500 xL ABI Genetic analyzer using version 3.1 of the big dye terminator method (Applied Biosystems, Foster City, CA). Contig assembly of the generated sequences was performed using CLC Bio Workbench 6 and the sequences aligned and analyzed using BioEdit version 7.1.3.0. All sequences were compared against the *pfcr* (Accession Number; XM\_001348968) and *pfmdr1* (Accession Number; XM\_001351751) 3D7 reference sequence published in the NCBI database.

### 2.3. *In vitro* drug susceptibility testing

The drugs used for the *in vitro* testing were artemether and lumefantrine which were supplied by the Division of Experimental Therapeutics of the WRAIR. Parasites were maintained in continuous culture as previously described (Trager and Jensen, 1976). SYBR Green I- based drug sensitivity assay was used for the *in vitro*

drug sensitivity testing as previously published (Bacon et al., 2007; Johnson et al., 2007; Rason et al., 2008). *P. falciparum* parasites in continuous culture attaining 3%–8% parasitemia were adjusted to 2% hematocrit and 1% parasitemia. Blood samples with >1% parasitemia were adjusted to 1% parasitemia at 2% hematocrit, and those with ≤1% parasitemia were used unadjusted at 2% hematocrit.

Drugs were prepared in 5 mL of 99.5% dimethyl sulfoxide to attain 5 mg/mL which were lowered to starting concentrations of 690 nM for AT and 378 nM for LU. This was followed by serial dilution across 10-concentrations ranging from (in nM) AT 670 to 0.65 and LU 378 to 0.37. For drug assaying, 3.2 µL of this drug was coated on to 384-well plates. The assay was initiated by the addition of 100 µL reconstituted parasite components added to drug coated plates and incubated at 37 °C as previously described (Bacon et al., 2007; Johnson et al., 2007; Rason et al., 2008). The assay was terminated after 72 h by adding 100 µL of lysis buffer containing SYBR Green I (1× final concentration) directly to the plates and kept at room temperature in the dark for 24 h. Parasite replication inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR Green I dye using the Tecan Genios Plus® with excitation and emission wavelengths of 485 nm and 535 nm. The IC<sub>50</sub> values for each drug were calculated as previously described (Johnson et al., 2007).

#### 2.4. Statistical analysis

Changes in prevalence of *pfcr*t and *pfmdr*1 individual SNPs and the combined haplotype pre- and post-ACT were analyzed using chi-square test for proportions and logistic regression with year included as a continuous covariate respectively. The odds ratios (OR) analysis was done using STATA version 11 (Stata, College Station, TX); OR were used to calculate the relative change of parasite genotype and the IC<sub>50</sub>s per year. Chi-square analysis was used to compare change in the prevalence of genotypes. This was done using the Prism program (version 5.0.2; GraphPad Software, Inc., San Diego, CA). The IC<sub>50</sub> estimates were expressed as medians with interquartile ranges (IQR). Further, IC<sub>50</sub> data for isolates with the different genetic polymorphism in *pfcr*t and *pfmdr*1 were compared by using nonparametric tests (1-way analysis of variance, Kruskal–Wallis test with Dunn's multiple comparison post test). Associations between IC<sub>50</sub> estimates and genetic markers were considered significant when p-value < 0.05.

### 3. Results

#### 3.1. Prevalence of SNPs in pre- and post-ACT parasites

A total of 792 field isolates collected from the study sites between 1995 and 2014 were successfully analyzed. There were 47 pre-ACT (1995–2003) samples and 745 post-ACT (2008–2014) samples analyzed; the pre-ACT sample size was significantly smaller than the post-ACT. For the post-ACT samples, 118 were from an efficacy study, of which 68 had parasite clearance half-life data and were used in subsequent analysis. Four SNPs were genotyped: *Pfcr*t 76 and *pfmdr*1 86, 184 and 1246. Analysis was performed for pre-ACT and post-ACT parasites, revealing statistically significant difference between pre- and post-ACT genotypes at the four codons (chi-square analysis; p < 0.0001). Data revealed that the mean prevalence of the wild-type allele in *pfcr*t codon 76 (K76) in pre-ACT parasites was 10.4% and 61.7% post-ACT, whereas the mean prevalence for the mutant allele in *pfcr*t codon 76 (76T) in pre-ACT parasites was 62.2% and 28.5% in post-ACT. In pre-ACT parasites, 27.4% carried the mixed genotype whereas in post-ACT, 9.8% carried the mixed genotype. Further analysis revealed K76 prevalence was

lowest in 1995–1996 at 6.4% and highest in 2014 at 93.2% whereas 76T was highest in 2002–2003 at 85.7% and lowest in 2014 at 2.3%.

For the wild-type allele in *pfmdr*1 codon 86 (N86), the mean prevalence in pre-ACT parasites was 16.7% and 73.0% in post-ACT whereas the mean prevalence for the mutant allele in *pfmdr*1 codon 86 (86Y) in pre-ACT parasites was 62.8% and 14.9% in post-ACT. The prevalence of mixed genotype was 20.5% and 11.4% in pre- and post-ACT parasites respectively. Further, data revealed N86 prevalence was lowest in 2002–2003 at 0.0% and highest in 2014 at 92.4%, whereas 86Y was highest in 2002–2003 at 92.3% and lowest in 2014 at 0.7%.

The mean prevalence of the wild-type allele in *pfmdr*1 codon 184 (Y184) in pre-ACT parasites was 11.6% and 35.0% in post-ACT whereas the mean prevalence for the mutant allele in *pfmdr*1 codon 184 (184F) in pre-ACT parasites was 67.8% and 40.8% in post-ACT. The prevalence of the mixed genotype was 17.1% and 24.2% in pre- and post-ACT parasites respectively. Further, data revealed Y184 prevalence was lowest in 2002–2003 at 8.3% and highest in 2008 at 50.0% whereas prevalence of 184F was highest in 2002–2003 at 83.3% and lowest in 2008 at 30.0%.

The mean prevalence of the wild-type allele in *pfmdr*1 codon 1246 (D1246) in pre-ACT parasites was 40.0% and 83.5% in post-ACT whereas the mean prevalence for the mutant allele in *pfmdr*1 codon 1246 (1246Y) in pre-ACT parasites was 43.4% and 13.8% in post-ACT. The prevalence of mixed genotype was 16.7% and 2.7% in pre- and post-ACT parasites respectively. Further, data revealed D1246 prevalence was lowest in 2002–2003 at 20.0% and highest in 2008 at 100.0% whereas prevalence of 1246Y was highest in 2002–2003 at 66.7% and lowest in 2013 at 5.5%.

#### 3.2. Prevalence of K + NFD and T + YYY haplotypes

Parasite haplotypes at codon 76 in *pfcr*t and codons 86, 184 and 1246 in *pfmdr*1 were evaluated. Data in all the four codons were obtained in 499 samples (70.2%) where 28 were pre-ACT and 471 post-ACT. Further, we analyzed K + NFD and T + YYY haplotypes. There were 142 parasite samples carrying K + NFD or T + YYY haplotype; 132 were K + NFD and 10 were T + YYY (Table 1).

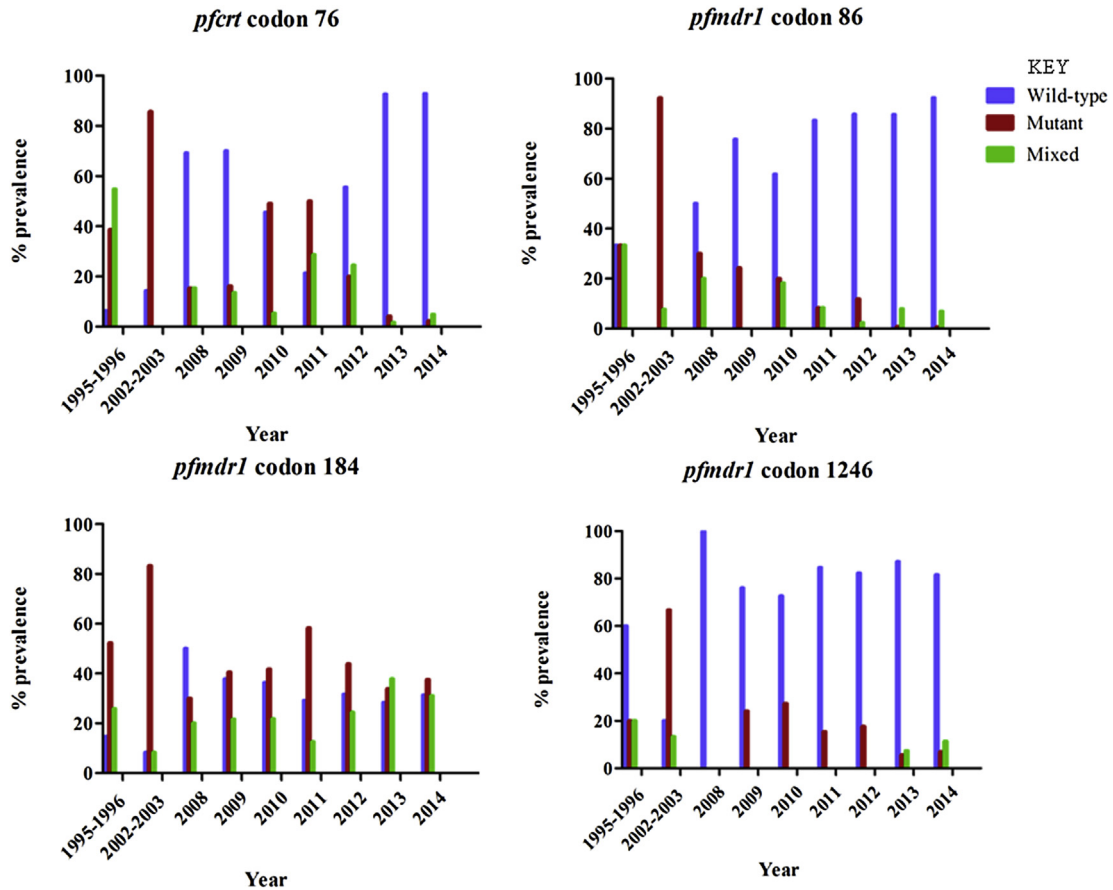
#### 3.3. Temporal trends of SNPs in *pfcr*t and *pfmdr*1 genes

Fig. 1 shows the temporal trend of SNPs in *pfcr*t and *pfmdr*1 genes from 1995 to 2014. The prevalence of *pfcr*t K76 increased from 6.4% in 1995–1996 to 93.2% in 2014 (Fig. 2). There was a steady increase over the 20-year period from 10.4% in pre-ACT parasites to 61.7% in post-ACT (yearly OR = 0.67 [95% CI 0.60 – 0.74; p < 0.001]). The mutant allele, *pfcr*t 76T was highest in 2002–2003 at 85.7%, decreasing to 2.3% in 2014. In 1995–1996, the majority of the parasites carried mixed alleles (54.8%) whereas in 2014, only 4.9% carried mixed genotype. In post-ACT, parasites collected in 2010, 2011 and 2012 had low prevalence of the *pfcr*t K76, with 2011 having the lowest prevalence at 37.0%. Interestingly, in 2013 and 2014, the prevalence of *pfcr*t K76 increased significantly to more than twice the previous year (2012), reaching 92.9% in 2014.

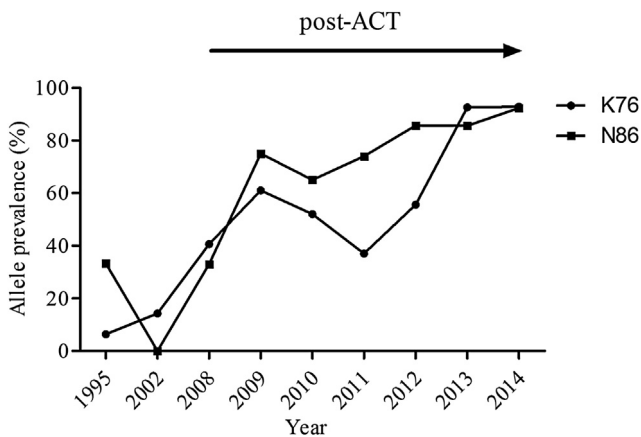
The wild-type allele in *pfmdr*1 codon 86 increased from 33.3% in 1995–1996 to 92.4% in 2014 (Fig. 2). This increment was steady over the 20-year period from 16.7% in pre-ACT parasites to 72.0% in post-ACT (yearly OR = 0.76 [95% CI 0.72 – 0.82; p < 0.0001]). The mutant allele, *pfmdr*1 86Y was highest in 2002–2003 at 92.3%, decreasing to 0.7% in 2014; there was no single parasite carrying the wild-type allele in 2002–2003. The highest percentage of the mixed alleles was in found 1995–1996 parasites at 33.3%, decreasing to 6.9% in 2014. The wild-type allele in *pfmdr*1 codon 184 increased from 14.8% in 1995–1996 to 31.4% in 2014. The highest prevalence of *pfmdr*1 Y184 was in 2008 at 50.0% which was followed by a

**Table 1**  
Number of parasites carrying K + NFD versus T + YYY haplotype from 1995 to 2014.

	1995–1996 n (%)	2002–2003 n (%)	2008 n (%)	2009 n (%)	2010 n (%)	2011 n (%)	2012 n (%)	2013 n (%)	2014 n (%)
K + NFD	0 (0.0)	0 (0.0)	3 (23.1)	6 (14.3)	3 (4.4)	0 (0.0)	10 (19.0)	29 (16.6)	81 (27.7)
T + YYY	2 (6.5)	7 (20.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.9)	0 (0.0)	0 (0.0)



**Fig. 1.** The prevalence of SNP in the *pfprt* and *pfmdr1* genes in the pre- and the post-ACTs parasite samples.



**Fig. 2.** The temporal trends of the *pfprt* K76 and *pfmdr1* N86 alleles in the pre- and the post-ACT periods (1995–2014). The percent prevalence of parasite samples carrying either K76 or N86 genotype over the years. The arrow indicates the post-ACT period.

downward trend reaching 31.4% in 2014. The highest prevalence of the mutant allele *pfmdr1* 184F was in 2002–2003 at 83.3% and lowest in 2008 at 30.0%. There was a steady increase of the mixed genotype at codon 184 from 20.0% in 2008 to 33.1% in 2014. With exception of 2008, the prevalence of *pfmdr1* 184F remained higher than *pfmdr1* Y184 in all post-ACT parasites (yearly OR = 1.1 [95% CI 1.04 – 1.16;  $p < 0.001$ ]).

The wild-type allele in *pfmdr1* codon 1246 increased from 60.0% in 1995–1996 to 81.6% in 2014 (yearly OR = 0.87 [95% CI 0.83 – 0.92;  $p < 0.001$ ]). The prevalence of the mutant allele, *pfmdr1* 1246Y was highest in 2002–2003 at 66.7% and lowest in 2008 at 0.0%. However, the prevalence of the mutant allele rebounded back in 2009 at 24.0% but steadily declined over time reaching 6.9% in 2014. There were no mixed genotypes between 2008 and 2012.

**3.4. Temporal trends of K + NFD and T + YYY haplotypes in pre- and post-ACT parasites**

Of the 28 pre-ACT parasite samples with complete genotype data at codon *pfprt* 76 and codons *pfmdr1* 86, 184 and 1246, analysis of parasite samples carrying pure alleles of K + NFD or T + YYY

haplotypes revealed that 100.0% of the pre-ACT parasite samples carried the T + YYY ( $n = 9$ ). In post-ACT parasite samples, of the 471 parasite samples successfully analyzed at all the codons, 28.2% ( $n = 133$ ) of the parasite samples carried K + NFD haplotype whereas only 0.2% ( $n = 1$ ) carried T + YYY haplotype; the single T + YYY haplotype in post-ACT parasite samples was present in 2012 (Table 1). K + NYD haplotype was not present in pre-ACT parasite samples but appeared in the post-ACT parasite samples, increasing significantly in 2013–2014; there were 3 parasite samples in 2009, 2 in 2011, 41 in 2013 and 67 in 2014 carrying this haplotype. Other haplotypes of interest analyzed include those carrying the mixed allele at *pfcr* 76 and pure single alleles at *pfmdr1* 84, 186 and 1246. For example, there was one pre-ACT parasite sample that carried K/T + NFD and eight in post-ACT parasite samples, and two parasite samples in 2009 that carried K/T + YYY. Mixed alleles at all the four codons were present only in pre-ACT parasite samples.

### 3.5. Associations of *in vitro* sensitivity with *pfcr* and *pfmdr1* polymorphisms

Artemether and lumefantrine  $IC_{50}$  values for the two reference strains, 3D7 and W2, were established and used as internal controls in subsequent experiments. The  $IC_{50}$  data was obtained for a total of 187 field isolates with genotype data at any one of the four codons. The profile for *in vitro* sensitivities per genetic polymorphism in all the four codons was assessed. At each codon, the correlation of the  $IC_{50}$ s (for artemether and lumefantrine) were done for parasites carrying the wild-type, mutant or mixed alleles. For the *pfcr* codon 76, the median (interquartile range [IQR]) of artemether  $IC_{50}$  for parasites carrying K76, 76T or K76T alleles were 3.2 nM (1.6–6.4 nM), 3.5 nM (2.4–9.5 nM) and 5.0 nM (2.6–12.5 nM), respectively. Although K76T had a slightly higher median  $IC_{50}$ , the difference did not reach statistical significance (1-way ANOVA, Kruskal–Wallis test  $p = 0.06$ ). Similarly, the difference in  $IC_{50}$ s for lumefantrine between the three different *pfcr* genotypes did not reach statistical difference ( $p = 0.29$ ). Analysis of the *pfmdr1* revealed there was significant correlation (1-way ANOVA, Kruskal–Wallis test  $p = 0.04$ ) between lumefantrine  $IC_{50}$  and polymorphism at codon 184. The median (IQR) lumefantrine  $IC_{50}$ s for parasites carrying Y184, 184F or Y184F alleles were 25.8 (11.2–43.2), 27.0 (11.9–59.5) and 12.0 (4.8–39.3), respectively. Dunn's multiple comparison test revealed the significance was between  $IC_{50}$ s for parasites carrying 184F vs. Y184F. There was no other genotype in *pfmdr1* that showed any correlation with either artemether or lumefantrine.

### 3.6. Associations of parasite clearance rates with *pfcr* and *pfmdr1* polymorphisms

Clearance half-lives were obtained for the 68 subjects with complete parasite haplotype profiles. Five different parasite haplotype profiles were analyzed including K + NFD, K + NYD, K + N Y/F D, K + N Y/F D/Y and all other profiles combined that did not fall to any of the indicated haplotype profiles. There was no difference in the parasite clearance half-lives based on genetic haplotype profiles (1-way ANOVA, Kruskal–Wallis test,  $p = 0.61$ ).

## 4. Discussion

The data shows a dramatic change in the prevalence of antimalarial molecular markers associated with changes in chloroquine drug pressure and deployment of AL in western Kenya. The significant changes are in the prevalence of the wild-type alleles in *pfcr* codon 76, and *pfmdr1* codons 86 and 1246 between pre-ACT

and the post-ACT era which coincided with withdrawal of chloroquine and the introduction of AL. The *pfcr* K76 and *pfmdr1* N86 alleles had the most dramatic increase in prevalence reaching to more than 92% in the post-ACT period from low points of 6.4% and 0.0% respectively in the pre-ACT period. Conversely, *pfcr* 76T and *pfmdr1* 86Y prevalence dramatically decreased 38- and 128-fold respectively from the highest point in the pre-ACT period to the lowest point in the post-ACT period. The prevalence of mixed genotypes in these two codons decreased as well. The *pfmdr1* D1246 also had dramatic change, increasing to a prevalence of 87.2% in 2013. Conversely, the prevalence of *pfmdr1* 1246Y decreased to 5.5% in 2013. The mixed genotype decreased from the pre-ACT period to the post-ACT period. The prevalence of *pfmdr1* Y184 allele and the mixed genotype increased in the post-ACT parasites compared to the pre-ACT parasites. Interestingly, haplotype analysis revealed the pre-ACT parasites carried only T + YYY haplotype whereas the post-ACT parasites, with exception of one parasite (1 out of 133), carried the K + NFD haplotype. Of interest was the K + NYD (wild-type in all the four alleles) which appeared in the post-ACT parasites, increasing dramatically in 2013 and 2014. Our data also revealed that there was a significant correlation between lumefantrine  $IC_{50}$  with polymorphisms at *pfmdr1* codon 184. However, there was no association between parasite clearance rates with polymorphisms at codons in *pfcr* or *pfmdr1* genes.

In line with previous studies (Kublin et al., 2003; Laufer et al., 2010; Eyase et al., 2013; Okombo et al., 2014) we have shown there was selection of the *pfcr* K76 after change of drug policy. Removal of chloroquine drug pressure results in resurgence of chloroquine sensitive parasite population. In Malawi, the re-emergence of chloroquine-sensitive *P. falciparum* malaria parasites reached 100% in less than 10 years after chloroquine was replaced with SP (Kublin et al., 2003). In our study, there was evidence of dramatic resurgence ( $p < 0.001$ ) of the *pfcr* K76 in 2008, 2 years after introduction of AL and close to 10 years after withdrawal of chloroquine. However, in 2002–2003, about 5 years after withdrawal of chloroquine and introduction of SP, the *pfcr* K76 had not dramatically resurged. In the Kenyan coastal region, studies have revealed a significant decrease of the *pfcr* 76T after the withdrawal of chloroquine. In samples analyzed from Kilifi (which is in the coastal region of Kenya), the *pfcr* 76T decreased from 94% to 63% from 1993 to 2006 and in a study conducted in Tiwi which is also at the Kenyan coast, the *pfcr* 76T decreased from 88% to 64% from 1999 to 2008 (Mwai et al., 2009; Mang'era et al., 2012). In a more recent study which also used samples from Kilifi, there was a more drastic change in the *pfcr* 76T from 96% in 1999 to 15% in 2012/2013 (Okombo et al., 2014). In our study, we saw a more dramatic drop of the *pfcr* 76T from a high of 85.7% in 2002/2003 to 2.3% in 2014. This is the lowest reported prevalence of the *pfcr* 76T in Kenya. At the same time, Okombo et al. (2014) reported significance resurgence of the *pfcr* K76 in Kilifi from 38% in 1995 to 81.7% in 2012/2013. In our study, the *pfcr* K76 resurged from 6.4% to 92.9% in 2014. There is obvious significant resurgence of the *pfcr* K76 in Kenyan parasites after the withdrawal of chloroquine. The rate of change in the *pfcr* K76 is not related to transmission intensity as the changes are almost identical between western and coastal regions of Kenya which have different transmission and disease endemicity. A recent study from western Kenya showed a directional selection of the *pfcr* K76 after treatment of patients with AL (Henriques et al., 2014). Our data demonstrates that after the removal of chloroquine pressure on the parasite population, there was a loss of the *pfcr* 76T genotype but at a slow rate with the introduction of SP. AL was adopted as the first-line treatment in Kenya in 2006 (Amin et al., 2007). The frequency of the *pfcr* 76T allele decreased after the introduction of AL but it is not until the 2013/2014 time-frame that there was a dramatic loss of the *pfcr*

76T allele which can be attributed to continued selection of the *pfcr* K76 allele by AL.

A recent pooled analysis from clinical trials showed patients infected with parasites carrying the *pfmdr1* N86 allele and/or increased the *pfmdr1* copy number (as independent risk factors) were at a significantly greater risk of AL treatment failure than those whose parasites carried the 86Y allele or a single copy of the *pfmdr1* (Venkatesan et al., 2014). In a recent study where we analyzed the presence and distribution of the copy number variation of the *pfmdr1* gene in the pre- and the post-ACT parasites, seven of eight parasite samples with multiple *pfmdr1* gene copy number were the post-ACT (Ngala et al., 2015). We also showed that the post-ACT parasites had significantly higher *pfmdr1* gene copy number compared to the pre-ACT parasites ( $p = 0.0002$ ). There was an overlap in the parasite samples analyzed in this study and the Ngalah et al. (2015) study. Although the *pfmdr1* gene amplification is rarely reported in African parasites (Uhlemann et al., 2005; Holmgren et al., 2006), a recent efficacy study showed amplification of the *pfmdr1* gene contributes to recurrent *P. falciparum* parasitemia following AL treatment (Gadalla et al., 2011). In this study, we have shown dramatic increase of the *pfmdr1* N86 from 0.0% in 2002/2003 to reaching over 92% in 2014 and the *pfmdr1* 86Y decreasing to less than 1% in 2014. This data presents evidence of strong selection of the *pfmdr1* N86 allele, as well as increased *pfmdr1* gene copy number in the post-ACT parasites which is rarely reported in African parasites. The increased prevalence of the N86 allele and the *pfmdr1* gene copy number are the most significant independent risk factors for recurrence of parasites in patients treated with AL (Venkatesan et al., 2014). Although adequate clinical and parasitological response to ACTs remain high in western Kenya (Ogutu et al., 2014), data suggest there is underlying selection of parasites taking place (Henriques et al., 2014) which might result in decreased parasite susceptibility to AL. Indeed, re-infecting parasites carrying the *pfmdr1* N86 have been shown to withstand lumefantrine 35-fold higher compared to those carrying the *pfmdr1* 86Y (Malmberg et al., 2013a). To the best of our knowledge, our data shows the highest prevalence of the *pfmdr1* N86 and increase of *pfmdr1* gene copy number that has ever been reported for western Kenya parasites.

In line with previous studies (Sisowath et al., 2007; Malmberg et al., 2013a,b), we showed there was also a significant increase of the *pfmdr1* D1246 in the post-ACT parasites compared to the pre-ACT parasites. In the post-ACT parasites, the *pfmdr1* 184F allele trend remained relatively constant but the mixed allele, *pfmdr1* Y184F significantly increased over time. Interestingly, when we analyzed the association of the *in vitro* parasites sensitivity with the *pfcr* and *pfmdr1* polymorphisms, only polymorphisms at the *pfmdr1* codon 184 showed significant association with lumefantrine  $IC_{50}$ . The clinical relevance of this finding is not clear but provides a basis to support close monitoring of parasite clearance in western Kenya since development of resistance to antimalarials is a dynamic process. In a study that assessed patient lumefantrine drug level and its association with the *pfmdr1* polymorphisms, the influence of polymorphisms at codon 184 was not clear since parasites carrying the Y184 allele, which is considered to be “sensitive”, were able to withstand high drug levels (Malmberg et al., 2013a).

The haplotype K + NFD has been shown to appear following AL treatment (Sisowath et al., 2005, 2007, 2009). In line with previous studies (Malmberg et al., 2013a,b; Okombo et al., 2014) K + NFD haplotype increased significantly in the post-ACT period. The haplotype T + YYY appeared only in the pre-ACT parasite samples and not in the post-ACT. Of interest was the appearance of the K + NYD haplotype which was not present in pre-ACT parasite samples but appeared and increased significantly longitudinally in

the post-ACT parasite samples. This is also in line with the recent study by (Okombo et al., 2014) where they showed significant increase of the K + NYD haplotype over time. Malmberg et al. (2013a,b) showed a trend of decreasing lumefantrine susceptibility in the order of NFD, NYD, YYY, and YYD. Our data present evidence that strong selection of these haplotypes is taking place in western Kenya due to use of AL. Interestingly, recent studies suggested selection for the NFD haplotype is exerted by the artemisinin component of the ACTs (Conrad et al., 2014; Henriques et al., 2014). Clear evidence has shown these selected haplotypes have decreased susceptibility to lumefantrine (Malmberg et al., 2013a,b) but no such evidence has been presented for artemisinin component for the ACTs. Whether selection of these genotypes will lead to reduced susceptibility to AL remains to be seen but calls for close monitoring.

Although polymorphisms at the *pfmdr1* codons 86, 184, 1034, 1042, 1246 are associated with altered sensitivity to artemisinins and mefloquine in SEA, the key determinant has been shown to be the copy number variation (Cowman et al., 1994; Price et al., 1999; Duraisingh et al., 2000; Price et al., 2004; Sidhu et al., 2005; Picot et al., 2009; Muhamad et al., 2011). Further, the discovery of mutations in K13-propeller in SEA but not in African parasites shows the multigenetic nature of artemisinin resistance in different parts of the world. AL and other ACTs remain highly efficacious in SSA including western Kenya (Ogutu et al., 2014; Venkatesan et al., 2014). Whether selection of haplotype K + NFD or K + NYD signifies apparent selection of AL resistant parasite is yet to be confirmed. Even with the emergence and spread of ACTs resistant parasites in SEA, ACTs remain the first-line treatment in this region. The *pfcr* K76 allele and *pfmdr1* N86 might soon reach saturation at 100% prevalence in western Kenya. Further, for the first-time in western Kenya, we have shown the prevalence of *pfmdr1* copy number is on the rise. The apparent recent increase in these molecular markers should be a cause to rethink the issue with the deployment of first-line treatment of uncomplicated malaria. Although *in vivo* efficacy of AL remains >95%, the malaria community should start thinking of strategies of deploying other ACTs that are currently available to ensure a longer useful therapeutic lifespan before other new molecules are available in the market.

With the prevalence of *pfcr* K76 allele well above 90%, a clear re-emergence of chloroquine-sensitive *P. falciparum* malaria in western Kenya, there is need to evaluate the role of chloroquine or compounds in the same class in the treatment of malaria. This is supported by the current molecular data that unambiguously show genetic polymorphisms in *pfcr* and *pfmdr1* genes are changing in favor of chloroquine sensitivity.

Based on our findings and data from other studies (Kublin et al., 2003; Laufer et al., 2010; Gadalla et al., 2011; Eyase et al., 2013; Malmberg et al., 2013a,b; Henriques et al., 2014; Ogutu et al., 2014; Okombo et al., 2014; Venkatesan et al., 2014), more studies are required to determine the genotypic or phenotypic parasite characteristics that are directly associated with artemisinin resistance. Further, studies on how to mitigate the emergence and spread of parasites that are resistance to ACTs are critical in SSA where majority of the deaths due to malaria occur.

## 5. Conclusion

We have shown significant increase in polymorphisms at key codons in the *pfcr* and *pfmdr1* genes that are selected for by AL and decrease of these key codons with the withdrawal of chloroquine. This is exemplified by the high prevalence of the K + NFD haplotype which although associated with reduced susceptibility to AL, is generally linked to increased susceptibility to chloroquine and amodiaquine (Humphreys et al., 2007). There was no association

between parasite clearance rates and the *pfprt* and *pfmdr1* polymorphisms. The findings call for close monitoring of parasite genotypic, phenotypic and clinical dynamics in response to current first-line treatment in western Kenya. Having been the first focus of chloroquine resistance in Africa (Fogh et al., 1979), western Kenya will be crucial in informing the next steps on the deployment of first-line treatment of uncomplicated malaria in the possible future era of attenuated response of artemisinin in SSA.

#### Author contributions statement

A.O.A. Performed experiments, data analysis, manuscript writing. P.M. Performed experiments. L.A.I. Performed experiments, data analysis. B.H.O. Performed experiments. D.W.J. Data analysis. R.Y. Performed experiments. B.S.N. Contributed data. B.R.O. Manuscript writing and review. B.A. Study PI, data analysis, manuscript review. H.M.A. Project design, data analysis, manuscript review. E.K. Conceived and design project, data analysis, manuscript writing.

#### Competing financial interests

The authors declare no competing financial interests.

#### Conflict of interest

None declared.

#### Acknowledgments

We would like to thank the patients, clinical and other support staff at the study sites. We are grateful to our colleagues in the Malaria Drug Resistance laboratory for their technical and moral support. We would also like to thank the Director of KEMRI for permission to publish this work. This work was supported by the Armed Forces Health Surveillance Center, Division of Global Emerging Infections Surveillance and Response System Operations. The opinions and assertions contained herein are private opinions of the authors and are not to be construed as reflecting the views of the U.S. Army Medical Research Unit-Kenya, the U.S. Department of the Army, the U.S. Department of Defense or the U.S. Government.

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