Limited Polymorphisms in *Plasmodium falciparum* Lines Exposed to Pure Artemisinin and *Artemisia Annua* Extracts

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Abstract: *Artemisinin-based combination therapy has been a vital tool in malaria control and elimination programmes. However, artemisinin resistant P. falciparum parasites have emerged in Southeast Asia, posing a major threat to the effectiveness of ACT. Resistance results in prolonged parasite clearance in vivo and enhanced survival of ring-stage parasites in vitro. Therefore, understanding the genetic basis of resistance would be critical to the success treatment and intervention strategies. This study aimed at identifying single nucleotide polymorphisms associated with artemisinin and Artemisia annua resistance. Genetic analysis was done on P. falciparum lines W2 and D6, previously selected under pure artemisinin and Artemisia annua extracts. Genomic DNA was extracted using QIAamp blood mini kit. Libraries were sequenced on Illumina Miseq platform using 151bp paired-end chemistry. Sequencing read data from each sample was mapped against P.falciparum reference sequence version 3.1. One non-synonymous (NS) mutation K189T was identified in K13 gene. The Pfmdr1 mutation N86Y was detected in W2 parasite exposed to pure artemisinin at IC_{50} equivalents and notably, the Pfcrt CQ sensitive CVMNK genotype was retained. The study also identified one background mutation in Pfcrt (I356T) in W2 parasites exposed to artemisinin at IC_{50} equivalents. In conclusion, K13 mutation described here has not been linked to reduced parasite clearance or in vitro artemisinin tolerance. Pfmdr1 gene may putatively play a role in artemisinin resistance.*

Keywords: Artemisinin-resistance, K13 gene, malaria, *Plasmodium falciparum*

1. Introduction

Artemisinin-based combination therapy (ACT) is the recommended standard treatment against *Plasmodium falciparum* malaria in majority of the countries where the disease is endemic (WHO, 2010). ACTs are safe, well tolerated, and effect rapid clearance of both asexual blood stages and transmissible sexual stages of *Plasmodium* parasites. Currently, several forms of ACTs regimens have been endorsed by WHO, including artemether-lumefantrine, dihydroartemisinin-piperaquine, artesunate-amodiaquine (Staedke *et al*., 2008) and artesunate-pyronaridine (Rueangweerayut *et al*., 2012). The worldwide deployment of ACT has been the key factor behind the current reduction in malaria morbidity, mortality and transmission in the endemic world (Peak *et al*., 2015). However, the emergence and spread of *P.falciparum* parasites resistant to artemisinins in Southeast Asia threatens to undermine these impressive gains.

Clinical resistance is characterized by delayed parasite clearance in artesunate or ACT-treated patients (Dondorp *et al*., 2010; WHO, 2015), and was correlated with *in vitro* artemisinin resistance (Witkowski *et al*., 2013). In ACT treatment, higher number of *Plasmodium* parasites survive exposure to artemisinin components during the 3-day regimen, leaving more for partner drug to clear (Fairhurst, 2015). This eventually leads to treatment failure and increases the risk of parasites selecting resistance to partner drugs. Artemisinin resistance in Southeast Asia has been shown to be a heritable genetic trait (Anderson *et al*., 2010),

linked to single nucleotide polymorphisms in the PF3D7_1343700 locus, which encodes a putative kelch protein (Ariey *et al*., 2014). Multiple mutations within the kelch repeat of the C-terminal K-13 propeller domain were shown to be associated with decreased susceptibility of ring stage parasites and slow parasite clearance. The M476I was selected *in vitro* with artemisinin pressure in African parasite line F32-ART5. Subsequent genetic analysis of artemisinin resistant parasites from Cambodia revealed an association of C580Y, R539T, I543T and Y493H mutations with prolonged parasite *ex vivo* survival. Further, a previous study confirmed that prolonged parasite clearing infections (parasite clearance half-life> 5hrs) were strongly associated with point mutations in *Pfk13* gene across Southeast Asia (Ashley *et al*., 2014), where C580Y mutation was the most prevalent.

Single nucleotide polymorphisms in *P. falciparum* multidrug resistance 1 gene (*Pfmdr1*) have been associated with modulation of parasite susceptibility to a number of antimalarials, including ACTs (Humphreys *et al*., 2007; Dahlostrom *et al*., 2014). Single nucleotide polymorphisms at codons N86Y, Y184F, S1034C, and D1246Y have been reported to have an effect on parasite responses to quinine (QN), mefloquine (MQ), halofantrine (HF), artemisinin (Sidhu *et al*., 2005), lumefantrine and amodiaquine (Li *et al*., 2014). Notably, recent findings have shown that artemether-lumefantrine (AL) selects parasite harboring 86N, 184F, and 1246D haplotypes, whereas artesunate-amodiaquine selects parasites with 86Y, 184Y and 1246Y genotypes (Otienoburu *et al*., 2016). *Pfmdr1* gene is a drug transporter (Sanchez *et*

al., 2011), studies have associated increased copy number in *Pfmdr1* with the treatment failure of artesunate-mefloquine combination (Alker *et al*., 2007).

P. falciparum chloroquine resistant transporter gene (*Pfcrt*) localized on chromosome 7 has been implicated to play a role in chloroquine resistance (Valderramos and Fidock, 2006). Chloroquine resistance has been correlated with K76T mutation (Sidhu *et al*., 2002) and interestingly, the selection of wild-type allele K76 has been associated with decreased susceptibility to lumefantrine (Sisowath *et al*., 2009). Evidence from *in vitro* studies have shown that *Pfcrt* mutations can also influence parasite susceptibility to multiple antimalarials including quinine, halofantrine and artemisinin (Johnson *et al*., 2004)

Resistance to antimalarial drugs including artemisinin is accompanied by compensatory and modulatory changes within the same or different genes (Jiang *et al*., 2008). More recently, a genome-wide study pinpointed six background genetic changes common in Southeast Asia, on which K13 mutations arise independently (Miotto *et al*., 2015). Sequence variation D193Y in *fd* gene, T481I in *mdr2*, V127M in *arps10*, N326S and I356T in *Pfcrt*, V1157L in *pph* and C1484F in *pibp* gene were suggested to play a compensatory role in artemisinin resistance. These background mutations form a genetic backbone which might reduce fitness cost associated with artemisinin resistant phenotype.

Despite the absence, thus far of *Pfk13* variants associated with artemisinin resistance in *P. falciparum* isolates of African origin (Kamau *et al*., 2014; Taylor *et al*., 2015), previous spread of CQ and sulphadoxine-pyrimethamine resistant parasites from Asia to Africa (Mita *et al*., 2011) suggest that artemisinin resistance is likely to spread. Identification of genetic determinants is therefore central to the understanding of molecular basis of artemisinin resistance and in effect potentiate the developments of strategies to manage it. In this study, we exploited whole genome sequencing of chloroquine resistant W2 and chloroquine sensitive D6 parasite lines, to identify genetic changes that occurred in the parasites lineage during the selection of artemisinin and *Artemisia annua* resistance.

2. Materials and Methods

2.1 Study site

Experimental procedures of the study were carried out at malaria laboratory, in Kenya Medical Research Institute, Nairobi, Kenya.

2.2 Parasite lines

Two *Plasmodium falciparum* parasite lines, W2 (CQ-sensitive clone from Indochina) and D6 (CQ-sensitive clone from Sierra-Leone) were used for the study. The parasite lines were previously selected under escalating doses of artemisinin and *Artemisia annua* for 3 years (Kang'ethe *et al* 2016). W2 and D6 plain (cultured for the same period without drug exposure) were used as controls.

Table 1: W2 and D6 parasite lines analysed in the study

Study ID	Parasite line	Number of drug pressure cycles	Drug selecting
W2	W2	-	-
D6	D6	-	-
1L	D6-A	C17	Artemisinin
2L	D6-B	C20	Artemisinin
3L	D6-C	C20	*Artemisia annua*
4L	D6-D	C20	*Artemisia annua*
6L	W2-1	C36	Artemisinin
7L	W2-2	C42	Artemisinin
8L	W2-3	C37	*Artemisia annua*
9L	W2-4	C45	*Artemisia annua*

D6-A, D6-B=D6 parasite exposed to artemisinin at IC_{50} and IC_{90} respectively. D6-C and D6-D=D6 parasites exposed to *A.annua* at IC_{50} and IC_{90} equivalents. W2-1, W2-2= W2 parasites exposed to ART at IC_{50} and IC_{90} respectively. W2-3, W2-4=W2 parasites exposed to *A. annua* at IC_{50} and IC_{90} respectively.

2.3 *Plasmodium falciparum* genomic DNA extraction

Plasmodium falciparum genomic DNA was extracted by first removing leukocytes to minimize human DNA contamination. A total of 1 to 2 ml parasite cultures was aliqouted for the assay and samples were depleted of leukocytes using plasmodipur filters (Euro-Diagnostica). Parasite DNA was then extracted using commercial QIAamp blood mini kit (Qiagen, Valencia, CA) according to protocol developed by the manufacturer.

The quantity of the extracted DNA was determined by fluorescence analysis using Qubit 3.0 flourometer (Thermo Fisher Scientific) and DNA was frozen at $-80^{o}c$ until use.

2.4 Whole genome sequencing

Whole genome sequencing of the parental strains and their resistant lines was performed using Illumina Miseq platform. Illumina paired-end libraries were constructed using Nextera XT DNA sample preparation kit, following standard Illumina sample preparation protocol. Briefly, genomic DNA was fragmented by Nextera XT transposome to a mean fragment distribution of 300-500bp. The adapter ligated fragments were PCR amplified with index primers index 1 (i7) and index 2 (i5). Inserts were size selected and purified using Agencourt AMpure XP beads (Beckman Coulter Genomics). This was followed by pooling and loading of the Illumina library onto the Miseq platform for cluster generation and sequencing. Each library was hybridized onto the flow cell surface bound with oligos complementary to the adapters and bridge amplification was performed to generate clusters. Sequencing generated paired-end reads of 151bp.

2.5 Data analysis

Sequencing read data obtained from 10 *P. falciparum* samples was subjected to standard Illumina quality control procedures. For initial data quality filtering, Trimmomatic *V0.35* software (Bolger *et al*., 2014) was used to trim Nextera XT adapter sequences, low quality bases at 5' and 3' ends and discard low quality reads (Phred< 33) .The

quality control checks were then done using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

2.5.1 Variant calling

We developed a pipeline based on GATK best practices recommendations (Depristo *et al*., 2011; Van Der Auwera *et al*., 2013). Each dataset was mapped independently against the *P. falciparum* 3D7 reference sequence version 3.1 (ftp://ftp.sanger.ac.uk/pub/project/pathogens/plasmodium/falciparum/3D7/3D7.latest_version3.1/2016), using the Burrow-Wheeler alignment (BWA) software, as described previously by Manske *et al*., 2012. Mapping statistics from each sample alignment files was computed using Samtools (Li *et al*., 2009), and the BAM files were further pre-processed using Picard tool. Specifically, potential PCR duplicates which arise during the PCR amplification step of the library preparation were mapped and marked using Picard MarkDuplicates tool (http://picard.sourceforge.net). Next, we performed local realignment of reads around possible Indels and areas of high entropy using GATK IndelRealigner (Mckenna *et al*., 2010). Raw base quality scores were adjusted using GATK BaseRecalibrator tool and known variants from *Plasmodium falciparum* crosses 1.0 data (MalariaGEN) as a training set (ftp://ngs.sanger.ac.uk/production/malaria/pf-crosses/1.0/3d7_hb3.combined.final.vcf.gz). Overall genome wide coverage and loci covered to a certain percentage was computed using GATK tool.

Variant calls were generated using GATK Haplotype caller *V3.6* (McKenna *et al*., 2010) with the parameters stand_emit_conf 10 and stand_call_conf 30. When merged across all samples, a set of 120,561 potential SNPs positions was obtained. This list of candidate SNPs was filtered using quality measures described by GATK developers (McKenna *et al*., 2010). Further, we excluded SNP positions located within the subtelomeric regions, hypervariable multigene families (*var*, *rifin* and *stevor*) and non-coding sequence retaining 34,520 biallelic SNPs. At each potential SNP, functional annotation was produced with snpEFF tool (Cingolani *et al*., 2012), using Ensembl functional annotation of the *Plasmodium falciparum* 3D7 as input.
The exonic sequences for parental and selected strains were compared and SNPs were confirmed by visual inspection in Artemis (Rutherford *et al*., 2000). Further, we analyzed the DNA and predicted amino acid sequences from each locus by alignment in Muscle (www.ebi.ac.uk) against a 3D7 reference sequence.

2.5.2 *Pfcrt* haplotype determination

The core *Pfcrt* haplotype coding for five amino-acid substitutions at position 72-76 was genotyped using a procedure previously described (Srimuang *et al*., 2016).

3. Results

3.1 Genome sequencing of *P. falciparum* parasite lines

Sequencing generated between 2.6 to 6.3 million paired-end reads per sample and the mean read length was 151bp (Table 2). After quality filtering, 2.3 to 5.8 million reads were obtained per sample. The average quality per read (Phred scores) was roughly 37 indicating that the base calls were of high quality and could be used for downstream processes. The initial data analysis showed that with BWA majority of the reads could be mapped against the reference genome and the average alignment rate was 95.8% (Table 2).

PCR duplicates were mapped and marked to reduce excessive coverage which can bias variant identification process. As summarized in table 2, the duplication rate was quite low and ranged from 2.0% (W2-2 C42) to 4.3 % (D6), with an average of 2.8%. D6 and W2 parental strains were sequenced to a genome wide coverage of 21.1X and 12.2X respectively (Table 2). The average coverage for the resistant parasites ranged from 9.57X (W2-3 C37) to 24.1X (D6-B C20) although it was variable in the subtelomeric regions. Moreover, in all the samples the majority of the genome was covered with reads with the coverage of at least 5X.

We called single nucleotide polymorphism from high quality datasets obtained from the 10 *P. falciparum* samples. In total, we identified 120,561 variable positions across all samples, which after quality filtering a list of 34,520 biallelic SNPs was produced.

3.2 *PfK13* propeller polymorphism

This study detected one non-synonymous mutation at the proximal end (upstream region) of K13 gene. Mutation K189T corresponding to nucleotide position A566C, was found in 7 out of the 10 samples (Table 3). The D6 parental parasite and its resistant progenies (D6-A C17, D6-B C20, D6-C C20 and D6-D C20) had the K189T mutation. Two W2 parasites (W2-2 C42 and W2-4 C45) had the K189T mutation, while the W2 parental parasite and two samples of the four sequenced (W2-1 C36 and W2-3 C37) haboured the wild type allele K189.

3.3 *Pfmdr1* polymorphism

This study identified one mutation in *Pfmdr1* gene (Table 3). The N86Y mutation corresponding to nucleotide position A256T was observed in W2-1 C36 parasites exposed to artemisinin at $1C_{50}$ equivalents. The parasites exposed to *Artemisia annua* extracts had wild type allele N86.

3.4 *Pfcrt* allelic types

The *pfcrt* wild-type CVMNK haplotype at codons 72-76 of CRT protein was confirmed present in 80% (8/10) samples (Table 3). The K76T mutation was identified in W2 parental parasite and one of its resistant progeny W2-1 C36. This study also identified 4 additional mutations on the *pfcrt* gene distinct from the CVIET genotypes commonly assessed. Mutations A220S, I356T and R371I were observed in W2-1 C36 parasites exposed to artemisinin at IC_{50} equivalents. The Q271E mutation corresponding to nucleotide position C811G was observed in W2 parental strain and W2-1 C36 parasites.

Table 2: The summary of the sequencing metrics of the parental strains and the resistant selections

Parasite line	Raw read pairs	Filtered reads	Mapped reads	%PCR duplicates	Coverage	% genome covered by at least 5 reads
W2	2,933,446	2,699,322	2,585,551 (95.94%)	2.2%	12.28X	70%
W2-1 C36	5,011,184	4,591,450	3,784,220 (82.84%)	3.0%	15.86X	79%
W2-2 C42	5,541,234	5,107,176	4,941,512 (96.85%)	2.0%	21.07X	84%
W2-3 C37	2,620,124	2,372,582	2,344,031 (98.83%)	3.2%	9.57X	65%
W2-4 C45	5,128,674	4,741,238	4,568,484 (96.46%)	3.2%	19.76X	84%
D6	6,354,848	5,857,358	5,700,867 (97.37%)	4.3%	21.15X	84.7%
D6-A C17	3,883,688	3,487,744	3,393,981 (97.38%)	2.1%	15.22X	78%
D6-B C20	6,052,700	5,631,426	5,531,750 (98.23%)	2.4%	24.13X	85%
D6-C C20	3,876,570	3,603,698	3,442,943 (95.67%)	2.9%	14.96X	77%
D6-D C20	3,973,492	3,667,966	3,618,676 (98.69%)	3.0%	14.33X	75%

W2= W2 control. W2-1 C36, W2-2 C42=W2 parasites exposed to artemisinin. W2-3 C37, W2-4 C45= W2 parasites exposed to *Artemisia annua* extracts. D6= D6 control. D6-A C17, D6-B C20= D6 parasites exposed to artemisinin. D6-C C20, D6-D C20=D6 parasites exposed to *Artemisia annua* extracts.

Table 3: *PfK13*, *Pfmdr1* and *Pfcrt* polymorphisms observed in *P. falciparum* parasite lines

Gene	Codon Position	Reference sequence Amino acid	Reference sequence Nucleotide	Mutant Sequence Amino acid	Mutant Sequence Nucleotide	Total number of samples %
K13 (Pf3D7_1343700)	189	K	A**A**A	T	A**C**A	7/10 (70)
Pfmdr1 (Pf3D7_0523000)	86	N	A**A**T	Y	**T**AT	1/10 (10)
Pfcrt (Pf3D7_0709000)	76	K	A**A**A	T	A**C**A	2/10 (20)
	220	A	**G**CC	S	**T**CC	1/10 (10)
	271	Q	AA**C**	E	AA**G**	2/10 (20)
	356	I	A**T**A	T	A**C**A	1/10 (10)
	371	R	A**G**A	I	A**T**A	1/10 (10)

Nucleotide changes from reference sequence to mutant are shown in bold letters

4. Discussion

The recent spread of artemisinin-resistant *P. falciparum* parasites across Southeast Asia (Ashley *et al*., 2014) is problematic and threatens the effectiveness of ACTs in treating malaria. Although, *in vivo* studies are a vital tool for assessing antimalarial drug efficacy, they are prohibitively expensive for large-scale surveillance purposes. This study filled this gap by analyzing mutations acquired specifically by laboratory-adapted clones selected *in vitro* under artemisinin and *Artemisia annua* extracts. Knowledge of the genetic basis of artemisinin resistance provides crucial information for the effective treatment strategies and measures to combat drug resistant.

Plasmodium falciparum kelch 13 (PF3D7_1343700) polymorphisms has been accounted for prolonged *in vivo* parasite clearance rates (>5h) and reduced susceptibility to artemisinin *in vitro* (Ariey *et al*., 2014; Ashley *et al*., 2014). Recent observations show that C580Y mutation has emerged independently in Cambodia and Thai-Myanmar border (Phyo *et al*., 2016). In addition, transfection studies inducing genetic modification of K13 locus of the *P. falciparum* parasites have confirmed it as the major gene conferring artemisinin resistance (Ghorbal *et al*, 2014). This research demonstrated that the introduction of K13 C580Y mutation into artemisinin sensitive *P. falciparum* strain caused a significant increase in ring-stage parasite survival in presence of artemisinin.

In the current study, there was a very limited variability within the coding sequence of K13 gene. We did not detect any of the polymorphisms associated with artemisinin resistance in Southeast Asia nor the M476I mutation associated with artemisinin tolerance *in vitro*. Only one non-synonymous mutation, located in the *Plasmodium*/Apicomplexa-specific domain was detected. This is in consistent with a recent study which did not find any K13 polymorphisms in parasites cultured under artemisinin drug pressure (Njoka *et al*., 2016). The K189T variant was detected in 70% of the samples. This mutation was previously reported in 42.2% (27/64) isolates from Dakar, Senegal (Torrentino-madam et *et al*., 2014), and in 4.7 % (8/169) isolates from Bangladesh (Mishra *et al*., 2016). As discussed elsewhere (Takala-harrison *et al*., 2015), the isolate harboring K189T mutation in Bangladesh was associated with prolonged parasite clearance half-life >5hrs. Conversely, isolates in Africa which had this mutation were associated with rapid parasite clearance half-life < 5hrs (Ashley *et al*., 2014). Although, the mutant allele K189T has been found at a comparatively high frequency in Africa, it is not associated with delayed parasite clearance or confirmed artemisinin resistance, suggesting that this polymorphism is a part of naturally-evolving parasite population.

Polymorphisms in *Pfmdr1* gene at codons 86, 184 and 1246 have served as valuable molecular markers for tracking and monitoring changes in parasite susceptibility to various drugs, including amodiaquine and artemether-lumefantrine (Dahlström *et al*., 2014). Previous results have indicated that artemisinin selects *Pfmdr1* N86 and D1246 mutations *in vitro* (Reed *et al*., 2000; Mwai *et al*., 2009). In the two resistant parasite lines used in the present study, only one allelic variation was identified in *Pfmdr1*. The N86Y mutation was observed in W2 parasites exposed to artemisinin drug at IC$_{50}$ equivalents. Parasites exposed to *Artemisia annua* extract did not harbor mutant allele in *Pfmdr1* gene.

Although *Pfmdr1* polymorphisms have been associated with reduced susceptibility to ART (Veiga *et al.*, 2011), other studies found no association between this gene and prolonged parasite clearance rates in patients from Cambodia (Imwong *et al.*, 2010). Importantly, no *Pfmdr1* selection was shown in parasites cultured under artemisinin drug pressure for 5years (Ariey *et al.*, 2014). This may suggests that *Pfmdr1* action may be dependent on other genetic factors.

Recent work have reported that ACT treatment can select for particular alleles in *Pfcrt* gene (Sondo *et al.*, 2016). The *Pfcrt* gene is involved in drug transport from the food vacuole lumen to cytoplasm an action important in the development of drug resistance (Ibraheem *et al.*, 2014; Juge *et al.*, 2015). In the present study, *Pfcrt* CQ sensitive CVMNK haplotype was the most favored allele. The *Pfcrt* K76T mutation, which is known to mediate CQ resistance, was observed inW2 parental line and W2 parasites exposed to artemisinin.

Antimalarial drug resistance is accompanied by reduced replicative fitness in *P.falciparum* parasites. A recent study reported that there is an interplay between K13 mutations and six background mutations; D193Y in *fd* gene, T481I in *mdr2*, V127M in *arps10*, N326S and 1356T in *Pfcrt*, V1157L in *pph* and C1484F in *pibp* gene (Miotto *et al.*, 2015). In this study, all parasites haboured wild type alleles in *mdr2*, *arps10*, *fd*, *pph* and *pibp* genes. *Pfcrt* 1356T mutation was observed in parasites exposed to artemisinin at IC_{50} equivalents. This compensatory mutation may evolve in response to artemisinin resistance phenotype. However, our resistant lines possess no polymorphisms in K13 gene associated with ART resistance, suggesting that these genetic markers play an important role in artemisinin resistance. Notably, the cysteine protease *falcipain* 2a gene, previously associated with *in vitro* response to artemisinin (Ariey *et al.*, 2014; Klonis *et al.*, 2011), remained unaltered during the 3 year selection.

Whole plant *Artemisia annua* is 5-fold more potent than a comparable dose of pure artemisinin (Elfawal *et al.*, 2012). The plant is rich in secondary metabolites, mainly flavonoids which may synergize the effects of artemisinin against malaria (Weathers *et al.*, 2015). Studies have shown that *Artemisia annua* extract mitigates against resistance development in rodent malaria parasites *P. yoelli* (Elfawal *et al.*, 2015). In the current study, no polymorphisms was associated with *A. annua* resistance.

Only a few cases of reduced parasite clearing infections or of prolonged $RSA_{0-3hours}$ survival rates have been reported in Sub-Saharan Africa to date (Ashley *et al.*, 2014; Sirima *et al.*, 2016; Menard *et al.*, 2016a), suggesting that the drug pressure has not yet selected for K13 mutations. A plausible explanation would be absence of a favorable genetic background that predispose the parasite populations to the emergence of artemisinin resistance. The A578S mutation is the most predominant in Africa (Ashley *et al.*, 2014; Kamau *et al.*, 2014; Taylor *et al.*, 2015), however, it is neutral and has not been associated with delayed parasite clearance or *in vitro* artemisinin resistance (Menard *et al.*, 2016b). Resistance causing K13 polymorphisms appear to have

multiple geographical origin (Takala-Harrison *et al.*, 2015), and it is clear from a recent work that *PfK13* alleles reported in Southeast Asia may not be the same alleles selected in Africa (Talundzic *et al.*, 2017). Therefore, there is need to characterize the functional significance of novel K13 mutations circulating in Africa.

5. Conclusion

The findings of this study suggest that artemisinin resistance will finally occur and there is need for increased surveillance of the resistant markers and ex vivo ring-stage assays to identify resistant *P. falciparum* parasites in areas where slow parasite clearance is suspected. Further, no polymorphisms were observed in parasites exposed to *Artemisia annua* extracts. *Artemisia annua* has a broad potential therapeutic power against *Plasmodium* parasites and it could be adopted locally thus reducing the cost of health care.

6. Acknowledgements

7. Competing interests

The authors declare that there is no competing interest.

References

[1] Alker, A. P., Lim, P., Sem, R., Shah, N. K., Yi, P., Bouth, D. M., Tsuyuoka, R., Maguire, J. D., Fandeur, T., Ariey, F., Wongsrichanalai, C., & Meshnick, S. R. (2007). *Pfmdr1* and *in vivo* resistance to artesunate-mefloquine in *falciparum* malaria on the Cambodian-Thai border. *American Journal of Tropical Medicine and Hygiene*, *76*(4), 641–647.

[2] Anderson, T. J. C., Nair, S., Nkhoma, S., Williams, J. T., Imwong, M., Yi, P., Socheat D., Das, D., Chotivanich, K., Day, N. P. J., White, N. J., & Dondorp, A. M. (2010). High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in Western Cambodia. *Journal of Infectious Diseases*, *201*(9), 1326–1330.

[3] Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C. M., Bout, D. M., Menard, S., Rogers, W. O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Bras, J. L., Berry, A., Barale, J. C., Fairhust, R. M., Benoit-Vical, F., Mercereau-Puijalon, O., & Didier, M. (2014). A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature*, *505*, 50–55.

[4] Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Kim, S., Suon, S., Sreng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chuor, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T. T., Thuy-Nhien, N. T., Thanh, N. V., Phu, N. H., Htut, Y., Han, K-T., Aye, K. H., Mokuolu, O. A., Olaosebikan,

R. R., Folaranmi, O. O., … and White, N. J. (2014). Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *New England Journal of Medicine*, *371*(5), 411–423.

[5] Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic : a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120.

[6] Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., Land, S. J., Ruden, D. M & Lu, X. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118 ; iso-2; iso-3. *Fly*, *6*(2), 80–92.

[7] Dahlström, S., Aubouy, A., Maïga-ascofaré, O., Faucher, J., Wakpo, A., & Ezinmègnon, S. (2014). *Plasmodium falciparum* polymorphisms associated with Ex Vivo drug susceptibility and clinical effectiveness of artemisinin-based combination therapies in Benin. *Antimicrobial Agents and Chemotherapy*, *58*(1), 1–10.

[8] Depristo, M. A., Banks, E., Poplin, R. E., Garimella, K. V, Maguire, J. R., Hartl, C., Philippakis, A. A., Angel. G. D., Rivas, M. A., Hanna, M. McKenna, A., Fennell.,T. J, Kernytsky, A. M., Sivachenko, A. Y., Cibulskis, K., Gabriel, S. B., Altshuler, D., & Daly, M. J. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics*, *43*(5), 491–498.

[9] Dondorp, A. M., Fairhurst, R. M., Slutsker, L., MacArthur, J. R., Breman, J. G., Guerin, P. J., Wellems, T. E., Ringwald, P., Newman, R. D., & Plowe, C. V. The threat of artemisinin-resistant malaria. *New England Journal of Medicine*, *365*(12), 1073-1075.

[10] Elfawal, M. A., Towler, M. J., Reich, N. G., Golenbock, D., Weathers, P. J., & Rich, S. M. (2012). Dried whole plant *Artemisia annua* as an Antimalarial Therapy. *PLoS One*, *7*(12), 1–7.

[11] Elfawal, M. A., Towler, M. J., Reich, N. G., Weathers, P. J., & Rich, S. M. (2015). Dried whole-plant *Artemisia annua* slows evolution of malaria drug resistance and overcomes resistance to artemisinin. *PNAS*, *112*(3), 821–826.

[12] Fairhurst, R. M. (2015). Understanding artemisinin-resistant malaria: what a difference a year makes. *Current Opinions in Infectious Diseases*, *28*(5), 417–425.

[13] Gadalla, N. B., Adam, I., Elzaki, S., Bashir, S., Mukhtar, I., Oguike, M., Gadalla, M., Mansour, F., Warhurst, D., El-Sayed, B. B., & Sutherland, C. J. (2011). Increased *Pfmdr1* copy number and sequence polymorphisms in *Plasmodium falciparum* isolates from Sudanese Malaria Patients treated with Artemether-Lumefantrine. *Antimicrobial Agents and Chemotherapy*, *55*(11), 5408–5411.

[14] Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., & Scherf, A. (2014). Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system. *Nature Biotechnology*, *32*(8), 819–821.

[15] Humphreys, G. S., Merinopoulos, I., Ahmed, J., Whitty, C. J. M., Mutabingwa, T. K., Sutherland, C. J., & Hallett, R. L. (2007). Amodiaquine and Artemether-Lumefantrine select distinct alleles of the *Plasmodium falciparum mdr1* gene in Tanzanian children treated for

Uncomplicated Malaria. *Antimicrobial Agents and Chemotherapy*, *51*(3), 991–997.

[16] Ibraheem, Z. O., Majid, R. A., Noor, S. M., Sedik, H. M., & Basir, R. (2014). Role of Different *Pfcr*t and *Pfmdr-1* Mutations in conferring resistance to antimalarial drugs in *Plasmodium falciparum*. *Malaria Research and Treatment*, *2014*, 1–17.

[17] Imwong, M., Dondorp, A. M., Nosten, F., Yi, P., Mungthin, M., Hanchana, S., Das, D., Phyo, A. P., Lwin, K. M., Pukrittayakamee, S., Lee, S. J., Saisung, S., Koecharoen, K., Nguon, C., Day, N. P. J., Socheat, D., & White, N. J. (2010). Exploring the contribution of candidate genes to artemisinin resistance in *Plasmodium falciparum*. *Antimicrobial Agents Chemotherapy*, *54*(7), 2886–2892.

[18] Jiang, H., Patel, J. J., Yi, M., Mu, J., Ding, J., Stephens, R., Cooper, R. A., Ferdig, M. T., & Su, X. (2008). Genome-wide compensatory changes accompany drug-selected mutations in the *Plasmodium falciparum CRT* gene. *PLoS One*, *3*(6), e2484.

[19] Johnson, D. J., Fidock, D. A., Mungthin, M., Bir, A., Sidhu, S., Bray, P. G., & Ward, S. A. (2004). Evidence for a central role for *Pfcrt* in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. *Molecular Cell*, *15*(6), 867–877.

[20] Juge, N., Moriyama, S., Miyaji, T., Kawakami, M., Iwai, H., & Fukui, T. (2015). *Plasmodium falciparum* chloroquine resistance transporter is a H + -coupled polyspecific nutrient and drug exporter. *PNAS*, *112*(11), 1–6.

[21] Kamau, E., Campino, S., Amenga-etego, L., Drury, E., Ishengoma, D., Johnson, K., Mumba, D., Kerkre, M., Yavo, W., Mead, D., Bouyou-Akotet, M., Apinjoh, T., Golassa, L., Randrianarivelojosia, M., Andagalu, B., Maiga-Ascofare, Oumou., Amambua-Ngwa, A., Tindana, P., Ghansah, A., Maclnnis, B., Kwiatkowski, D., & Djimde, A. A. (2014). K13-Propeller Polymorphisms in *Plasmodium falciparum* Parasites from Sub-Saharan Africa. *Journal of Infectious Diseases*, *211*, 1352-1355.

[22] Kangethe, L. N., Sabar, O., Nganga, J. K., Kimani, F., Kinyua, J., & Hassanali, A. (2016). Comparative effects of repeated *in vitro* exposures of *Plasmodium falciparum* to sub-lethal doses of pure artemisinin and *Artemisia annua* phytochemical blend. *Scientific Pages of Infectious Diseases*, *1*(1), 1–5.

[23] Klonis, N., Crespo-Ortiz, M. P., Bottova, I., Abu-bakar, N., & Kenny, S., (2011). Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. PNAS, *108* (28), 11405-11410.

[24] Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., & Durbin, R. (2009). The Sequence Alignment / Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079.

[25] Li, J., Chen, J., Xie, D., Urbano, J., Eyi, M., Matesa, R. A., Obono, M. M. O., Ehapo, C. S., Yang, L., Lu, D., Yang, H., Yang, H. T., & Lin, M. (2014). High prevalence of *Pfmdr1* N86Y and Y184F mutations in *Plasmodium falciparum* isolates from Bioko island , Equatorial Guinea. *Pathogens and Global Health*, *108*(7), 339–343.

[26] Manske, M., Miotto, O., Campino, S., Auburn, S., Zongo, I., Ouedraogo, J., Doumbo, O., Zongo, I.,

Puedraogo, J. –B., Michon, P., Mueller, I., Siba, P., Nzila, A., Borrmann, S., Kiara, S. M., Marsh, K., Jiang, H., Su, X. –Z., Amaratunga, C., Fairhurst, R., … Kwiatkowski, D. P. (2012). Analysis of *Plasmodium falciparum* diversity in natural infections by deep sequencing. *Nature*, *487*(7407), 375–379.

[27] Mckenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., & Depristo, M. A. (2010). The Genome Analysis Toolkit : A Map reduce framework for analyzing next-generation DNA sequencing data. *Genome Research*, *20*, 1297–1303.

[28] Menard, D., Khim, N., Beghain, J., Adegnika, A. A., Shafiul-Alam, M., Amodu, O., Rahim-Awab, G., Barnadas, C., Berry, A., Boum, Y., Bustos, M. D., Cao, J., Chen, J. –H., Collet, L., Cui, L., Thakur, G. –D., Dieye, A., Djalle, A., Dorkenoo, M. A., … Mercereau-Puijalon, O. (2016). A Worldwide map of *Plasmodium falciparum* K13-popeller polymorphisms. *The New England Journal of Medicine*, *374*, 2453–2464.

[29] Menard, S., Tchoufack, J. N., Maffo, C. N., Nsango, S. E., Iriart, X., Abate, L., Tsapi, T. T., Awono-Ambene, P. H., Mekongo, F. A., Molais, I. & Berry, A. (2016). Insight into K13 propeller gene polymorphism and ex vivo DHA ‑ response profiles from Cameroonian isolates. *Malaria Journal*, *15*(572), 1–7.

[30] Miotto, O., Amato, R., Ashley, E. A., Macinnis, B., Maclnnis, B., Almagro-Garcia, J., Amaratunga, C., Lim, P., Mead, D., Oyola, S. O., Dhorda, M., Imwong, M., Stalker, J., Drury, E., Campino, S., Amenga-Etego, L., Thanh, T.-N. N., Tran, H. T., Ringwald, P., Bethell, D., Nosten, F., Phyo, A. P. … Kwiatkowski, D. P. (2015). Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nature Genetics*, *47*(3), 226–234.

[31] Mishra, N., Bharti, R. S., Mallick, P., Singh, O. P., Srivastava, B., Rana, R., Phookan, S., Gupta, H. P., Ringwald, P., & Valecha, N. (2016). Emerging polymorphisms in *falciparum* Kelch 13 gene in Northeastern region of India. *Malaria Journal*, *15*(583), 1–6.

[32] Mita, T., Venkatesan, M., Ohashi, J., Culleton, R., Takahashi, N., Tsukahara, T., Ndounga, M., Dysoley, L., Endo, H., Hombhanje, F., Ferreira, M. U., Plowe, C. V & Tanabe, K. (2011). Limited geographic origin and global spread of sulfadoxine-resistant *dhps* alleles in *Plasmodium falciparum* populations. *Journal of Infectious Diseases*, *204,* 1980-1988.

[33] Mwai, L., Kiara, S. M., Abdirahman, A., Pole, L., Rippert, A., Diriye, A., Bull, P., Marsh, K., Borrmann, S., & Nzila, A. (2009). *In vitro* activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in *Pfcrt* and *Pfmdr1*. *Antimicrobial Agents and Chemotherapy*, *53*(12), 5069–5073.

[34] Njokah, M. J., Kang'ethe, J. N., Kinyua, J., Kariuki, D., & Kimani, F. T. (2016). *In vitro* selection of *Plasmodium falciparum Pfcrt* and *Pfmdr1* variants by artemisinin. *Malaria Journal*, *15*(381), 1–9.

[35] Otienoburu, S. D., Ascofaré, O. M., Schramm, B., Jullien, V., Jones, J. J., Zolia, Y. M., Houze, P., Ashley, E. A., Kiechel, J. –R., Guérin, P. J. Bras, J. L., & Houze, S. (2016). Selection of *Plasmodium falciparum Pfcrt* and *Pfmdr1* polymorphisms after treatment with artesunate – amodiaquine fixed dose combination or artemether – lumefantrine in Liberia. *Malaria Journal*, *15*(452), 1-6.

[36] Peak, C. M., Thuan, P. D., Britton, A., Nguyen, T. D., Wolbers, M., Thanh, N. V., Buckee, C. O., & Boni, M. F. (2015). Measuring the association between artemisinin-based case management and malaria incidence in Southern Vietnam, 1991 – 2010. *American Journal of Tropical Medicine and Hygiene*, *92*(4), 811–817.

[37] Phyo, A. P., Ashley, E. A., Anderson, T. J. C., Bozdech, Z., Carrara, V. I., Sriprawat, K., Nair, S., White, M. M., Dziekan, J., Ling, C., Proux, S., Konghahong, K., Jeeyapant, A., Woodrow, C. J., Imwong, M., McGready, R., Lwin, K. M., Day, N. P. J., White, N. J., & Nosten, F. (2016). Declining Efficacy of artemisinin combination Therapy against *P. falciparum* malaria on the Thai – Myanmar Border (2003 – 2013): The Role of Parasite Genetic Factors. *Clinical Infectious Diseases, 63* (6), 784-791.

[38] Reed, M. B., Saliiba, K. J., Caruana, S. R., Kirk, K., & Cowman, A. F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, *403*, 8–11.

[39] Rueangweerayut, R., Phyo, A. P., Uthaisin, C., Poravuth, Y., Binh, T. Q., Tito, H., Penali, L. K., Valecha, N., Tien, N. T., Abdulla, S., Borghini-Fuhrer, I., Duparc, S., Shin, C., & Fleckenstein, L. (2012). Pyronaridine–Artesunate versus Mefloquine plus Artesunate for Malaria. *The New England Journal of Medicine*, *366*(14), 1298–1309.

[40] Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., & Rice, P. (2000). Artemis : sequence visualization and annotation. *Bioinformatics*, *16*(10), 944–945.

[41] Sanchez, C. P., Mayer, S., Nurhasanah, A., Stein, W. D., & Lanzer, M. (2011). Genetic linkage analyses redefine the roles of *PfCRT* and *PfMDR1* in drug accumulation and susceptibility in *Plasmodium falciparum*. *Molecular Microbiology*, *82*(4), 865–878.

[42] Sidhu, A. B. S., Verdier-Pinard, D., & Fidock, D. A. (2002). Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *Pfcrt* mutations. *Science*, *298*(5591), 210–213.

[43] Sidhu, A. B. S., Valderramos, S. G., & Fidock, D. A. (2005). *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Molecular Microbiology*, *57*(4), 913–926.

[44] Sirima, S. B., Ogutu, B., Lusingu, J. P. A., Mtoro, A., Mrango, Z., Ouedraogo, A., … Carn, G. (2016). Comparison of artesunate – mefl oquine and artemether – lumefantrine fi xed-dose combinations for treatment of uncomplicated *Plasmodium falciparum* malaria in children younger than 5 years in Sub-Saharan Africa : a randomised , multicentre , phase 4 trial. *Lancet Infectious Diseases*, *16*, 1123–1133.

[45] Sisowath, C., Petersen, I., Veiga, M. I., Mårtensson, A., Premji, Z., Björkman, A., Fidock, A & Gil, J. P. (2009). In Vivo Selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible *pfcrt* K76 Allele after treatment with Artemether-Lumefantrine in Africa. *The Journal of Experimental Biology*, *199*, 750–757.

[46] Sondo, P., Derra, K., Nakanabo, S. D., & Tarnagda, Z. (2016). Artesunate-Amodiaquine and Artemether-Lumefantrine Therapies and Selection of *Pfcrt* and *Pfmdr1* Alleles in Nanoro, Burkina Faso. *Plos ONE*, *11*(3), e0151565.

[47] Srimuang, K., Miotto, O., Lim, P., Fairhurst, R. M., Kwiatkowski, D. P., Woodrow, C. J., & Imwong, M. (2016). Analysis of antimalarial resistance markers in *Pfmdr1* and *Pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration. *Malaria Journal*, *15*(541) 1-12.

[48] Staedke, S. G., Jagannathan, P., Yeka, A., Bukirwa, H., Banek, K., Maiteki-sebuguzi, C., Clark, T. D., Nzarubara, B., Njama-Meya, D., Mpimbaza, A., Rosenthal. P. J., Kamya, M. R., Wabwire-Mangen, F., Dorsey. G., & Talisuna, A. O. (2008). Monitoring antimalarial safety and tolerability in clinical trials : A case study from Uganda. *Malaria Journal*, *7*(107) 1-10.

[49] Takala-harrison, S., Jacob, C. G., Arze, C., Cummings, M. P., Silva, J. C., Dondorp, A. M., Fukuda, M. M., Hien, T. T., Mayxay, M., Noedl, H., Nosten, F., Kyaw, M. P., Nhien, T. T., Imwong, M., Bethell, D., Se, Y., Lon, C., Tyner, S. D., … Plowe, C. V. (2015). Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. *The Journal of Infectious Diseases*, *211*, 670–679.

[50] Talundzic, E., Ndiaye, Y. D., Deme, A. B., Olsen, C., Patel, D. S., Biliya, S., Daniels R., Vannberg, F. O., Volkman, S. K., Udhayakumar, V., & Ndiaye, D. (2017). The Molecular epidemiology of *Pfk13* mutations in Senegal using targeted amplicon deep sequencing. *Antimicrobial Agents and Chemotherapy*, *61*(3), 1-8.

[51] Taylor, S. M., Parobek, C. M., Deconti, D. K., Kayentao, K., Coulibaly, S. O., Greenwood, B. M., Tagbor, H., Williams, J., Bojang, K., Njie, F., Desai, M., Kariuki, S., Gutman, J., Mathanga, D. P., Mårtensson, A., Ngasala, B., Conrad, M. D., Rosenthal, P. J., & Juliano, J. J. (2015). Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in Sub-Saharan Africa : A molecular epidemiologic study. *The Journal of Infectious Diseases*, *211*, 680–688.

[52] Torrentino-madamet, M., Fall, B., Benoit, N., Camara, C., Amalvict, R., Fall, M., Dionne, P., Ba Fall, K., Nakoulima, A., Diatta, B., Diemé, Y., Menard, D., Wade, B., & Pradines, B. (2014). Limited polymorphisms in *k13* gene in *Plasmodium falciparum* isolates from Dakar, Senegal in 2012 – 2013. *Malaria Journal*, *13*(472) 1-5.

[53] Valderramos, S. G., & Fidock, D. A. (2006). Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci*, *27*(11), 594–601.

[54] Van Der Auwera, G. A., Carneiro, M. O., Hartl, C., Poplin, R., Levy-moonshine, A., Jordan, T., Shakir, K., Roazen, D., Thibault, J., Banks, E., Garimella, K. V., Altshuler, D., Gabriel, S., & Depristo, M. A. (2013). From FastQ data to high confidence variant calls: the Genome Analysis Tool kit best practices pipeline. *Current Protocols in Bioinformatics*, *11*(1110), 1–43.

[55] Veiga, M. I., Ferreira, P. E., Jornhagen, L., Malmberg, M., Kone, A., Schmidt, B. A., Petzold, M., Bjorkman, A., Nosten, F., & Gil. J. P. (2011). Novel polymorphisms in *Plasmodium falciparum* ABC transporter genes are associated with major ACT antimalarial drug resistance. *PLoS One*, *6*(5), e20212.

[56] Weathers, P. J., Towler, M., Hassanali, A., Lutgen, P., & Engeu, P. O. (2015). Dried-leaf *Artemisia annua*: A practical malaria therapeutic for developing countries? *World Journal of Pharmacology*, *3*(4), 39–55.

[57] Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Lim, P., Mao, S., Sopha, C., & Sam, B. (2013). Novel phenotypic assays for the detection of artemisinin resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infectious Diseases*, *13*(12), 1043–1049.

[58] World Health Organization. (2010). Guidelines for the treatment of malaria 2nd edition. Geneva: *World Health Organization, Geneva, Switzerland*.

[59] World Health Organization. (2015). Status report on artemisinin and ACT resistance. *World Health Organization*, *Geneva, Switzerland*. Retrieved from http://www.who.int/malaria/ publications/atoz/status-rep-artemisinin-resistancesept2015.pdf