Lack of K13 mutations in *Plasmodium falciparum* persisting after artemisinin combination therapy treatment of Kenyan children

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**Abstract**

**Background:** Studies in Southeast Asia reported a strong relationship between polymorphisms at the propeller domain of the Kelch 13 (K13) protein encoded by the *Plasmodium falciparum* k13 (*pfk13*) gene and delayed parasite clearance after artemisinin treatment. In Africa, *P. falciparum* remains susceptible and combination therapy regimens which include an artemisinin component display good efficacy. Using quantitative real-time PCR (qPCR), sub-microscopic persistence of *P. falciparum* has previously been reported in one-third of children treated with artemisinin combination therapy (ACT) in western Kenya. In this study, further investigation was made to evaluate whether these sub-microscopic residual parasites also harbour mutations at the propeller region of *pfk13* and whether the mutations, if any, affect treatment outcome.

**Methods:** The *pfk13* propeller domain was genotyped in DNA samples obtained in 2009 from Kenyan children treated with artemether–lumefantrine (AL) and dihydroartemisinin–piperaquine (DP). Paired samples at pre-treatment (day 0) and day of treatment failure (day 28 or 42) for 32 patients with documented recurrent parasitaemia were available for genotyping. Additional day 3 DNA samples were available for 10 patients.

**Results:** No mutation associated with artemisinin resistance in Southeast Asia was observed. Only one DP-treated patient harboured a non-synonymous mutation at codon 578 (A578S) of *pfk13*-propeller gene in the day 0 sample, but this allele was replaced by the wild-type (A578) form on day 3 and on the day of recurrent parasitaemia. The mutation at amino acid codon 578 showed no association with any phenotype. Polymorphisms in *pfk13* were not responsible for parasite persistence and gametocyte carriage in the children treated with ACT.

**Conclusion:** This study contributes to the ongoing surveillance of suspected artemisinin resistance parasites in Africa by providing baseline prevalence of k13-propeller mutations in western Kenya with samples collected from a longitudinal study.

**Clinical Trials Registration** NCT00868465.

**Keywords:** k13-propeller, Artemisinin resistance, Western Kenya, Africa, Slow clearance, Sub-microscopic, qPCR
Background
Artemisinin combination therapy (ACT) is widely used to treat uncomplicated Plasmodium falciparum malaria in endemic areas. Significantly reduced susceptibility to artemisinins, characterized by delayed parasite clearance in vivo and enhanced survival of early stage parasites in vitro, has been reported in Southeast Asia [1, 2]. Most recently, these parasite phenotypes have been associated with mutations in the pfk13 gene of P. falciparum, encoding the kelch-domain protein K13 [3]. Further studies reported direct evidence of resistance to artemisinin using genetic modification of different pfk13 loci [4]. This work has led to an operational definition of partial artemisinin resistance: ≥5 % of patients carrying K13 resistance-associated mutations, all of whom have been found to have either persistent parasitaemia by microscopy on day 3, or a parasite clearance half-life of ≥5 h after treatment with ACT or artesunate monotherapy [5].

In Africa, ACT is the first-line anti-malarial treatment and remains efficacious and safe [3, 6]. However, resistance to previous generations of anti-malarial drugs such as chloroquine, sulfadoxine-pyrimethamine (SP) emerged in the 1970s in Southeast Asia and eventually spread to the Indian sub-continent and then to Africa [7]. It is, therefore, critical that continuous monitoring of the therapeutic response of ACT is carried out in endemic areas in order to detect early warning signs and effectively track and contain the development and spread of artemisinin resistance. Currently, delayed clearance of microscopically detectable parasites has not been observed in Africa. In addition, there are limited data about the in vitro susceptibility of ring stage of parasite development against artemisinins as well as the presence and prevalence of pfk13 mutations [8]. Recently, a cross-sectional survey was carried out to determine the prevalence of pfk13 mutations in 14 sub-Saharan African countries. The study reported the absence of mutations that were associated with artemisinin resistance in Southeast Asia, with the exception of codon 543 [9]. In previous study, the presence of residual sub-microscopic P. falciparum parasites in western Kenyan children on day 3 after ACT treatment was reported and this was associated with subsequent recrudescence and transmission [10]. In addition, the authors have shown in the same Kenyan patient that P. falciparum parasites carrying certain genotypes at pfmdr1, pfcr, pfubp1, and pfap2mu genes were found to survive more often after ACT treatment at sub-microscopic level [11]. The aim of this study was to investigate whether these sub-microscopic residual parasites also harboured mutations at the propeller region of pfk13 and whether the mutations, if any, affect treatment outcome.

Methods
DNA samples were previously obtained from filter-paper blood spots from children (age 8 months–10 years) treated with either artemether–lumefantrine (AL) or dihydroartemisinin–piperaquine (DP) in a clinical trial carried out in 2009 in Mbita, western Kenya and fully described elsewhere [6, 10]. The study was carried out 5 years after Kenya had officially changed the first line of treatment from SP to AL [12]. The protocol was approved by the Kenya Medical Research Institute Ethical Review Committee and the London School of Hygiene and Tropical Medicine Ethics Committee (reference 5455). Written informed consent was obtained from a parent or guardian of each participating child. This is an ancillary study of pfk13 propeller domain polymorphism in DNA samples available from the 2009 study at the following time points: for 32 patients with documented recurrent parasitaemia, paired samples at day 0 (pretreatment) and day of treatment failure (28 or 42, PCR-uncorrected); for 10 patients, additional day 3 DNA samples (Table 1).

The K13-propeller gene fragment (coordinates 1726169–1726997 on chromosome 13 of 3D7 isolate (PF3D7_1343700) was amplified by nested PCR using previously published primers [13]. For the first round of PCR, 250 nM primer (Eurofins, Germany), 1X hot fire pol® blend master mix (Solis Biodyne, Estonia) and 5 μl of DNA in a total volume of 20 μl was run under the following cycling conditions: 95 °C for 15 min then 30 cycles at 95 °C for 30 s, 58 °C for 2 min and extension at 72 °C for 2 min and final extension at 72 °C for 10 min. For the second round of PCR, a total volume of 25 μl made of 18.75 μl nuclease free water, 250 nM primer, 1X hot fire pol® blend master mix (Solis Biodyne, Estonia) final concentration and 5 μl of DNA was run under the following cycling conditions: 95 °C for 15 min then 40 cycles at 95 °C for 30 s, 60 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min. A positive control (K13_5) with one of the mutations identified in Cambodia (provided by D Ménard) was included in every experiment.

Polymorphisms at pfk13 were determined by direct sequencing of amplicons using ABI BigDye Terminator v3.1 cycling sequencing kit and analysis on an ABI 3730 sequencer (Applied Biosystems, USA) as described previously [11] and chromatogram sequences were analysed using Geneious v6.1.5 (Biomatters, USA) in comparison to pfk13-propeller sequence region of 3D7 isolate (PF3D7_134700).

Results
Pfk13 sequences for 32 samples on day 0, seven samples on day 3 and 30 samples on day of failure were successfully obtained and analysed for sequence variation. Of
these 69 sequenced isolates, 68 harboured \textit{pfk13} loci encoding propeller domains identical to the reference. A single DP-treated patient (K1368) harboured a non-synonymous mutation at codon 578 of \textit{pfk13}-propeller gene in the day 0 sample, but this allele was replaced by the wild-type form on day 3 and on the day of recurrent parasitaemia (day of failure) (Table 2). The K13-propeller sequence was deposited in Gene Bank (accession number KT261646). To understand the clearance dynamics of the mutant parasites in the patient, \textit{pfk13} was retrospectively sequenced in day 1 and day 2 parasites from this individual. Sequence analysis of day 0, day 1 and day 2 samples revealed the presence of mutation at codon A578S, suggesting that the mutant parasite was present 2 days after DP treatment (Figure 1). The treatment outcome and other phenotype and genotype data for the patient are found in Table 2. The patient cleared parasites as measured by microscopy on day 1 after DP treatment and no gametocytes were detected on day 7. Recurrent parasitaemia was observed on day 42 but was classified as new infection after PCR-correction. One additional patient carried a synonymous mutation at \textit{pfk13} codon P553P on day of failure but that was not observed on day 0 or day 3.

| Sample ID | D0 | D1 | D2 | D3 | D28 | D42 |
|-----------|----|----|----|----|-----|-----|
| K0025     | X  |    |    |    |     |     |
| K0344     | X  |    |    |    |     |     |
| K0385     | X  |    |    |    |     |     |
| K0544     | X  |    |    |    |     |     |
| K0598     | X  |    |    |    |     |     |
| K0701     | X  |    |    |    |     |     |
| K0719     | X  |    |    |    |     |     |
| K0774     | X  |    |    |    |     |     |
| K0804     | X  |    |    |    |     |     |
| K0840     | X  |    |    |    |     |     |
| K0875     | X  |    |    |    |     |     |
| K0881     | X  |    |    |    |     |     |
| K1010     | X  |    |    |    |     |     |
| K1149     | X  |    |    |    |     |     |
| K1152     | X  |    |    |    |     |     |
| K1307     | X  |    |    |    |     |     |
| K1348     | X  |    |    |    |     |     |
| K1368     | X  | X  | X  | X  |     |     |
| K1438     | X  |    |    |    |     |     |
| K1478     | X  |    |    |    |     |     |
| K1521     | X  |    |    |    |     |     |
| K1861     | X  |    |    |    |     |     |
| K1917     | X  |    |    |    |     |     |
| K1943     | X  |    |    |    |     |     |
| K2023     | X  |    |    |    |     |     |
| K2024     | X  |    |    |    |     |     |
| K3077     | X  |    |    |    |     |     |
| K3212     | X  |    |    |    |     |     |
| K3215     | X  |    |    |    |     |     |
| K3404     | X  |    |    |    |     |     |
| K3762     | X  |    |    |    |     |     |
| K3801     | X  |    |    |    |     |     |

Table 1 Sample availability for \textit{k13} genotyping

Table shows sample which were available for \textit{k13} genotyping on different time points. Days 0 and day of fail (days 28 or 42) samples were available for \textit{k13} genotyping for 32 patients. Of the 32, ten patients had additional samples on day 3 while for patient K3168, additional samples on days 1 and 2 were included retrospectively for further \textit{k13} genotype analysis.
Phenotype
Genotype
K1368 S A NFD CVMNK S

elling, the authors suggested that it has an effect on tertiary
observed in clinical isolates and using computational mod-
in Bangladesh, the mutation at codon 578 has also been
site clearance half-life in patients treated with ACT [3].

msp1 the same patient as short as 6 h apart [10, 14]. In fact, the
that there were genetic changes in samples collected in
ings broadly agree with Farnert et al., who also showed
of the assay. The importance of multiplicity of infection
present on days 0, 1 and 2 but below the detection limit
However, it is not clear whether the wild type was already
in this individual, and persistent parasites detected at day
3 and day 42 harboured the wild type A578 allele only. However, it is not clear whether the wild type was already
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the same patient as short as 6 h apart [10, 14]. In fact, the
msp1 and msp2 genotyping of the day 0 sample showed
that the patient was infected with at least three different
parasite clones. Blood from this patient was presented to
Anopheles gambiae mosquitoes at day 7 by membrane
feeding, as reported in previous study, but no infected
mosquitoes were generated [6].

The A578S polymorphism has previously been reported in a cross-sectional study in western Kenya, Kisumu, and in four other African countries [9], but the current study is the first study to investigate the role of this variant in gametocyte carriage and infectiousness to mosquito. These findings are consistent with recent published multisite studies in Southeast Asia and Africa that showed mutation at codon 578 in pfk13 and its lack of association with parasite clearance half-life in patients treated with ACT [3]. In Bangladesh, the mutation at codon 578 has also been observed in clinical isolates and using computational mod-
eelling, the authors suggested that it has an effect on tertiary
structure of the protein [15]. Although the parasite har-
bouring the mutant A578S allele was successfully cleared
by DP; the contribution of piperaquine in clearing these
parasites is not clear. This polymorphism lies adjacent to
C580Y, and further in vivo and in vitro studies to clarify
the significance of the A578S mutation are warranted,
particularly in different genetic backgrounds. Mutations
in pfcrt, pfmdr1, pfap2mu, and pfubp1 also contribute to
treatment outcomes after either artemisinin monotherap-
Y treatment [11, 16]. Studies introducing transgenic
variants of pfk13 and other candidate loci into parasite
isolates with an African genetic background could clarify
whether pfk13 mutations or polymorphisms in other genes
are most relevant in determining artemisinin sensitivity in
clinical isolates in Africa [4, 17].

The rarity of pfk13 variants in the current small study pre-
cludes from making meaningful assessment of the role of
A578S and other propeller domain mutations in treatment
outcomes in Africa. However, it can be confirmed that the
sub-microscopic clearance phenotype observed in west-
ern Kenya is not directly related to the parasite clearance
half-life observed in Southeast Asia. The sub-microscopic
residual parasites have been shown previously to be impor-
tant phenotypes as children harbouring those parasites were
significantly more likely to be infectious to mosquitoes and
were more likely to have recurrent asexual parasitaemia on
day 28 or 42 [10]. Factors associated with the host, drug and
parasite should be investigated to determine the exact cause of
the sub-microscopic residual parasites.

This study contributes to the ongoing surveillance of
suspected artemisinin resistance parasites in Africa by
providing baseline prevalence of k13-propeller mutations
in western Kenya with samples collected from a longitudi-
nal study, and thus one of the first to relate genotype
and phenotype. This information from samples collected
in 2009 does not reflect the current status of ACT, nor
the prevalence of k13-propeller mutations now. There-
fore, continuous monitoring of ACT in western Kenya
and the collation of phenotypic and genotypic data to

Discussion
No evidence was found that artemisinin resistance-asso-
ciated mutations in the pfk13-encoded propeller domain,
including the major resistance-associated mutation
C580Y, contribute to ACT treatment failure in Mbita,
western Kenya. Only one of the 32 patients from this
longitudinal study had mutation in the propeller region
of pfk13 gene, despite evidence of parasite persistence in
over 30 % of children [10]. One patient transiently car-
rried parasites harbouring mutation at codon 578 (A578S)
on enrolment, and on days 1 and 2 after DP treatment.
However, these parasites were apparently cleared by DP
in this individual, and persistent parasites detected at day
3 and day 42 harboured the wild type A578 allele only. However, it is not clear whether the wild type was already
present on days 0, 1 and 2 but below the detection limit of the assay. The importance of multiplicity of infection
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and phenotype. This information from samples collected
in 2009 does not reflect the current status of ACT, nor
the prevalence of k13-propeller mutations now. There-
therefore, continuous monitoring of ACT in western Kenya
and the collation of phenotypic and genotypic data to
track the emergence and spread of parasites with reduced susceptibility to artemisinins is recommend.

Abbreviations
ACT: artemisinin combination therapy; AL: artemether–lumefantrine; DP: dihydroartemisinin–piperaquine; EDCTP: European and developing countries clinical trials partnership; pfk13: Plasmodium falciparum kelch 13 gene; qPCR: quantitative (real-time) PCR.

Authors’ contributions
PS, TB, CJS, and KBB contributed to study design. PS collected clinical data. JM, GH, CJS and KBB did laboratory work. JM, CJS and KBB analysed the data. JM, CJS and KBB wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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Fig. 1 DNA and amino acid sequence of a propeller region of PfK13 showing mutation at codon 578. Sequence of samples from patient K1368 at day 0, 1 and 2 show mutation at amino acid position 578 while the samples on day 3 and day 42 have no mutations. DNA amplification of sample on day 3 using pfk13[13] and pgm1 primers[18] repeatedly failed. DNA and amino acid sequence of 3D7 isolate was used as a reference. Each sample has a sequence using forward (F) and reverse (R) primers. Arrow indicates mutation and top numbers indicate amino acid position

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