**In vivo** selection of *Plasmodium falciparum* Pfcrt and Pfmdr1 variants by Artemether-Lumefantrine and Dihydroartemisinin Piperaquine in Burkina Faso

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ABSTRACT

Plasmodium falciparum Pfcrt-76 and Pfmdr1-86 gene polymorphisms were determined during a clinical trial in Burkina Faso comparing the efficacy of dihydroartemisinin-piperaquine (DHA-PPQ) and artemether-lumefantrine (AL). A significant selection of Pfcrt-K76 was observed after exposure to AL and DHA-PPQ, as well as of Pfmdr1-N86 after AL but not after DHA-PPQ treatment, suggesting a reverse selection on the Pfcrt gene by PPQ. These results support the rational use of DHA-PPQ in settings where CQ resistance is high.
Emergence of *Plasmodium falciparum* resistance to artemisinin-based combination therapy (ACT) in Asia [1,2] represents a major threat to the recent gains in malaria control efforts. Partner drugs in ACTs increase the elimination of late clearing parasites; however, if resistance develops against these partner drugs, treatment failures will probably increase [3].

Artemether-Lumefantrine (AL) is currently the most widely used ACT in Africa [4] and Dihydroartemisinin-Piperaquine (DHA-PPQ) was recently recommended by the WHO for treating uncomplicated *falciparum* malaria [5]. Piperaquine (PPQ) monotherapy was extensively used in China until 1978 when it was abandoned due to widespread resistance [6]. PPQ has a long half-life of 3-4 weeks; therefore its use may expose re-infecting and late clearing parasites to sub-optimal blood concentration of PPQ, potentially leading to selection of resistant strains [6].

Chloroquine (CQ) resistance transporter mutation at codon 76 (*Pfcrtr-K76T*) has been associated with CQ and amodiaquine (AQ) resistance [7,8]. Due to structural similarities between PPQ and CQ, there have been attempts to identify common markers of resistance [9,10]. However, the limited number of studies available did not observe selection of *Pfcrtr* SNPs [11,12].

Mutations in the multidrug resistance gene 1 codon 86 (*Pfmdr1-N86Y*) are associated with altered response to structurally unrelated antimalarials, including 4-aminoquinolines and aryl-aminoquinolines [13,14]. Significant selection of *Pfmdr1*-N86 have been consistently reported with AL in several settings[11,12,15]. However, there is so far no *in vivo* evidence of *Pfmdr1* selection after DHA-PPQ treatment with the exception of the reported borderline selection of *Pfmdr1*-D1246 [12].
In Burkina Faso, artesunate-amodiaquine (AS-AQ) and AL were introduced in 2005 as first-line treatment of uncomplicated malaria [16], following high CQ resistance (CQR) [17]. As part of a multi-centric study on the safety and efficacy of DHA-PPQ, a clinical trial was conducted in Burkina Faso to test the non-inferiority of DHA-PPQ compared to AL [18]. We present here Pfert and Pfmdr1 SNPs analysis in P. falciparum recurrent infections in an attempt to assess whether DHA-PPQ selected for known polymorphisms in this area of known high CQR.
A total of 301 children aged 6-59 months were randomly allocated to either DHA-PPQ or AL treatment at a ratio of 2:1 and followed up for 42 days as published elsewhere [18]. Bloodspot filter paper specimens were collected on the day of enrollment and during follow-up. Genotyping to distinguish recrudescent from new infections was done as previously described [19]. The detection of the Pfcrt-76 and Pfmdr1-86 SNPs were carried out by PCR followed by RFLP as previously described [7]. A total of 272 (91 AL vs 181 DHA-PPQ) Day0 as well as 24 and 37 samples for recurrent P. falciparum infections in AL and DHA-PPQ groups respectively were analysed. Chi-square with Yates correction or Fisher Exact test were used for categorical variables as required. The strength of association between markers and treatment outcomes was evaluated by odds ratios (OR). Mixed alleles were treated as mutant while unsuccessful PCR were excluded from the analysis. Statistical significance was set at $\leq 0.05$.

A total of 267 (98.2%) and 272 (100%) Day0 blood samples were successfully genotyped for the Pfcrt-K76T and Pfmdr1-N86Y polymorphisms, respectively (Figure 1). At baseline, the prevalence of Pfcrt-76T mutants was similar between the two study arms, about 50% (48.3% for AL and 48.9 for DHA-PPQ) when considering infections with only the mutant alleles, and almost 70% when considering infections with mixed alleles (Table 1). The prevalence of the Pfmdr1-N86 allele at baseline was higher, 59.3% for AL and 64.1% for DHA-PPQ.

The prevalence of the Pfcrt-K76 was significantly higher in recurrent infections than at Day0, before treatment, both in the AL (79.2% vs 30.3%) ($p<0.001$) and in the DHA-PPQ arm (67.6% vs 29.2%) ($p<0.001$). Similarly, after treatment with AL, the Pfmdr1-N86 prevalence
in recurrent infections was significantly higher than that at Day0 (95.8% vs 59.3%) (p<0.001) while there was no difference in the DHA-PPQ arm (Table 1). No association between Pfcr-100 K76T or Pfmdr1-N86Y SNPs and treatment outcome was observed (Table 2).
Our results showed a high baseline prevalence of the Pfcrt-76T alleles which corroborates previous reports from Burkina Faso [8]. Interestingly, significant selection of the Pfcrt-K76 after DHA-PPQ treatment was unexpectedly observed as this is contrary to the hypothesis that PPQ and CQ share similar mechanisms of resistance given their structural similarities [20]. In comparison, DHA-PPQ did not select for Pfcrt (K76) polymorphisms in previous studies carried out in Burkina Faso [11,12]. Reasons for the observed differences are unknown. However, an earlier in vitro study from Kenya [10] found that PPQ Inhibitory Concentration (IC$_{50}$) values were not associated with Pfcrt or Pfmdr1 mutations. Conversely, Pfcrt CQR haplotypes and a novel Pfcrt mutation (C101F) were associated with resistance to PPQ in other studies [20, 21]. Overall, available evidence suggests high in vitro susceptibility of the CQR parasites to PPQ [9,10,22]. This could be explained by the large bis-quinolone structure of PPQ postulated to inhibit the transporter-mediated drug efflux thus maintaining high potency against CQR strains [23]. Indeed, clinical trials data confirm outstanding efficacy of DHA-PPQ despite high CQR levels [18,24]. Thus, as DHA-PPQ becomes increasingly available endemic settings for treatment and chemoprevention, Pfcrt polymorphism and its clinical implications should be further monitored.

Unlike recently reported results from Uganda [25], there was no significant selection of Pfmdr1-86Y allele observed in our study after DHA-PPQ treatment consistent with previous reports from Burkina Faso [11,12]. These observed differences may be explained by different genetic backgrounds of the parasites in West and East Africa. Our results further confirmed that AL treatment selects for Pfcrt-K76 and Pfmdr1-N86 as previously observed [15,26]. At present, AL remains efficacious though the selected wild-type alleles are repeatedly associated with diminished sensitivity to lumefantrine [12,15,27].
In conclusion, we observed significant selection of Pfert-K76 following DHA-PPQ and AL treatment, suggesting that, despite structural similarities between PPQ and CQ, the drugs exert a different mechanism of selection on the Pfert gene. These results support the rational use of DHA-PPQ in settings where CQ resistance is high.

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Table 1: Selection of Pfcrt and Pfmdr1 SNPs among P. falciparum recurrent infections by AL and DHA-PPQ

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pfcrt and Pfmdr1 alleles</th>
<th>Day 0 n/N (%)</th>
<th>Recurrent infections n/N (%)</th>
<th>χ² (P-value)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Pfcrt-K76</td>
<td>27/89 (30.3)</td>
<td>19/24 (79.2)</td>
<td>16.7 &lt;0.001</td>
<td>Selection for K76</td>
</tr>
<tr>
<td></td>
<td>Pfcrt-76T</td>
<td>43/89(48.3)</td>
<td>1/24 (4.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfcrt mixed (K76T)</td>
<td>19/89(21.4)</td>
<td>4/24 (16.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfmdr1-N86</td>
<td>54/91(59.3)</td>
<td>23/24 (95.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfmdr1-86Y</td>
<td>17/91(18.7)</td>
<td>1/24 (4.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfmdr1 mixed (N86Y)</td>
<td>20/91(22.0)</td>
<td>0/24 (0 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHA-PPQ</td>
<td>Pfcrt-K76</td>
<td>52/178(29.2)</td>
<td>25/37 (67.6)</td>
<td>18.0 &lt;0.001</td>
<td>Selection for K76</td>
</tr>
<tr>
<td></td>
<td>Pfcrt-76T</td>
<td>87/178(48.9)</td>
<td>9/37 (24.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfcrt mixed (K76T)</td>
<td>39/178 (21.9)</td>
<td>3/37 (8.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfmdr1-N86</td>
<td>116/181 (64.1)</td>
<td>24/37(64.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfmdr1-86Y</td>
<td>32/181(17.7)</td>
<td>9/37(24.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pfmdr1 mixed (N86Y)</td>
<td>33/181(18.2)</td>
<td>4/37(10.8)</td>
<td>0.0 0.929 No evidence</td>
<td></td>
</tr>
</tbody>
</table>

SNP: single-nucleotide polymorphism
Pfcrt: P. falciparum chloroquine resistance transporter gene (Pfcrt K76= wild type, Pfcrt 76T=mutant type)
Pfmdr1: P. falciparum multidrug resistance gene (Pfmdr1 N86=Wild type, Pfmdr1 86Y=mutant type)
NA= not applicable
Significant p-values are in bold
Table 2: Association between SNPs in Pfcrt-76 and in Pfmdr1-86 in P. falciparum isolates and treatment outcomes with AL and DHA-PPQ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SNPs</th>
<th>Treatment Outcome*</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pfcr/pfmdr1 alleles</td>
<td>Failed, n/N (%)</td>
<td>Cured, n/N (%)</td>
</tr>
<tr>
<td>AL</td>
<td>K76</td>
<td>2/7 (28.6)</td>
<td>25/82 (30.5)</td>
</tr>
<tr>
<td></td>
<td>76T</td>
<td>5/7 (71.4)</td>
<td>57/82 (69.5)</td>
</tr>
<tr>
<td></td>
<td>Pfmdr1</td>
<td>N86</td>
<td>7/7 (100.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86Y</td>
<td>0/7 (0.0)</td>
</tr>
<tr>
<td>DHA-PPQ</td>
<td>K76</td>
<td>3/13 (23.1)</td>
<td>49/165 (29.7)</td>
</tr>
<tr>
<td></td>
<td>76T</td>
<td>10/13 (76.9)</td>
<td>116/165 (70.3)</td>
</tr>
<tr>
<td></td>
<td>Pfmdr1</td>
<td>N86</td>
<td>8/13 (61.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86Y</td>
<td>5/13 (38.5)</td>
</tr>
</tbody>
</table>

The denominators represent number of samples for which that SNP was analysable. Mixed alleles of the Plasmodium falciparum chloroquine resistance transporter (Pfcrt) gene or multidrug resistance 1 (Pfmdr1) genes were treated as mutants.

AL = Artemether Lumefantrine; DHA-PPQ = Dihydroartemisinin Piperaquine; OR = odds ratio; CI = confidence interval, NA = Not applicable.

*PCR adjusted treatment failure was defined according to the World Health Organization [28].
Figure 1. Trial profile indicating the number of samples analyzed at Day0 and among recurrences by treatment arm. Treatment outcomes are all PCR corrected.
Total enrolled in the trial (n=301)
Total isolates analysed = 272/301 (90.4%)

DHA PPQ
181/201 (90%) samples analysed
Day 0: Successful RFLP for PfCRT = 178/181 (98%)
Successful RFLP for Pfmdr1 = 181/181 (100%)

Recurrent infections = 37/38 (95%) analyzed
Successful RFLP for PfCRT = 37/37 (100%)
Successful RFLP for Pfmdr1 = 37/37 (100%)

Treatment failure = 13/13 (100%) analyzed
New infections = 24/24 (100%) analyzed

AL
91/100 (91%) samples analysed
Day 0: Successful RFLP for PfCRT = 89/91 (97%)
Successful RFLP for Pfmdr1 = 91/91 (100%)

Recurrent infections = 24/45 (53%) analyzed
Successful RFLP for PfCRT = 24/24 (100%)
Successful RFLP for Pfmdr1 = 24/24 (100%)

Treatment failures = 7/7 (100%)
New infections = 17/17 (100%)