In Vivo Selection of *Plasmodium falciparum* pfmdr1 86N Coding Alleles by Artemether-Lumefantrine (Coartem)

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Artemisinin derivative–based combination therapy is expected to suppress the development of *Plasmodium falciparum* drug resistance in Africa. We have performed an artemether-lumefantrine (Coartem; Novartis) follow-up clinical trial in Zanzibar, in which *pfcr* K76T and *pfmdr1* N86Y frequencies were determined before drug administration and in all recurrent parasites during a follow-up period of 42 days. A significant increase in *pfmdr1* 86N was observed after exposure to the drug. This points to 86N as a potential marker of lumefantrine resistance in vivo, while suggesting that Coartem is not robust enough to avoid selection of resistance-associated mutations in some malarial settings.

The development and expansion of antimalarial drug resistance is a main cause of the increased morbidity and mortality associated with malaria during the past decade in Africa. The progressive failure of chloroquine has led several countries to promote sulfadoxine-pyrimethamine (SP) as a first-line drug. Regrettably, the molecular nature of resistance to SP—that is, amino acid alterations in the drug target proteins—generally leads to a rapid increase in clinical failures when applied on a large scale. New combinations of drugs have been proposed to be the next strategy for the control of malaria. Among these, artemisinin derivative–based combination therapy (ACT) is being increasingly advocated. ACT promises high efficacy and protection against the development of resistance to each component [1]. The concept behind ACT is that artemisinin derivatives, because of their high parasite-reduction rate, quickly reduce the number of the parasites in an infection, leaving its long–elimination half-life partner drug with a significantly lower parasite population to eliminate, thereby minimizing the risk of selecting parasites resistant to the partner drug.

Since artemisinin derivatives typically have a very short elimination half-life, the weak link in the combination is the period during which the unprotected partner drug remains alone during its elimination period, particularly at subtherapeutic concentrations. At this point, selection of reinfecting resistant parasites may occur. In settings with low transmission (e.g., Southeast Asia), this might constitute a minor flaw that does not affect the effectiveness of the combination. In fact, the ACT strategy (fixed artesunate-mefloquine combination) has been applied with significant success in Thailand [2]. The question remains as to whether this approach will have the same success in African regions with high transmission.

Zanzibar is one of the first regions in Africa to have officially changed its drug policy toward ACT, with amodiaquine-artesunate representing the first-line alternative and artemether-lumefantrine (Coartem; Novartis) representing the second line. In the particular case of artemether-lumefantrine, the parasites are expected to be naïve to both partner drugs.

Single-nucleotide polymorphisms (SNPs) in the *pfcr* and *pfmdr1* genes are known to be involved in the development of in vitro and in vivo resistance to quinoline antimalarial drugs [3]. We investigated, in an in vivo follow-up study, whether the 2 main mutations of these genes would be drug selected after administration of artemether-lumefantrine. In particular, we were interested to know whether *pfmdr1* 86N–carrying parasites would be selected, since this mutation has been associated with decreased sensitivity to lumefantrine in vitro [4, 5].

**Subjects, materials, and methods.** Between October 2002 and February 2003, 200 children attending the public health care units in Kivunge (Unguja Island) and Micheweni (Pemba Island) who had microscopically confirmed, uncomplicated *Plasmodium falciparum* malaria were enrolled in the present study. Inclusion criteria were as follows: age ≥12 months and <59 months, body weight ≥9 kg, fever or fever history during the preceding 24 h, and parasitemia levels of $2.0 \times 10^5$–$2.5 \times 10^5$ parasites/μL. Exclusion criteria were as follows: signs of severe malaria, other danger signs (including impaired consciousness, inability to sit or stand, severe anemia [hemoglobin concentration ≤5 g/dL]), recent history of convulsions, shock [systolic blood pressure <50 mmHg], inability to drink or breastfeed, or persistent vomit-
Malaria Control Program. The children were checked routinely for parasite presence on study days 1, 2, 3, 7, 14, 21, 28, 35, and 42 and at any time fever was recorded between the scheduled days. Giemsa-stained thick blood film analyses were performed, and parasitemia was quantified (as number of parasites per microliter) by a standard approximation method. A positive smear was defined as a smear with at least 1 asexual parasite form seen after examination of 100 thick-smear fields under ×1000 magnification. Quality control was performed by re-reading 10% of the slides in a central laboratory.

If a patient again received a clinical and parasitological diagnosis of malaria, he or she was re-treated with parenteral quinine and was transported to the referral hospital. Finger-prick blood samples were collected on filter paper at every examination, except on study days 2 and 3.

Molecular analysis of filter paper–preserved blood samples was performed at the Karolinska Institute (Stockholm). The pfmdr1 N86Y and pfcrt K76T SNPs were analyzed by established polymerase chain reaction (PCR)–based protocols, with modifications [6]. Primer sequences used for the amplification of the pfcrt fragment harboring the K76T SNP were as follows: first amplification, 5′-CCG TTA ATA ATA AAT ACA CGC AG-3′ (forward) and 5′-CGG ATG TTA CAA AAC TAT AGT TAC C-3′ (reverse); nest amplification, 5′-TGT GCT CAT GTG TTT AAA CTT-3′ (forward) and 5′-CAA AAC TAT AGT TAC CTT TG-3′ (reverse). For pfmdr1, the primer sequences used for the analysis of the N86Y SNP were as follows: first amplification, 5′-ATG GGT AAA GAG CAG AAA GAG-3′ (forward) and 5′-CGT ACC AAT TCC TGA ACT CAC-3′ (reverse); nest amplification, 5′-TGT ATT ATC AGG AGG AAC ATT AC-3′ (forward) and 5′-GTA ATT ACA TCC ATA CAA TAA CTT G-3′ (reverse). All oligonucleotide primers were synthesized by the Thermoelectron Corporation, and Taq polymerase and dNTPs were from Promega. All PCRs were performed in Biometra Tpersonal thermocyclers. Both pfcrt- and pfmdr1-specific PCR products were restricted with Apol (New England Biolabs) for the analysis of K76T and N86Y, respectively. Restriction fragments were analyzed on 2%–2.5% agarose gels (Promega) containing 0.1 μg/mL ethidium bromide and were visualized by UV transillumination in a BioRad GelDoc 2000.

The genetic diversity of P. falciparum infections was defined by PCR-based genotyping of pfmsp2 [7], a single-copy gene that is highly polymorphic both in sequence and in size, through analysis of the variable number of tandem repeats. The outer conserved region of polymorphic repetitive block 3 of pfmsp2 is amplified in an initial reaction, followed by 2 nested reactions with oligonucleotide primers specific for the FC27 and IC/3D7 allelic types of pfmsp2. The sequences of the primers and the PCR protocol are described elsewhere [7]. The PCR products were separated by gel electrophoresis on 2% MetaPhor agarose (BMA Bioproducts) and were visualized by UV transillumination after staining with ethidium bromide. Length differences were determined using a 100-bp molecular-weight ladder (Amersham Biosciences) and the BioRad GelDoc 2000 image-analysis program. The number of products of the 2 respective allelic types denotes the number of pfmsp2 genotypes—that is, the number of clones per infection.

Calculation of confidence intervals (CIs) was performed by use of the Confidence Interval Analysis program (version 1.1) [8]. Yates’s corrected χ2 testing was performed by use of Microstat software (release 4; Ecosoft).

Results. Complete parasite clearance within 48 h was observed in all patients except 1, including normal and stable body temperatures within 72 h. In 1 patient, clearance was complete after 72 h. No serious adverse event was recorded. The PCR-adjusted cure rates were 100% at day 14, 98.5% at day 28, and 94.8% at day 42.

The patients were followed for 42 days after artemether-lumefantrine treatment. The earliest breakthrough infections were detected parasitologically by day 21. Between days 21 and 42, 45 events of parasitemia were observed.

For 39 of the 45 patients with recurrent parasitemia, successful analyses for both the pfcrt K76T and pfmdr1 N86Y SNPs were performed. A total of 189 infections before treatment were also successfully analyzed, including for all of the aforementioned 39 children.

Since a mixed infection contributes equally to the frequency of each of the studied alleles, 185 of 190 distinguishable parasite strains carried the pfcrt 76T allele (frequency, 0.974; 95% CI, 0.940–0.991) before treatment. This allele was present in all baseline isolates, except for 4 pure 76K–carrying infections. After artemether-lumefantrine treatment, all parasites found were 76T carriers. The high frequency of this allele precludes any meaningful analysis of its selection after artemether-lumefantrine exposure.

Similar to pfcrt 76T, the pfmdr1 86Y allele was present in 160 of 209 distinguishable parasite strains (frequency, 0.766; 95% CI,
A statistically significant selection of the pfmdr1 86N allele was observed after administration of artemether-lumefantrine. The frequency of this allele in pure form increased 2.7-fold, from 15.3% to 41.0% ($\chi^2 = 11.35; P = .00075$), among the parasites detected after artemether-lumefantrine treatment (table 1). When the dual contribution of the observed mixed infections is taken into account, this difference still shows statistical significance ($\chi^2 = 9.32; P = .002$). The majority of the parasites carrying pfmdr1 86N were identified between 20 and 30 days after the administration of artemether-lumefantrine ($\chi^2 = 9.595; P = .002$) (table 1).

In 8 of the children, pfmsp2 analysis showed evidence of true recrudescence between days 28 and 42. In these children, 6 infections carried pfmdr1 86Y, whereas 2 harbored 86N (table 2).

Discussion. In this study, we analyze for the first time, to our knowledge, the effects of artemether-lumefantrine use on pfcrT and pfmdr1 SNP selection in Africa. The observed prevalence of the pfcrT 76T allele was too high, both before and after treatment, to allow any conclusions to be made concerning the possible involvement of this SNP in artemether-lumefantrine response. In any case, the tendency for there to be an increase in the frequency of pfcrT 76T among the posttreatment parasitemias suggests that further studies should be conducted, to confirm whether selection of this SNP actually occurs in response to this drug combination.

For the pfmdr1 86Y SNP, the baseline frequency observed (76.6%) was similar to that recently observed in another location in East Africa [9]. A significant increase in the prevalence of pfmdr1 86N in pure form was observed, from 15.3% before the administration of artemether-lumefantrine to 41.0% afterward—that is, a 2.7-fold increase in frequency. To our knowledge, this is the first time that selection of a SNP previously associated with quinoline antimalarial drug resistance has been documented in ACT.

Analysis of the biodiversity marker gene pfmsp2 showed that the observed selection of pfmdr1 86N mainly occurred upon reinfecting parasites (table 2), when the remaining drug concentration of lumefantrine was probably too low to eliminate the mutated parasites and when it was no longer protected by artemether. If pfmdr1 86N were involved in an ongoing development of high-grade resistance to lumefantrine, we might expect the majority of the recrudescing parasites to harbor this allele. This observation is also plausibly related to the recent observations showing that the lumefantrine-artemisinin synergy essentially affects pfmdr1 86N carriers [10].

The observed selection of the pfmdr1 86N allele supports previously published in vitro data showing a significant increase in the IC50 of lumefantrine on laboratory clones carrying this version of the SNP [4]. This leads us to suggest pfmdr1 86N as a probable factor in the development of in vivo resistance to lumefantrine, in accordance with previous proposals [10]. This selection appears to be operating mainly in reinfections occurring during the elimination phase of lumefantrine, 20–30 days after the initiation of the therapy (average elimination half-life, 4.5 days [11]), when the residual lumefantrine is expected to be at low concentrations and is no longer protected by artemether (table 1). Finally, the fact that the frequency increase of pfmdr1 86N was essentially confined to reinfection events suggests that the application of this concept in African high-transmission malarial settings may not have the same success as that witnessed in Southeast Asia, which is characterized by a significantly lower transmission.

These observations raise a new additional point: can ACT accelerate the development of resistance to artemisinin deriv-

### Table 1. pfmdr1 Y86N single-nucleotide polymorphism (SNP) frequencies in isolates before (day 0) and after the administration of artemether-lumefantrine.

<table>
<thead>
<tr>
<th>Study day(s)</th>
<th>86Y</th>
<th>86N</th>
<th>86Y + 86N</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>140</td>
<td>29</td>
<td>20</td>
<td>189</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 21</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Days 26 + 28</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Day 35</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Days 39 + 40 + 42</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Total follow-up no.</td>
<td>20</td>
<td>16</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td>Total follow-up frequency (95% CI)</td>
<td>0.513 (0.348–0.676)</td>
<td>0.410 (0.256–0.549)</td>
<td>0.077 (0.016–0.209)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of isolates with the given SNP, unless otherwise noted. CI, confidence interval.

### Table 2. The pfmdr1 N86Y single-nucleotide polymorphism (SNP) in recrudescences vs. reinfections after artemether-lumefantrine treatment.

<table>
<thead>
<tr>
<th>pfmdr1 N86Y SNP</th>
<th>Recrudescences</th>
<th>Reinfections</th>
<th>Uncertain</th>
</tr>
</thead>
<tbody>
<tr>
<td>86Y</td>
<td>6</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>86N</td>
<td>2</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>86Y + 86N</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

0.708–0.823) before treatment. A statistically significant selection of the pfmdr1 86N allele was observed after administration of artemether-lumefantrine. The frequency of this allele in pure form increased 2.7-fold, from 15.3% to 41.0% ($\chi^2 = 11.35; P = .00075$), among the parasites detected after artemether-lumefantrine treatment (table 1). When the dual contribution of the observed mixed infections is taken into account, this difference still shows statistical significance ($\chi^2 = 9.32; P = .002$). The majority of the parasites carrying pfmdr1 86N were identified between 20 and 30 days after the administration of artemether-lumefantrine ($\chi^2 = 9.595; P = .002$) (table 1).

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These observations raise a new additional point: can ACT accelerate the development of resistance to artemisinin deriv-
atives? The presence of the pfmdr1 86N allele has also been shown to confer less sensitivity to artemisinin in vitro [4, 5]. Although artemisinin or its derivative may not select pfmdr1 86N, lumefantrine pressure may provide the main selective force on the allele and, thus, co-drive a decrease in sensitivity to both compounds. The results of our study reinforce the idea that an understanding of the molecular basis of drug resistance of ACT components is fundamental for its maintained efficacy in malaria control.

References