ASSOCIATION BETWEEN MUTATIONS IN PLASMODIUM FALCIPARUM CHLOROQUINE RESISTANCE TRANSPORTER AND P. FALCIPARUM MULTIDRUG RESISTANCE 1 GENES AND IN VIVO AMODIAQUINE RESISTANCE IN P. FALCIPARUM MALARIA-INFECTED CHILDREN IN NIGERIA


Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria; Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts; Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Springs, Maryland; Special Program for Research and Training in Tropical Diseases, World Health Organization, Geneva, Switzerland

Abstract. This study investigated the association between Plasmodium falciparum chloroquine resistance transporter (pfcrt) T76 and P. falciparum multidrug resistance gene 1 (pfmdr1) Y86 alleles and in vivo amodiaquine (AQ) resistance, as well as the clearance of parasites harboring these two alleles in children treated with AQ in southwest Nigeria. One hundred one children with acute uncomplicated P. falciparum malaria infections were treated with the standard dosage of AQ and followed-up for 28 days. Blood samples were collected on filter paper samples at enrollment and during follow-up for identification of parasite genotypes and pfcrt and pfmdr1 mutations using polymerase chain reaction and restriction fragment length polymorphism approaches. Parasitologic assessment of response to treatment showed that 87% and 13% (RI) of patients were cured and failed treatment, respectively. Although infections in patients were polyclonal (as determined by merozoite surface protein 2 genotyping), the presence of both mutants pfcrtT76 and pfmdr1Y86 alleles in parasites is associated with in vivo AQ resistance (odds ratio = 7.58, 95% confidence interval = 1.58–36.25, P = 0.006) and is selected by the drug in children who failed AQ treatment. Treatment failure with the combination of mutant pfcrtT76 and pfmdr1Y86 alleles as well as the ability of patients to clear these resistant parasites is dependent on age, suggesting a critical role of host immunity in clearing AQ-resistant P. falciparum. The combination of mutant pfcrtT76 and pfmdr1Y86 alleles may be useful markers for monitoring the development and spread of AQ resistance, when combining this drug with other antimalarials for treatment of malaria in Africa.

INTRODUCTION

The emergence and spread of parasites resistant to antimalarial drugs continues to be a major public health problem in the management of Plasmodium falciparum infections in many malaria-endemic countries. Resistance to chloroquine (CQ), the most widely used and affordable antimalarial drug, has contributed to increased mortality and morbidity caused by P. falciparum infections. Increasing CQ and sulfadoxine-pyrimethamine resistance in P. falciparum has led to renewed search for alternative effective drugs and efforts to counter the problems of resistance to these two major antimalarial drugs. Many malaria-endemic countries in Africa are currently facing the crucial issue of switching drug regimens. There is an increasing acceptance that the ideal approach to antimalarial treatment is the use of combination of two or more drugs, rather than a single antimalarial drug, preferably with artemisinin derivative as one of the drugs. Amodiaquine (AQ) in combination with artesunate (AS) has been introduced as first-line treatment of malaria to replace CQ in Nigeria and other malaria-endemic countries of Africa. Although the role of AS in this combination is to prevent the development of AQ resistance, parasites may quickly develop resistance to AQ in areas where extensive CQ resistance has been documented. In addition, little is known about the mechanism or epidemiology of AQ resistance. Cross-resistance between CQ and AQ has been reported both in vitro and in vivo. Furthermore, increasing use of AQ will increase emergence of P. falciparum strains with reduced sensitivity to this drug. Therefore, a tool to monitor the development and spread of resistance to AQ is greatly needed.

Despite reports of cross-resistance between CQ and AQ, the molecular mechanisms of AQ resistance and the role of mutations in the P. falciparum chloroquine resistance transporter (pfcrt) or P. falciparum multidrug resistance 1 (pfmdr1) genes remain unclear. The similarity in the chemical structures of CQ and AQ and their likely common mode of action is suggestive that molecular markers of CQ resistance may be useful for detection of parasites resistant to AQ. Two parasite proteins associated with the lysosomal membrane, Pgh1 (the protein product of the pfmdr1 gene) and PFCRT (the protein product of the pfcrt gene), are thought to be important to the process of CQ resistance. Point mutations in two P. falciparum genes encoding these proteins have been associated with in vitro CQ resistance in laboratory lines or field isolates, and have been used as molecular markers of CQ resistance in vivo in many malaria-endemic countries. In this study, the association between pfcrtT76 and pfmdr1Y86 alleles and AQ treatment outcome in children with acute uncomplicated P. falciparum malaria was investigated in Ibadan in southwestern Nigeria. We showed that the combination of mutant pfcrtT76 and pfmdr1Y86 alleles was associated with in vivo AQ resistance and that this association was age dependent. In addition, analysis of post-treatment samples obtained from patients who failed therapy showed that AQ selects parasites with pfcrtT76 and pfmdr1 alleles. Furthermore, we explored the possibility of using these markers as tools for monitoring the emergence and spread of resistance to AQ in countries that have introduced AQ in combination with other antimalarials as first-line treatment of malaria.

* Address correspondence to C. T. Happi, Malaria Research Laboratories IMRAT, College of Medicine, University of Ibadan, Nigeria. E-mail: christianhappi@hotmail.com or chappi@hsph.harvard.edu
METHODS

Study area. The study was carried out at the Malaria Research Laboratories, College of Medicine, University of Ibadan, Nigeria, from April to September 2005. Malaria in Ibadan is hyperendemic and transmission occurs year round, but is more intense from April to October during the rainy season.

The protocol for the study was reviewed and approved by the Joint University of Ibadan/University College Hospital Institutional Review Committee at the University of Ibadan, Nigeria and the Harvard School of Public Health Human Subject Committee. Documented informed consent was obtained from parents and guardians.

Patient treatment and follow-up. Children were eligible to join the study if they were ≤12 years of age, had symptoms of acute uncomplicated malaria, with pure *P. falciparum* parasitemia between 2,000 and 500,000 asexual forms/µL of blood, a temperature ≥37.5°C or recent history of fever, absence of other concomitant illness, no history of antimalarial use in the two weeks preceding presentation, negative urine test results for antimalarial drugs (Dill-Glazko and Ligin), and written informed consent given by parents or guardians. Patients with severe malaria,35 severe malnutrition, serious underlying diseases (renal, cardiac, or hepatic), and known allergy to study drugs were excluded from the study. The disease history was recorded by asking patients or their parents when the present symptomatic period had started, and was followed by a full physical examination.

Enrolled children were treated with standard regimen of AQ (30 mg/kg of body weight over a three-day period). The drug was given orally in the clinic and all patients were observed for at least 1 hour after drug administration to ensure the drug was not vomited. Children who vomited the medication were excluded from the study. If necessary, children were provided with antipyretics (paracetamol tablets, 10–15 mg/kg every 8 hours for 24–48 hours). A physician controlled drug administration. Follow-up with clinical and parasitologic evaluation was done daily for seven days (days 1–7) and then on days 14, 21, and 28. Thick and thin blood films prepared from a finger prick were stained with Giemsa and examined by light microscopy under an oil-immersion objective at 1,000× magnification by two independent assessors. Parasitemia (axexual or sexual) in thick films was estimated by counting asexual or sexual parasites relative to 1,000 leukocytes. From this figure, the parasite density was calculated assuming a leukocyte count of 8,000/µL of blood. Two drops of blood was also blotted onto 3MM filter paper (Whatman International Ltd., Maidstone, United Kingdom) for extraction and analysis of parasitic DNA.

Classification of responses to treatment was done according to World Health Organization criteria.6 The cure rate on day 28 of follow-up was defined as the percentage of children who remained free of parasitemia. Treatment failures rates were corrected by merozoite surface protein 2 (*msp-2*) genotyping of parasites at enrollment and recrudescence of infections.33,37 Children who failed treatment (within 14 days) with AQ were re-treated with artemether (9.6 mg/kg over a five-day period and were regarded as treatment failures). Children were re-treated whenever they became symptomatic (usually between 14 and 21 days after initial enrollment).

Extraction of DNA from samples collected on filter paper. Parasite genomic DNA was extracted from blood samples collected on filter paper using the Chelex extraction method as described by Plowe and others.38 Part of the DNA extracted from each sample was used immediately for polymerase chain reaction (PCR) and the rest was stored at −20°C.

Amplification of *P. falciparum* genes and characterization of parasites population in infected patients. The regions of the *pfcrtr* and *pfmdr*-1 genes surrounding the polymorphisms of interest (in the smear-positive samples collected pre-treatment and post-treatment) were amplified by nested PCR and then subjected to restriction fragment length polymorphism (RFLP) analysis as previously described.32,33 In addition, each *P. falciparum* infection in this study was characterized on the basis of *msp-2* polymorphism performed on paired pre-treatment and post-treatment parasites samples obtained from patients.33,37 This genetic marker was chosen because a recent report33 from the same study site has demonstrated that *msp-2* is the best and most reliable marker to evaluate diversity and complexity of *P. falciparum* infections in both pre-treatment and post-treatment isolates because it showed more clones than other markers (*msp-1* or glutamate-rich protein). Thus, *msp-2* analyses of paired pre-treatment and post-treatment parasites were used to distinguish true treatment failures from new infections. The complexity of infection was calculated as the average number of distinct fragments of *FC27 and ICI/3D7* per PCR-positive sample.33,37

Statistical analysis. For a best assessment of genetically determined parasite phenotypes, only parasitologic treatment failures were used to define AQ resistance. For analysis purposes, each isolate was coded based on the presence or absence of a resistance-associated allele. For example, infections with mixed wild-type/mutant alleles of *pfcrtr* or *pfmdr1* were treated as mutants. Data were analyzed using the statistical programs SPSS for Windows version 10.01 (SPSS, Chicago, IL) and Epi-Info version 6.4 (Centers for Disease Control and Prevention, Atlanta, GA). For univariate analysis, frequencies were compared by calculating chi-square values with Yates’ correction and Fisher’s exact tests. Normally distributed, continuous data were compared by Student’s *t*-tests and analysis of variance. Paired samples were compared using the paired *t*-test. Data not conforming to a normal distribution were compared by the Mann-Whitney U test (or Wilcoxon ranked sum test) and the Kruskal-Wallis test. Multiple logistic regression analysis was performed to assess the weight of all variables on treatment outcome and clearance of resistant parasites. All tests of significance were two-tailed. *P* values <0.05 indicated statistical significance.

RESULTS

Patient treatment outcomes. A total of 106 children with acute uncomplicated malaria were enrolled into the study and treated with AQ. Characteristics and demographic data of the children are shown in Table 1. Of these children, 101 (95%) successfully completed the 28-day follow-up. Data from the remaining 5% of the patients who did not complete the 28-day follow-up were excluded from analysis. Treatment failures were confirmed by *msp-2* genotyping. Overall, 87% (88 of 101) of the patients were cured by AQ, and 13% (13 of 101) of the patients failed AQ treatment. Resistance levels in all
Plasmodium falciparum msp-2 and pfcrt mutations in 16 isolates collected at enrollment and amodiaquine at codon 76 (n = 54 (53.5) ± 38.14 ± 1.16 pfcrt locus. Presence of multidrug-resistance 1; pfcrtT76FC27pfcrtT76pfmdr1Y86/H11505 and pfcrtT76 and pfmdr1 allelic families of P. falciparum mutations evaluated are shown in Table 2. The prevalence of mutant pfcrtT76 (62%) allele was higher than that of the mutant pfmdr1Y86 (29%) allele. However, mixed pfmdr1Y86 + pfmdr1N86 (28%) or pfcrtT76 + pfcrT76 (16%) alleles were common in many samples. The prevalence of both mutant pfcrtT76 and pfmdr1Y86 alleles (46%) was also evaluated in samples collected before drug treatment (Table 2).

Association between pfcrt and pfmdr1 point mutations and failure to treatment with AQ. To test our hypothesis whether allelic variations in P. falciparum pfcrt or pfmdr1 are associated with outcome of patients treated with AQ, the presence of single pfcrtT76 or pfmdr1Y86 or the combination of these two mutations (pfcrtT76 + pfmdr1Y86) in samples collected prior to treatment with AQ were examined for their association with patients treatment outcome. Each isolate was coded based on the presence or absence of a resistance-associated allele. For example, infections with mixed wild-type/mutant alleles were treated as mutant. The mutant pfcrtT76 (P = 0.065) allele was not independently associated with AQ treatment failure (Table 3). The mutant pfmdr1Y86 allele was weakly associated (odds ratio [OR] = 5.02, 95% confidence interval [CI] = 1.051–23.98, P = 0.036) with AQ treatment failure. However, when the relationship between the combination pfcrtT76 + pfmdr1Y86 alleles and AQ treatment failure was investigated, a stronger association was observed between

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Treatment outcome (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>pfcrt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T76</td>
<td>13/13 (100)</td>
<td>67/88 (76)</td>
<td>1.31</td>
</tr>
<tr>
<td>K76</td>
<td>0/13 (0)</td>
<td>21/88 (24)</td>
<td>(1.16–1.47)</td>
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<tr>
<td>pfmdr1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y86</td>
<td>11/13 (85)</td>
<td>46/88 (52)</td>
<td>5.022</td>
</tr>
<tr>
<td>N86</td>
<td>2/13 (15)</td>
<td>42/88 (48)</td>
<td>(1.051–23.98)</td>
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<tr>
<td>pfmdr1 and pfcrt</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Y86</td>
<td>11/13 (85)</td>
<td>37/88 (42)</td>
<td>7.58</td>
</tr>
<tr>
<td>pfcrtT76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ pfcrT76</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pfcrtN86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ pfcrT76</td>
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</table>

† Treatment failure was based on the World Health Organization 28-day drug efficacy test and monitoring and corrected by merozoite surface protein 2 genotyping of infections. All infections with mixed wild-type/mutant alleles of the Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene or P. falciparum multidrug resistance 1 (pfmdr1) gene were treated as mutants. OR = odds ratio; CI = confidence interval.

1 Statistically significant.
between children infected with parasites harboring the combination of mutant pfcrT76 and pfmdr1Y86 (OR = 7.58, 95% CI = 1.58–36.25, P = 0.006) (Table 3). To further examine the role of these mutations on AQ treatment failure, we compare the prevalence of mutations in baseline samples (samples obtained from all patients) and post-treatment samples obtained from patients who failed treatment. The analysis showed that the pfcrT76 allele (P = 0.067) was not specifically selected by AQ treatment, while the mutant pfmdr1Y86 (χ² = 14.44, P = 0.00024) and the combination of mutant pfcrT76 and pfmdr1Y86 (χ² = 10.08, P = 0.0015) were strongly selected by the drug (Table 4).

Impact of age and pfcrT76/pfmdr1 mutations on patient treatment outcome. The effect of age on the association between pfcrT76 and pfmdr1 mutations and treatment outcome was evaluated in patient samples from two different age groups (≤ 5 years of age and > 5 years of age). Only 9 (9%) of 101 samples obtained at enrollment prior to treatment had no mutation in both genes, and all patients with these infections were cured with AQ regardless of the age category. The associations between the presence of both mutant pfmdr1Y86 and pfcrT76 alleles and treatment failures was significant (OR = 10.95% CI = 1.07–93.43, P = 0.035) only in children ≤ 5 years of age and not in older children (> 5 years of age) (OR = 2.26, 95% CI = 1.33–3.92, P = 0.405).

Pfcrt/pfmdr1 mutants and parasites clearance. The role of some patient characteristics on the ability to clear the infections with pfcrT76/pfmdr1 mutant parasites was evaluated by analyzing the potential association between parasites clearance rates, age, parasites density or fever (body temperature ≥ 37.5°C) at enrollment as described previously by Djimde and others.39 Univariate analysis showed that the clearance of pfcrT76 and pfmdr1Y86 mutants parasites by patients was significantly associated with age (P = 0.0017), and fever at enrollment (χ² = 7.36, P = 0.0066) (Table 5). There was no association between pfcrT76/pfmdr1 mutant parasites clearance and parasite density (P = 0.7). However, when logistic regression analysis was performed to confirm the weight of univariate analysis results (age and fever at enrollment), only age was independently associated with the clearance of the resistant phenotype (P = 0.012). Children ≤ 5 years of age showed the lowest rate (55%, n = 11) of clearance of resistant parasites compared with much older children who showed a higher rate (84%, n = 26) of clearance of pfcrT76/pfmdr1 mutant parasites.

DISCUSSION

Overall, the current study shows an increase in resistance to AQ from 4.8%11 to 13% in Nigeria between 2001 and 2005. Data from the study also showed a selection of mutant pfcrT76 and pfmdr1Y86 alleles by AQ in children who failed treatment. More importantly, the present study demonstrated the association between the presence of both mutant pfcrT76 and pfmdr1 in combination and in vivo AQ resistance and the important role of age (the best surrogate marker for immunity) in clearing AQ-resistant parasites in an area of intense malaria transmission in west Africa.

The data from this study show an increase in rate of resistance to AQ in the study area. This increase might have been exacerbated by the extensive use of CQ in Nigeria. Cross-resistance between CQ and AQ has been observed in the study area10 and other disease-endemic areas.40 The increased in vivo AQ resistance in Nigeria observed in this study is of great concern because this drug is currently being used in this country in combination with AS for the treatment of acute uncomplicated malaria. A study in the Gambia has shown using reverse transcriptase-PCR that low density subpopulations of asexual parasites can escape short-acting drugs such as AS. Resistant parasites then likely to recrudesce un-

### Table 4

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Prevalence of point mutations in patient samples (%)</th>
<th>χ²</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Recrudescence</td>
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<tr>
<td>pfmdr1 codon 86</td>
<td></td>
<td></td>
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<tr>
<td>Wild-type N86</td>
<td>43% (44/101)</td>
<td>8% (1/13)</td>
<td>5.91</td>
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<tr>
<td>Mutant Y86</td>
<td>29% (29/101)</td>
<td>84% (11/13)</td>
<td>13.44</td>
</tr>
<tr>
<td>Mixed N86 + Y86</td>
<td>28% (28/101)</td>
<td>8% (1/13)</td>
<td>1.49</td>
</tr>
<tr>
<td>pfcr codon 76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type K76</td>
<td>22% (22/101)</td>
<td>0% (0/13)</td>
<td>2.25</td>
</tr>
<tr>
<td>Mutant T76</td>
<td>62% (63/101)</td>
<td>92% (12/13)</td>
<td>3.31</td>
</tr>
<tr>
<td>Mixed K76 + T76</td>
<td>16% (16/101)</td>
<td>8% (1/13)</td>
<td>0.13</td>
</tr>
<tr>
<td>pfmdr1 codon 86 + pfcr codon 76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N86 + K76</td>
<td>12% (12/101)</td>
<td>0% (0/13)</td>
<td>0.70</td>
</tr>
<tr>
<td>Y86 + T76</td>
<td>46% (47/101)</td>
<td>92% (12/13)</td>
<td>10.08</td>
</tr>
<tr>
<td>Mixed (N86 + T76/Y86 + K76)</td>
<td>42% (41/101)</td>
<td>8% (1/13)</td>
<td>4.28</td>
</tr>
</tbody>
</table>

*For definitions of abbreviations, see Table 2.
†Statistically significant.
under the selective force of the second drug in the combination and be transmitted to mosquitoes.\textsuperscript{41} Therefore, we cannot rule out the possibility of increasing selection of AQ-resistant parasites with the increasing use of AQ in combination with artemisinin in the future in Nigeria.

Molecular methods that detect genetic markers of drug resistance are potentially powerful tools for tracking drug-resistant malaria. In this study, the combination of \textit{pfcrt} and \textit{pfmdr1} mutations in isolates obtained from children prior to treatment was associated with \textit{in vivo} AQ resistance during a 28-day clinical efficacy trial. Mutant \textit{pfcrtT76} and \textit{pfmdr1Y86} alleles were observed in 62% and 29% of the samples, respectively. The mutant \textit{pfcrtT76} allele was not a useful predictor of clinical outcome, and the mutant \textit{pfmdr1Y86} allele weakly predicted patients' clinical outcome. The data from this study are different from a previous study in Sudan,\textsuperscript{32} which found that the mutant \textit{pfcrtT76} allele is associated with AQ treatment failure. This may be explained by differences in the epidemiology of CQ resistance between the two study sites and the high prevalence of the \textit{pfcrtT76} allele observed in Ibadan in southwestern Nigeria.

The high prevalence of the mutant \textit{pfcrtT76} allele (62%) observed in Ibadan, Nigeria confirms recent reports of the high prevalence rate of this allele in parasites obtained from same study site,\textsuperscript{32,33} and is also consistent with rates ranging from 60% to 100% reported in other malaria-endemic regions.\textsuperscript{25,29–31,34} However, analysis of baseline samples (samples collected from all patients at enrollment) and post-treatment samples from patients who failed AQ treatment showed the selection of parasites with mutant \textit{pfcrtT76} or \textit{pfmdr1Y86} or both mutant alleles by the drug (Table 4), although this selection was significant only for parasites with the mutant \textit{pfmdr1Y86} allele or both mutant \textit{pfcrtT76} and \textit{pfmdr1Y86} alleles. This selection process was further confirmed by \textit{msp-2} analysis, which showed a reduction in the average number of distinct clones of \textit{P. falciparum} per infection from 4.38 at enrollment to 2.23 at recrudescence.

The non-significant selection of the mutant \textit{pfcrtT76} by AQ may be due to the high prevalence of this allele in the \textit{P. falciparum} population from Ibadan, Nigeria.\textsuperscript{32} Selection of \textit{pfmdr1Y86} by AQ has also been reported previously in the Gambia\textsuperscript{19} and Kenya.\textsuperscript{43} Although the importance of point mutations in \textit{pfcrt} in producing CQ resistance is beyond dispute,\textsuperscript{44–47} recent transfection studies of \textit{pfcrt} have shown that isolates expressing the mutant \textit{pfcrtT76} allele retain sensitivity to AQ while showing a reduced susceptibility to monodeethyl AQ, the active metabolite of AQ.\textsuperscript{44} Therefore, it is possible that during treatment with AQ, monodeethyl AQ may be the driving force behind the selection of the mutant \textit{pfmdr1Y86} or \textit{pfcrtT76} alleles or the combination of these mutant alleles as observed in post-treatment samples of patients who failed treatment. The selection of the mutant \textit{pfcrtT76} and \textit{pfmdr1Y86} alleles indicates the primary involvement of these two genes in the mediation of AQ resistance. Most importantly, the absence of the wild-type \textit{pfcrtK76} allele in post-treatment samples of patients who failed treatment (Table 4) may indicate the critical role of the mutant \textit{pfcrtT76} allele in AQ resistance. Thus, similar to CQ resistance, AQ resistance in \textit{P. falciparum} may depend primarily on mutation(s) in \textit{pfcrt} and additional mutations in \textit{pfmdr1} or other \textit{Plasmodium} genes may also have significant roles in increasing resistance to the drug.

The combination of \textit{pfcrtT76} and \textit{pfmdr1Y86} mutations was associated with AQ treatment failure. These two alleles have been shown to be in linkage disequilibrium in CQ-resistant isolates of \textit{P. falciparum} from The Gambia\textsuperscript{38} and Nigeria\textsuperscript{49} (Happi CT and others, unpublished data). The similarity in the chemical structures of CQ and AQ\textsuperscript{13} and their likely common mode of action\textsuperscript{13,14} suggests that the molecular basis of resistance to these two drugs may be similar. These previous findings may explain the strong association ($P = 0.006$) between the combination of \textit{pfcrtT76} and \textit{pfmdr1Y86} mutant alleles and \textit{in vivo} AQ failure observed in this study, although, age-stratified analysis of the effect of these two mutations on treatment outcome did not show a significant association with treatment failure in children more than five years of age. One possible explanation for this finding is the immunopotentiating of AQ efficacy by the hosts. Previous studies have shown that in areas of moderate or high malaria transmission, drugs with suboptimal efficacy could cure infections in patients (>5 years of age) who have developed a degree of antiparasitic immunity to malaria (due to repeated exposure to various circulating drug-sensitive and drug-resistant strains of parasites).\textsuperscript{36,50,52}

The ability of some children in this study to clear infections with parasites with both \textit{pfcrtT76} and \textit{pfmdr1Y86} mutations was strongly associated with age (> 5 years) and fever at enrollment. The association between fever at enrollment and clearance of drug-resistant parasites is most likely related to an appropriate cytokine response (i.e., tumor necrosis factor-$\alpha$, interferon-$\gamma$) in the children clearing peripheral parasitemia. However, the synergy between the appropriate fever response of the host and drug treatment is unclear in light of this finding. The data from this study further show that children less than five years of age are highly vulnerable to malaria and cannot clear their parasites as efficiently as other age groups (Table 5). These data are similar to those in a previous report from Mali.\textsuperscript{39}

Overall, the findings from this study have significant bearings and implications on drug discovery and public health in Nigeria where AQ is combined with other antimalarial drugs (including artemisinin derivatives) for treatment of acute uncomplicated malaria. First, the evidence of selection of mutant \textit{pfcrtT76} and \textit{pfmdr1Y86} alleles by AQ may provide some insight in the previously observed cross-resistance between CQ and AQ \textit{in vivo}.\textsuperscript{11,40} Second, mutant \textit{pfcrtT76} and \textit{pfmdr1Y86} alleles currently used as molecular markers of CQ resistance can also be useful for monitoring the spread of AQ resistance in areas of low resistance to the drug such as west Africa. Third, clearance of the AQ-resistant phenotype by children less than five years of age suggests that for countries that have not yet changed their antimalarial drug policy, changes aimed at switching first-line treatment to drug combinations could be initially targeted to children less than five years of age, especially when the cost of these combinations is an obstacle to implementing the policy.

Ultimately, there is a need to validate mutant \textit{pfcrtT76} and \textit{pfmdr1Y86} alleles as markers for AQ resistance as proposed in this study in other malaria-endemic areas. Such an approach is necessary because it may harness the efforts aimed at the management and control of drug-resistant malaria.

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Authors’ addresses: C. T. Happi, Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115. G. O. Gbotohso, O. A. Folarin, O. M. Bolaji, and A. Sowunmi, Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria. D. E. Kyle and W. Milhous, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Springs, MD 20910. D. F. Wirth, Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115. A. M. J. Oduola, Special Program for Research and Training in Tropical Diseases, World Health Organization, Geneva, Switzerland.

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