Rapid Selection of *Plasmodium falciparum* Chloroquine Resistance Transporter Gene and Multidrug Resistance Gene-1 Haplotypes Associated with Past Chloroquine and Present Artemether-Lumefantrine Use in Inhambane District, Southern Mozambique

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Abstract. Chloroquine (CQ) use in Mozambique was stopped in 2002 and artemether-lumefantrine (AL) was implemented in 2008. In light of no use of CQ and extensive use of AL, we determined the frequency of molecular markers of *Plasmodium falciparum* drug resistance/tolerance to CQ and AL in persons living in Linga-Linga, an isolated peninsula and in Furvela village, which is located 8 km inland. The *P. falciparum* chloroquine resistance transporter gene CVMNK wild type increased in frequency from 43.9% in 2009 to 66.4% in 2010 ($P \leq 0.001$), and combined *P. falciparum* multidrug resistance gene 1 N86-184F-D1246 haplotype increased significantly between years ($P = 0.039$). The combination of *P. falciparum* chloroquine resistance transporter gene CVMNK and *P. falciparum* multidrug resistance gene NFD increased from 24.3% (2009) to 45.3% in (2010, $P = 0.017$). The rapid changes observed may largely be caused by decreased use of CQ and large-scale use of AL. In the absence of a clear AL-resistance marker and the (almost) continent-wide use of AL in sub-Saharan Africa, and when considering CQ reintroduction, continued monitoring of these markers is needed.

INTRODUCTION

Malaria remains one of the major killers in the tropical and sub-tropical world today, even though recent years have shown progress in its control. The World Health Organization estimated a decrease in malaria cases from 244 million to 216 million during 2005–and 2010, and estimated mortality decreased from 781,000 in 2009 to 655,000 in 2010.[1,2] This significant decrease in malaria-associated morbidity and mortality is largely attributed to large-scale malaria control efforts such as distribution of insecticide-treated nets, indoor residual spraying, intermittent preventive treatment in vulnerable groups, and implementation of highly efficacious artemisinin-based combination therapies (ACT) for the treatment of uncomplicated *Plasmodium falciparum* malaria in most malaria-endemic countries. The large-scale improvements are highly dependent on continued reliability of efficacious ACTs.

However, *P. falciparum* ACT resistance has emerged along the Thailand-Cambodia and Thailand-Myanmar borders,[3–6] and it might eventually be found in Africa, as happened with chloroquine (CQ) and sulfadoxine-pyrimethamine (SP).[7–9] Resistance to CQ in malaria-endemic Africa became as prevalent as malaria and the drug has not been officially used for several years in most malaria-endemic countries. Depending on fitness costs in the parasites associated with acquired drug resistance, the latent period without a certain drug pressure may result in the reemergence of drug-sensitive *P. falciparum* parasites.[10]

Resistance to CQ is mainly associated with a single nucleotide polymorphism (SNP) in the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene, resulting in an amino acid change from threonine to lysine mutation at codon 76 (K76T).[11,12] There are three main haplotypes in codons 72–76 of the *Pfcrt* gene, resulting in wild type CVMNK and CQ-resistant haplotypes CVIET and SVMNT.[13] In Africa, the CVIET haplotype is the dominant mutant haplotype.[14]

By monitoring the temporal prevalence of *Pfcrt* K76, Kublin and others showed the reemergence of fully CQ-sensitive parasite populations after several years since cessation of CQ use in Malawi.[15] Since this study, studies in Tanzania,[16] Kenya,[17] Senegal,[18] and Mozambique[19] have shown similar trends of reemergence of CQ sensitivity, and it is tempting to consider reintroduction of CQ in combination with another antimalarial drug in areas where CQ resistance has decreased and possibly reserved for malaria treatment of targeted populations, such as pregnant women, as has been suggested by others.[20]

Another marker of antimalarial drug resistance is the *P. falciparum* multidrug resistance gene-1 (*Pfmdr-1*) implicated in resistance/tolerance to almost all antimalarial drugs including CQ, amodiaquine (AQ) and most importantly, the artemisinins. It has recently been shown that certain combinations of SNPs in the *Pfmdr-1* gene, mainly at codons 86, 184, and 1246, are emerging in areas where the ACT drug combination artemether-lumefantrine (AL) is being widely used[21,22] and suggested that certain *Pfmdr-1* haplotypes may be markers of emergence of ACT tolerance.[23]

The Ministry of Health of Mozambique introduced SP-AQ in late 2002 to replace CQ monotherapy as first-line treatment against uncomplicated malaria.[24] In 2006, this combination was replaced with the ACT combination artesunate–SP. However, already in 2008, the policy was changed to AL because of widespread SP resistance in the country.[19]

Chloroquine resistance in Mozambique was reported for the first time in 1983, followed by a number of studies reporting it throughout most of the country.[25,26] In 1999, before abandonment of CQ, a study found that the *Pfcrt* K76T mutation was prevalent in 90% of infected children in Mozambique.[27] In 2001–2002, a trial conducted in southern Mozambique estimated a clinical efficacy for CQ of only

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47%.[26] Another study conducted in the same district in 2002–2003 demonstrated a frequency of the mutant CVIET haplotype to be > 90%.[24] Since CQ was officially abandoned in 2002, the CQ drug pressure has most likely waned in subsequent years. However, since the 4-aminoquinoline analog AQ (combined with SP) replaced CQ, this may have ensured some level of sustained drug pressure.

In a recent report by Raman and others over a five-year period (2006–2010), the prevalence of the *Pfcrt* K76T mutation was determined in children living in Gaza Province in southern Mozambique.[19] Overall, there was a striking decrease in the prevalence of the K76T mutation from > 95% in the four zones of Gaza Province in 2006 to 17.5–37.3% in 2010. The study also examined the prevalence of SNPs in the *Pfmdr1*-gene, but only regarding the N86Y mutation, in which a reduction was observed from > 70% to 25.8–48.8%.[19] Other studies from Mozambique have, to the best of our knowledge, not assessed SNP prevalence changes in the *Pfmdr-1* gene. Therefore, temporal changes in selection of polymorphisms in this gene remains to be elucidated. This need is especially important in light of the suggested relationship between certain *Pfmdr-1* haplotypes and emergence of ACT tolerance. We therefore analyzed the distribution and investigated short-term temporal change of SNPs in the *Pfcrt* codons 72–76 and *Pfmdr-1* codons 86, 184, and 1246 in persons living in Linga Linga, an isolated peninsula of Mozambique and in the village of Furvela located 8 km inland from Linga Linga.

MATERIALS AND METHODS

Study site. The peninsula of Linga Linga (23°43′1.29″S, 35°24′15.04″E) is located in Inhambane District and 500 km north of Maputo and opposite the district capital of Morrumbene, which is 6 km west (across the Morrumbene Bay). The residents are mainly fishermen or involved in the artisanal manufacture of raffia baskets, hats and bags. Furvela Village is 8 km west of Linga Linga on the mainland. Furvela has approximately 4,500 inhabitants, and Linga Linga is somewhat smaller with approximately 1,000 inhabitants. At the onset of the study in 2007, there was no health center on the peninsula proper, but one was established in 2009. Otherwise, the nearest health centers were situated in the village of Coche, 5 km north of Linga Linga, or in Morrumbene. The project received ethical clearance from the National Bioethics Committee of Mozambique (reference 123/CNBS/06) on August 2, 2006.

Sample collection and preparation. After an initial census, an all-age malaria prevalence survey was performed. Seven locations in Linga Linga based on local knowledge were chosen for establishment of the survey. At each location, residents were informed the day before the survey. In addition, a survey of school-age children was undertaken. After informed consent was obtained, survey teams collected cross-sectional samples from as many volunteers as possible, including small children whose parents consented. Blood samples were collected in March–April 2009 and April 2010 in the village of Linga Linga, and in May 2010 in the village of Furvela. A similar protocol was adopted in the latter village,[28–30] and five locations were used as sites for the survey.

Finger prick blood was used for preparation of thick and thin blood films and added to 1.5-mL Eppendorf tubes containing EDTA (Militom-14; VWR-Bie & Berntsen, Denmark). Blood samples were allowed to separate into serum and blood clot until clear separation was observed. Plasma was transferred into Eppendorf tubes and the blood clot was used for various molecular analyses of the parasites. Blood slides stained with 5% Giemsa for 20 minutes were read by technicians at the malaria reference laboratory in Maputo. Two hundred fields were examined before a slide was declared negative. Numbers of parasites per 500 leukocytes were counted and converted to densities per microliter of blood, assuming a density of 8,000 leukocytes/μL. Only blood slide–positive samples for *P. falciparum* were used for molecular analysis. The age of donors ranged from 1 to 79 years, and the degree of *P. falciparum* positivity varied markedly between years and when age groups were compared.

DNA extraction and SNP analysis of *Pfcrt* and *Pfmdr-1* genes. DNA was extracted by using the NucleoSpin Genomic DNA Bloodpure Kit (Macherey-Nagel, Düren, Germany). Extraction was performed according to the manufacturer's instructions.

Pfcrt genotyping was performed by using a nested polymerase chain reaction, followed by sequence-specific oligonucleotide probe (SSOP)–enzyme-linked immunosorbent assay as described.[31] A set of *P. falciparum* laboratory isolates were used for positive controls: 3D7 and HB3 as CVMNK controls, FCR3 and DD2 as CVIET controls, and 7G8 as an SVMNT control. Genotyping of *Pfmdr-1* SNPs was performed by using published polymerase chain reaction–restriction fragment length polymorphism protocols,[32,33] with minor modifications as described[23] and 3D7 (N86-Y184-D1246), FCR3 (86Y-Y184-1246D), DD2 (86F-184Y-1246D), and 7G8 (N86-184F-1246Y) used as positive controls. Blood donors from Denmark who were never exposed to malaria were used as *P. falciparum*-negative controls.

Statistical analysis. Statistical analysis was performed in 2×2 contingency tables, and chi-square test statistics or Fisher's exact test were applied when appropriate. For analysis of *Pfcrt* haplotypes, samples were considered to be mixed, but as containing a majority haplotype, when the optical density (OD) value of the weakly reacting *Pfcrt* SSOP was less than half the OD value of the strongly reacting *Pfcrt* SSOP. Conversely, if the OD value of the weakly reacting *Pfcrt* SSOP was higher than half the OD value of the strongly reacting *Pfcrt* SSOP, the infection was categorized as mixed with no dominant haplotype. To analyze for a possible temporal change in the frequency of the *Pfcrt* CVMNK haplotype, all infections containing CVMNK only or as the majority in mixed CVMNK/CVIET infections were tested against single CVIET infections. The analysis of temporal change in the prevalence of *Pfcrt* CVMNK haplotype were performed by comparing all infections containing CVMNK including all mixed CVMNK/CVIET haplotype infections against single CVIET infections.

Prevalence of *Pfmdr-1* SNPs was examined individually for codons 86, 184, and 1246 where the genotypes N86, 184F, and D1246 including mixed infections were compared against single 86Y, Y184, and 1246Y genotype infections, respectively. For frequency analysis, all mixed infections were omitted. Possible changes in frequency of constructed 86–184–1246 haplotypes were analyzed by excluding infections with one or more mixed genotype. Finally, analysis of the temporal frequency of constructed *Pfcrt-Pfmdr-1* haplotypes was performed by omitting all mixed *Pfmdr-1* infections and for

Pfcrt, mixed infections where a majority haplotype could not be determined.

RESULTS

Sample collection. In 2009 and 2010, of 435 and 385 samples collected from donors in Linga Linga, 159 (36.6%) and 108 (28.1%) were *P. falciparum* positive by microscopy, respectively. In addition, 336 samples were collected in Furvela in 2010, of which 111 (33.0%) were *P. falciparum* positive by microscopy.

Frequency and prevalence of codon72–76 haplotypes of the *Pfcrt* gene in study sites of Mozambique in 2009–2010. Of the *P. falciparum*-positive sample set, 136 (85.5%) and 195 (91.1%) samples were successfully haplotyped at codon 72–76 of the *Pfcrt* gene in samples from 2009 (Linga Linga only) and 2010 (Linga Linga, n = 97 and Furvela, n = 98), respectively. For the 2010 samples, no significant difference in frequency and prevalence of the *Pfcrt* haplotypes between Linga Linga and Furvela was observed ($\chi^2 = 0.01$, $P = 0.91$ and $\chi^2 = 1.07$, $P = 0.30$ for comparison of frequency and prevalence, respectively), wherefore the samples from the two villages were pooled.

The frequency of *P. falciparum* infections carrying the *Pfcrt* wild type CVMNK haplotype (including mixed CVMNK/CVIET infections in which CVMNK was the majority haplotype) versus mutant CVIET haplotype infections showed a significant increase of CVMNK haplotype from 43.9% in 2009 to 66.4% in 2010 ($\chi^2 = 13.1$, $P \leq 0.001$) (Figure 1A). Likewise, the prevalence of infections carrying the *Pfcrt* wild type CVMNK haplotype including mixed CVMNK/CVIET infections versus pure mutant CVIET haplotype infections increased significantly from 60.0% in 2009 to 74.9% in 2010 ($\chi^2 = 8.02$, $P = 0.005$) (Figure 1B).

Prevalence and frequency of SNPs at codons 86, 184, and 1246 of the *Pfmdr-1* gene. The temporal prevalence of SNPs at codons 86, 184, and 1246 was analyzed by comparing the distribution from 2009 and 2010 for codons 86 and 184 (Figure 2A and B). Except for codon 184 (see below), the data from Linga Linga and Furvela in 2010 were pooled because of a lack of significance between the settings. Prevalence of *P. falciparum* infections carrying the N86 wild type (including mixed 86N/Y infections) increased significantly from 64.7% in 2009 to 84.1% in 2010 ($\chi^2 = 16.3$, $P \leq 0.001$), and prevalence of the D1246 wild type genotype remained > 98% ($\chi^2 = 0.163$, $P = 0.67$). For the 184F mutant type (including mixed 184F/Y infections), the prevalence was 21.5% in Linga Linga in 2009, which increased to 34.3% in 2010 ($\chi^2 = 4.41$, $P = 0.036$) and to 51.0% in Furvela.

The frequency of the *Pfmdr-1* genotypes (disregarding mixed genotype infections) from Linga Linga and Furvela in 2010 was pooled because data was not significant between the settings. The frequency of the N86 wild type increased significantly from 52.9% to 73.0% ($\chi^2 = 8.93$, $P = 0.003$), whereas for the 184F mutant type, only an insignificant increase from 18.5% to 26.3% was seen ($\chi^2 = 2.07$, $P = 0.150$), and no change was observed in D1246, which remained stable at 98% between the years ($\chi^2 = 0.161$, $P = 0.688$).

Frequency of constructed haplotypes at codon 86, 184, and 1246 of the *Pfmdr-1* gene. The construction of *Pfmdr-1* 86–184–1246 haplotypes (excluding mixed SNPs at one or more codons) showed several different haplotypes and tem-
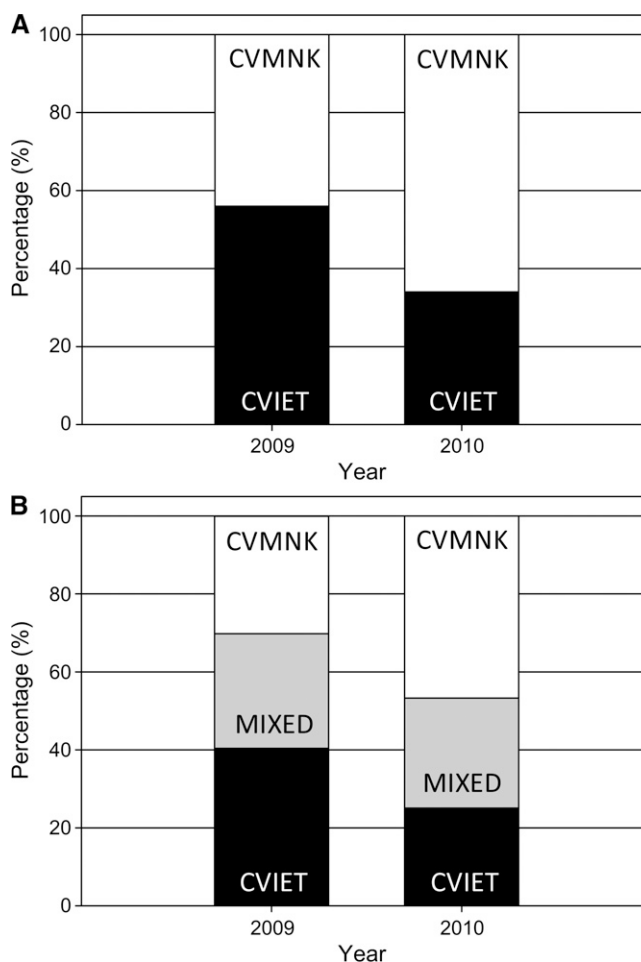

FIGURE 1. **A**, Frequency and **B**, Prevalence of *Plasmodium falciparum* chloroquine resistance transporter gene codon 72–76 haplotypes, CVMNK (wild type) and CVIET (mutant type) in Linga Linga (2009) and Linga Linga with Furvela village (2010) of Mozambique.

poral changes in the distribution (Figure 2C). The frequency of the single mutant 86Y–Y184–D1246 (YYD) haplotype decreased significantly from 47.8% in 2009 to 24.5% in 2010 ($\chi^2 = 10.32$, $P = 0.001$), whereas the frequency of the single mutant NFD haplotype increased significantly between the years ($\chi^2 = 4.27$, $P = 0.039$).

Frequency of combined *Pfcrt-Pfmdr-1* haplotypes. The *Pfcrt* haplotypes (CVMNK or CVIET) were combined with the constructed *Pfmdr-1* haplotypes omitting samples that were mixed with no clear majority infection (for *Pfcrt*), or mixed or negative in one or more of the *Pfmdr-1* codons. Of the remaining 165 samples (2009: n = 76, 2010: n = 89), analysis showed a significant increase in infections carrying the *Pfcrt-Pfmdr-1* combination CVMNK-NFD from 24.3% in 2009 to 45.3% in 2010 ($\chi^2 = 5.66$, $P = 0.017$).

DISCUSSION

The use of CQ to treat uncomplicated malaria in Mozambique was officially abandoned in 2002. Most likely, as everywhere else in the malaria-endemic world where CQ has been replaced by other antimalarial drugs, some informal use of CQ has subsequently been ongoing because of the low price
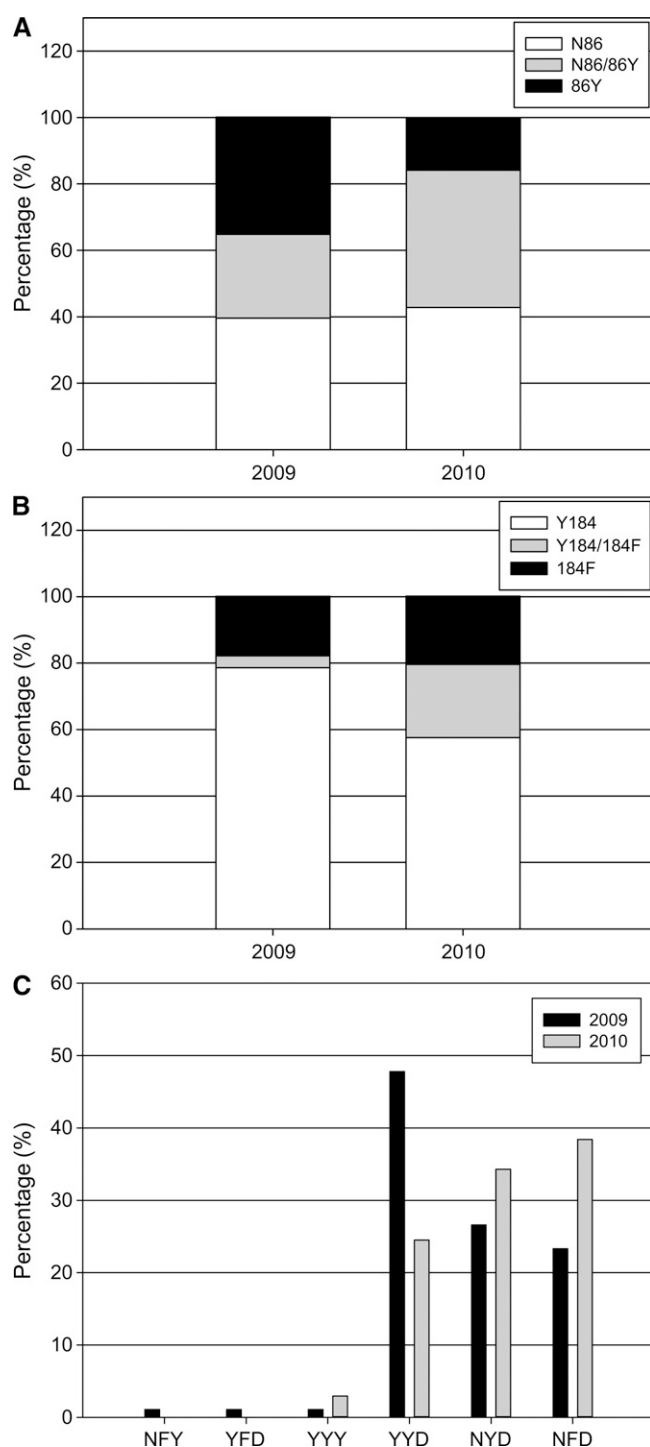
FIGURE 2.   Prevalence of *Plasmodium falciparum* multidrug resistance gene-1 codon 86 and 184 genotypes and frequency of the combined codon 86–184–1246 haplotypes in Linga Linga (2009) and Linga Linga with Furvela village (2010) of Mozambique. **A**, Prevalence of codon 86 genotypes. **B**, Prevalence of codon184 genotypes. **C**, Frequency of 86–184–1246 haplotypes (excluding mixed infections).

bly lower malaria prevalence, CQ drug pressure would decrease. Given that there are fitness costs for malaria parasites associated with CQ resistance,[34] it is expected that the prevalence of sensitive parasites *in vivo* will increase.

Although the validity of the *Pfcrt* 76T mutation as a predictive marker of CQ treatment failure remains doubtful because of confounding factors such as host immunity, monitoring the emergence of wild type *Pfcrt* 76K parasites in indigenous *P. falciparum* populations more adequately illustrates the temporal advancement of parasite sensitivity to CQ. A study in southern Mozambique in 2001 and 2003 reported frequencies of the mutant *Pfcrt* CVIET haplotype > 90% at the time of official abandonment of CQ.[24] In the present study, from a remote setting in Mozambique, the frequency of the *Pfcrt* CVMNK wild type increased from 44% to 66% within a single year. This finding is consistent with the recent study by Raman and others, in which the prevalence of the pure K76 wild type in the southern province of Gaza, Mozambique, increased from < 5% at baseline in 2006 to 65–80% in 2010.[19] Thus, both studies confirms the trend of a substantial increase in *P. falciparum* susceptibility to CQ in Mozambique, similarly to other studies conducted in other parts of the east African region such as Malawi,[15] Kenya,[35] and Tanzania.[16] However, recent studies in 2009–2010 in Mwanza, Tanzania, and Iganga, Uganda found a striking difference of 59.5% and 0% in the prevalence of *Pfcrt* CVMNK wild types, respectively.[36] Thus, the re-emergence of CQ susceptibility appears to evolve at different rates probably because of co-varying factors such as treatments given (also dependent on differences in diagnostic practices and transmission intensity) and the continued use of CQ and/or related drugs maintaining the drug pressure on *Pfcrt*, e.g., amodiaquine. In adition, the ACT drug combination AL has been shown to select for *Pfcrt* wild types.[37] Therefore, large-scale implementation of AL as first-line treatment in most sub-Saharan countries may as well facilitate re-emergence of CQ sensitivity in *P. falciparum*.

In this study we also describe a selection of N86 and 184F and the combined N86–184F–D1246 *Pfmdr-1* haplotype NFD. This finding is similar to our previous findings in Tanzania, where the N86 and 184F prevalence increased significantly over a five-year period.[23] Recently, Baliraine and Rosenthal determined the prevalence of single *Pfmdr-1* N86, 184F, and D1246 and combined NFD haplotype before either AL, artesunate-AQ, or AQ + SP treatment, and compared with prevalence up to 120 days after treatment in primarily new infections.[38] Only in the AL group, the prevalence of the N86, 184F and D1246, but as well the NFD haplotype were much higher compared with pre-treatment prevalence[38] indicating a survival advantage of these parasites. This finding does not indicate an immediate potential risk of clinical failures after AL treatment; AL still remains highly efficacious in Africa. However, the NFD haplotype in particular may be considered as a marker of increased tolerance to AL.

When combining the *Pfcrt* and the *Pfmdr-1* haplotypes, the present study showed a strong selection of the *Pfcrt-Pfmdr-1* CVMNK-NFD haplotype. This finding might be caused by decreased use of CQ. However, we propose that selection of this particular haplotype is as well largely a consequence of large-scale AL use. Baring in mind the small scale of this study and several confounding factors such as impact of other drugs, our findings are only indicative. Therefore, there is a continued need and urgency to monitor these two markers in

of CQ and good fever compliance. Furthermore, in Mozambique, the analog 4-aminoquinoline amodiaquine combined with SP replaced CQ, for a few years, which might have impacted *Pfcrt* and *Pfmdr-1* haplotypes. However, because of improved malaria diagnostics such as the use of rapid diagnostic tests, and since 2008 better treatment options, e.g., ACTs, and possi-

the light of a possible reintroduction of CQ in combination with another drug or alone for vulnerable groups such as pregnant women and because of the (almost) African-wide use of AL, in the absence of a better molecular marker for AL resistance.

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