## Title

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# Selection of Drug Resistance-Mediating *Plasmodium falciparum* Genetic Polymorphisms by Seasonal Malaria Chemoprevention in Burkina Faso

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**Seasonal malaria chemoprevention (SMC), with regular use of amodiaquine plus sulfadoxine-pyrimethamine (AQ/SP) during the transmission season, is now a standard malaria control measure in the Sahel subregion of Africa. Another strategy under study is SMC with dihydroartemisinin plus piperaquine (DP). *Plasmodium falciparum* single nucleotide polymorphisms (SNPs) in *P. falciparum crt* (*pfcrt*), *pfmdr1*, *pfdhfr*, and *pfdhps* are associated with decreased response to aminoquinoline and antifolate antimalarials and are selected by use of these drugs. To characterize selection by SMC of key polymorphisms, we assessed 13 SNPs in *P. falciparum* isolated from children aged 3 to 59 months living in southwestern Burkina Faso and randomized to receive monthly DP or AQ/SP for 3 months in 2009. We compared SNP prevalence before the onset of SMC and 1 month after the third treatment in *P. falciparum* PCR-positive samples from 120 randomly selected children from each treatment arm and an additional 120 randomly selected children from a control group that did not receive SMC. The prevalence of relevant mutations was increased after SMC with AQ/SP. Significant selection was seen for *pfcrt* 76T (68.5% to 83.0%, $P = 0.04$), *pfdhfr* 59R (54.8% to 83.3%, $P = 0.0002$), and *pfdhfr* 108N (55.0% to 87.2%, $P = 0.0001$), with trends toward selection of *pfmdr1* 86Y, *pfdhfr* 51I, and *pfdhps* 437G. After SMC with DP, only borderline selection of wild-type *pfmdr1* D1246 (mutant; 7.7% to 0%, $P = 0.05$) was seen. In contrast to AQ/SP, SMC with DP did not clearly select for known resistance-mediating polymorphisms. SMC with AQ/SP, but not DP, may hasten the development of resistance to components of this regimen. (This study has been registered at ClinicalTrials.gov under registration no. NCT00941785.)**

Malaria remains one of the greatest causes of morbidity and mortality in African children (1). In many African countries, malaria transmission is markedly seasonal, with most of the disease burden occurring during a distinct rainy season. Among available malaria control methods, intermittent preventive therapy in children (IPTc) has been widely studied (2, 3), and a variant of this approach, seasonal malaria chemoprevention (SMC), with full-treatment regimens of amodiaquine plus sulfadoxine-pyrimethamine (AQ/SP) provided monthly to at-risk children during the transmission season, has shown excellent protective efficacy in West Africa (4–6). Based on these results, SMC was recently endorsed by the WHO for use in at-risk children 3 to 59 months of age in areas of seasonal malaria transmission across the Sahel subregion of Africa (7). However, SMC is not recommended elsewhere, even for areas with seasonal transmission, due to known high levels of resistance to AQ and SP in eastern and southern Africa. For these areas, there is currently no standard recommendation for IPTc or SMC.

One alternative for SMC is dihydroartemisinin-piperaquine (DP), an artemisinin-based combination therapy that has demonstrated excellent efficacy in the treatment of uncomplicated malaria in Asia (8, 9) and Africa (10, 11) and, for chemoprevention, benefits from the long half-life of piperaquine. When administered monthly, DP offered a protective efficacy of 98% against malaria in Thai adults (12). In Senegalese children, the incidences of malaria were the same with monthly DP and monthly AQ/SP, although protective efficacies could not be measured due to the lack of a control group (13). In Uganda, monthly DP administered without directly observed therapy to children 6 to 24 months of age had a protective efficacy against malaria of 58%, significantly greater than that of monthly SP or daily trimethoprim-sulfamethoxazole (14), and monthly DP administered to school children 6 to 14 years of age for 1 year had a protective efficacy of 96% (15). In Burkina Faso, intermittent preventive therapy with DP in children aged 3 to 59 months showed 77% protective efficacy compared to 83% for AQ/SP (I. Zongo, unpublished data). DP is thus of promise as an alternative to AQ/SP for SMC, especially if resistance to AQ or SP worsens in West Africa, and, more broadly, as an efficacious regimen for IPTc across Africa.

A concern with all malaria therapy, and in particular with chemoprevention, is the selection by therapy of parasites with decreased drug sensitivity. In the case of AQ/SP, mediators of drug resistance are well characterized. For AQ, resistance is mediated principally by the 76T mutation in *Plasmodium falciparum crt* (*pfcrt*) (16–18), which encodes a putative drug transporter, and is augmented by polymorphisms in a second predicted transporter gene, *pfmdr1*. The *pfmdr1* 86Y and 1246Y mutations are common

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in Africa and mediate decreased sensitivity to AQ (16, 19–21); the Y184F allele is also highly polymorphic but is of uncertain significance. Additional *pfmdr1* polymorphisms at positions 1034 and 1042 and amplification of the gene are common in other areas but rare in Africa. For SP, mutations in *pfdhfr* and *pfdhps*, the genes encoding the drug targets, mediate resistance to both components of the drug, with increasing numbers of mutations leading to increasing resistance (22–24). In much of Africa, a set of 5 mutations is common, leading to marked limitations on SP efficacy, but in parts of West Africa, one of these mutations, *pfdhps* 540E, is rare, and SP efficacy remains good (25, 26).

Considering this background, it is of concern that increasing use of AQ/SP for SMC will jeopardize the efficacy of this regimen. In contrast to resistance to AQ/SP, resistance to DP does not appear to be a problem in Africa, despite the fact that heavy piperaquine use in China some decades ago apparently led to widespread resistance (27). Further, unlike other antimalarials, including AQ/SP, DP has not selected for known resistance-mediating polymorphisms in recurrent infections soon after prior therapy in West Africa. Specifically, in infections occurring within 28 to 42 days of prior therapy in Burkina Faso, AQ/SP selected for mutations in *pfdhfr*, *pfdhps*, *pfcrt*, and *pfmdr1* associated with drug resistance, but DP did not select for any of these polymorphisms (20, 26, 28). However, in a recent study in East Africa, prior use of DP selected in recurrent infections for polymorphisms in *pfmdr1* that were also selected for by AQ/SP (29); the reasons for differences in selection between East and West Africa are unknown. To evaluate the relative selective pressures of AQ/SP and DP administered as SMC, we evaluated the prevalences of key resistance-mediating *P. falciparum* polymorphisms in parasites collected from children before the initiation of the intervention, from children a month after the completion of three monthly treatments, and from a control group of children not subject to the intervention.

## MATERIALS AND METHODS

**Study sites.** We conducted this study in three rural health centers (Satiri, Balla, and Kadomba), all located within ~10 km of each other in the district of Lena, 40 to 50 km north of Bobo-Dioulasso, the major city in southwestern Burkina Faso. Malaria transmission is holoendemic in the region, with a sharp peak in transmission during the rainy season (May to October) and with the peak malaria incidence from August to October.

**Study design and participants.** The clinical trial that provided samples for this analysis is to be described separately. Briefly, 1,500 children aged 3 to 59 months whose parents or guardians provided informed consent were randomized 1:1 to receive either monthly DP (Duocotecxin, Holley Cotec) (dihydroartemisinin at 2.1 mg/kg of body weight and piperaquine phosphate at 17 mg/kg administered once daily for 3 days) or monthly AQ (Camoquin; Parke-Davis) (10 mg/kg syrup administered once daily for 3 days) plus SP (Fansidar; Roche) (25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine administered as one dose) (AQ/SP) in August, September, and October 2009. All drug administrations were directly observed. Children and guardians were encouraged to return to the clinic at the onset of any illness and were followed through November 2009. Children who presented with fever or a recent history of fever received a malaria rapid diagnostic test, and children with positive tests were treated with artemether-lumefantrine (Coartem) according to national guidelines. To estimate the incidence of malaria and prevalence of polymorphisms of interest in children not receiving SMC, a separate control cohort of 250 children from the study area was enrolled and followed in the same manner from September to November 2009.

The study was registered (ClinicalTrials.gov no. NCT00941785) and approved by Institutional Review Boards of the London School of Hygiene and Tropical Medicine and Centre Muraz, Bobo-Dioulasso, Burkina Faso. The laboratory studies described here were approved by the University of California, San Francisco Committee on Human Research.

**Samples for study.** Blood samples from finger sticks at enrollment and from a cross-sectional survey of participants in November, 1 month after the last dose of SMC, were placed on filter paper (Whatman 3MM), labeled with study numbers and dates, air-dried, and stored in sealed sample bags at ambient temperature with desiccant. We randomly selected for PCR 120 paired samples from each SMC group and 120 paired samples from the control group, with each paired set representing the enrollment and final survey samples for a single participant. PCR-positive samples from participants who experienced clinical malaria during the study were removed from the analysis because of their treatment with other antimalarial drugs during the study period.

**Laboratory methods.** DNA was isolated from selected dried blood spots with Chelex, as previously described (30). Known *P. falciparum* polymorphisms were assessed at the following alleles: *pfcrt* K76T; *pfmdr1* N86Y, Y184F, S1034C, N1042D, and D1246Y; *pfdhfr* N51I, C59R, S108N, and I164L; and *pfdhps* S436A, A437G, and K540E. Sequences were evaluated using nested PCR followed by restriction fragment length polymorphism (RFLP) analysis, as described elsewhere (31–33). Digestion products were resolved by 2.5% gel electrophoresis, and results were classified as wild type, mixed (presence of both alleles), or mutant, based on migration patterns. Investigations were performed in a blind manner with respect to treatment group and clinical outcomes during the molecular analysis.

**Data analysis.** We compared the differences in the prevalences of the polymorphisms of interest between the baseline samples and those collected at the end of study, excluding samples from those who developed clinical malaria during follow-up to avoid the influence of therapy with artemether-lumefantrine and also excluding pairs for which at least one sample was negative for *P. falciparum* by PCR. Data were entered using Microsoft Access, managed with SPSS 18, and analyzed using Stata SE 11.0. Categorical variables were compared using the chi-square test. $P < 0.05$ was considered to represent statistical significance.

## RESULTS

Among the samples from the 1,500 children included in the clinical trial, we randomly selected 120 paired samples from each SMC group and 120 paired samples from the control group, with each paired set representing the enrollment and final survey samples for a single participant (Fig. 1). From 120 randomly selected samples in the AQ/SP group, 77 (64.2%) had positive PCR for *P. falciparum* at both enrollment and the final survey; 48 (62.3%) of these PCR-positive participants did not develop malaria during the study and therefore received three rounds of SMC with AQ/SP. From 120 randomly selected samples in the DP group, 71 (59.2%) had positive PCR at both enrollment and follow-up; 46 (64.8%) of these PCR-positive participants did not develop malaria and therefore received three rounds of SMC with DP. Relevant SNPs in *pfcrt*, *pfmdr1*, *pfdhfr*, and *pfdhps* were assessed. At enrollment, samples showed a high prevalence of mutations known to be associated with resistance to CQ and AQ (for *pfcrt*, 76T; for *pfmdr1*, 86Y) and to SP (for *pfdhfr*, 51I, 59R, and 108N; for *pfdhps*, 437G) (Table 1). Consistent with most other available data, the *pfmdr1* 1246Y mutation was much less prevalent than in other parts of Africa, and we did not identify mutations at position 1034 or 1042 in *pfmdr1*, 164 in *pfdhfr*, or 540 in *pfdhps*.

Our primary interest was to compare the prevalences of key SNPs between the time of enrollment and the end of the study in the AQ/SP, DP, and control groups (Table 1). Prevalences of the SNPs of interest did not differ significantly between the three study arms before the intervention (not shown). In the AQ/SP

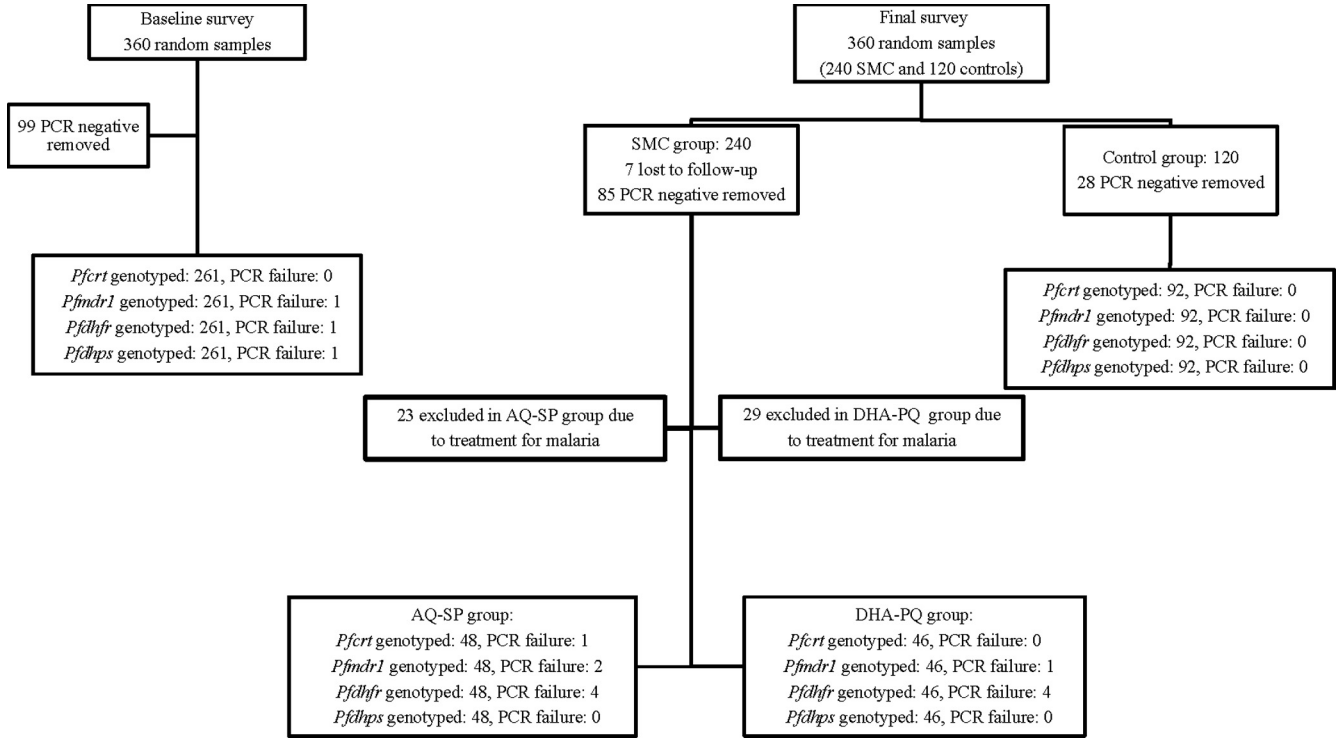


**FIG 1** PCR genotyping profile.

group, significant selection over the course of the trial was seen for *pfcrt* 76T (68.5% to 83.0%, $P = 0.04$), *pfdhfr* 59R (54.8% to 83.3%, $P = 0.0002$), and *pfdhfr* 108N (55.0% to 87.2%, $P = 0.0001$), with trends toward selection of *pfmdr1* 86Y, *pfdhfr* N51I, and *pfdhps* 437G. In the DP group, no significant selection of alleles was seen, except for borderline selection of wild-type *pfmdr1* D1246 (mutant, 7.7% to 0%, $P = 0.05$). For the control group, differences in prevalence between enrollment samples and those collected at the end of the study were not seen for any alleles.

## DISCUSSION

SMC is a promising strategy for malaria control in West Africa, and the use of monthly AQ/SP in at-risk children during the malaria transmission season is now endorsed by the WHO for the

**TABLE 1** Selection of *P. falciparum* polymorphisms by SMC regimens[a]

| Gene | SNP | Prevalence of mutation (%) in baseline samples ($n = 261$) | Prevalence of mutation (%) at the end of the study | | | *P* value for selection of mutant (M) or wild type (WT) | | |
|---|---|---|---|---|---|---|---|---|
| | | | AQ/SP ($n = 48$) | DP ($n = 46$) | No SMC ($n = 92$) | AQ/SP | DP | No SMC |
| *pfcrt* | K76T | 68.5 | 83.0 | 65.2 | 62.0 | **0.04 (M)** | 0.66 | 0.25 |
| *pfmdr1* | N86Y | 29.1 | 37.5 | 32.6 | 33.0 | 0.24 (M) | 0.62 | 0.52 |
| | Y184F | 58.5 | 58.3 | 56.5 | 67.0 | 0.98 | 0.79 (WT) | 0.13 (M) |
| | S1034C | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | N1042D | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | D1246Y | 7.7 | 4.2 | 0 | 8.0 | 0.5 | **0.05 (WT)** | 0.97 |
| *pfdhfr* | N51I | 58.1 | 69.6 | 54.6 | 59.0 | 0.14 (M) | 0.66 | 0.91 |
| | C59R | 54.8 | 83.3 | 45.7 | 48.0 | **0.0002 (M)** | 0.25 | 0.25 |
| | S108N | 55.0 | 87.2 | 51.1 | 53.0 | **0.0001 (M)** | 0.62 | 0.77 |
| | I164L | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| *pfdhps* | S436A | 35.1 | 35.4 | 30.4 | 34.0 | 0.97 | 0.53 | 0.8 |
| | A437G | 56.8 | 62.5 | 69.6 | **65.0** | 0.45 | 0.1 (M) | 0.15 (M) |
| | K540E | 0 | 0 | 0 | 0 | 1 | 1 | 1 |

[a] Both pure mutant and mixed samples were considered mutant for these analyses. *P* values are based on the chi-square test. Significant values are in bold type.

Sahel subregion of Africa. In the trial that provided samples for this study, administration of SMC as 3 monthly treatments to children in Burkina Faso offered protective efficacies against malaria of 83% for AQ/SP and 77% for DP (Zongo, unpublished). However, there is concern that regular use of AQ/SP or DP will select for parasite polymorphisms that mediate resistance to these regimens. To evaluate how readily key polymorphisms were selected, we compared genotypes of parasites collected at enrollment and 1 month after the third monthly SMC dose. We found that, after SMC, parasites from children who received monthly AQ/SP treatment had selection of multiple SNPs associated with resistance to both AQ and SP. In contrast, selection was not seen in parasites from children who received monthly DP. Thus, DP offered an efficacious alternative to AQ/SP for SMC, and it had the important advantage of not selecting for known resistance determinants.

The effects of AQ/SP on parasite markers of resistance were not surprising. A large body of literature has shown that after treatment for malaria with regimens that included AQ, subsequent infections showed selection of parasites with *pfcrt* and *pfmdr1* polymorphisms associated with AQ resistance (19, 20, 26, 34, 35) and with decreased *in vitro* AQ sensitivity (36). Similarly, after treatment with regimens that included SP, subsequent infections showed selection of parasites with *pfdhfr* and *pfdhps* polymorphisms associated with resistance to sulfadoxine and pyrimethamine (20, 26, 35). Our current findings expand observations to the use of AQ/SP for SMC rather than for the treatment of malaria, and they offer a sense of the scale of selection. During one transmission season, after 3 treatments with AQ/SP, the extent of selection was large, especially for the *pfcrt* 76T mutation that principally mediates resistance to AQ and for *pfdhfr* mutations that mediate resistance to pyrimethamine. Thus, it is likely that regular use of AQ/SP will profoundly affect the drug resistance profiles of parasites circulating in affected regions, although the impacts of these changes on clinical responses to treatment or SMC are uncertain.

In contrast to the situation with AQ/SP, DP did not select for polymorphisms associated with resistance to aminoquinolines. These results might seem surprising, as piperaquine is an aminoquinoline closely related to chloroquine (CQ) and AQ. However, earlier studies from Burkina Faso showed that prior treatment with DP did not select for *pfcrt* and *pfmdr1* polymorphisms associated with resistance to CQ and AQ (28). These results differ from those in a recent study in Uganda, in which therapy with DP selected in recurrent infections for *pfmdr1* polymorphisms (86Y and 1246Y) also selected by AQ-containing regimens (29). The reasons for different results between Uganda and Burkina Faso and for differential effects of the different aminoquinolines in Burkina Faso are not known. Piperaquine is a much larger molecule than CQ or AQ, and its mechanisms of antimalarial action and resistance are unknown (27). It seems that, if resistance to piperaquine currently exists, it may be mediated by factors other than the *pfcrt* and *pfmdr1* polymorphisms that mediate resistance to the other aminoquinolines. Interestingly, we saw borderline selection of the wild-type *pfmdr1* D1246 allele after SMC with DP consistent with borderline selection after treatment with DP in one but not in another prior trial in Burkina Faso (28). These modest selections were in the direction opposite that of those seen for DP in Uganda. In any event, the selective impact of piperaquine appears to be fundamentally different from that of CQ or AQ.

In comparing parasite genotypes before and after SMC, we excluded children who experienced an episode of malaria during the trial. This was done to limit the influence on parasite genetics of the current standard antimalarial treatment regimen in Burkina Faso, artemether-lumefantine. This exclusion obviously limited our analysis to only a subset of trial subjects. However, to obtain adequate sample size, our comparator group that did not receive SMC included children treated for malaria with artemether-lumefantrine during the study (76 of the 92 evaluable children experienced malaria). This information highlights the remarkably high incidence of malaria in our study population but also the important limitation that our control group differed from the children receiving the intervention. It may also be of interest to consider the competing impacts of SMC and treatment regimens. In fact, the standard treatment (artemether-lumefantrine) selects in the direction opposite that seen with CQ and AQ for *pfcrt* and *pfmdr1* polymorphisms (20, 37–39), potentially offsetting effects of SMC. Our results may also have been impacted by community changes in parasite genotypes over time. It has been shown elsewhere in West Africa that parasites are more likely to harbor resistance-mediating genotypes during the malaria transmission season, compared to the dry season, presumably due to the selective pressure of frequent malaria therapy (40). However, samples from control subjects who did not receive SMC had the same proportions of tested polymorphisms before and after the period of study, suggesting that community changes in parasite genetics through the transmission season were not responsible for the changes seen before and after SMC.

In summary, we observed that monthly AQ/SP, an effective SMC regimen for the prevention of malaria in Burkina Faso, readily selected for parasites with known SNPs associated with resistance to both AQ and SP. In contrast, monthly DP, which was nearly as efficacious, offered minimal selection of known resistance-mediating polymorphisms. These results highlight the importance of considering drug resistance selection when evaluating chemoprevention regimens. In addition, they identify a potential advantage of DP for SMC in West Africa and, more broadly, for chemoprevention of malaria in other regions.

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