

Molecular Markers of Resistance to Sulfadoxine-Pyrimethamine during Intermittent Preventive Treatment for Malaria in Mozambican Infants

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Background. Intermittent preventive treatment in infants (IPTi) with sulfadoxine-pyrimethamine (SP) is a potential malaria control strategy. There is concern about the impact that increasing in vivo resistance to SP has on the efficacy of IPTi, as well as about the potential contribution of IPTi to increases in resistance.

Methods. We compared the frequency of clinical episodes of malaria caused by *P. falciparum* parasites with mutations in *dhfr* and *dhps* among sick children who received SP or placebo in the context of a randomized, double-blind, placebo-controlled IPTi trial in Mozambique.

Results. Half of the children who received placebo harbored quintuple-pure mutant parasites. Nevertheless, the protective efficacy of IPTi within the 35 days after the third dose was 70.8% (95% confidence interval [CI], 40.7%–85.6%). Between month 2 after the third IPTi dose and the end of the follow-up period, children receiving SP harbored more *dhps* codon 437 mixed infections (odds ratio [OR], 10.56 [95% CI, 1.30–86.14]) and fewer *dhps* double-pure mutant parasites (OR, 0.43 [95% CI, 0.22–0.84]) than did placebo recipients.

Conclusions. IPTi appears to be associated with some changes in the prevalence of genotypes involved in SP resistance. In the face of a high prevalence of quintuple-mutant parasites, SP exhibited a high level of efficacy in the prevention of new episodes of malaria in infants.

Trial registration. ClinicalTrials.gov identifier: NCT00209794.

Intermittent preventive treatment in infants (IPTi) with sulfadoxine-pyrimethamine (SP) is a potential malaria control strategy consisting of the administration of SP

treatment doses, irrespective of the presence of parasites or symptoms, in conjunction with the Expanded Programme on Immunization (<http://www.ipti-malaria.org>). Currently, IPTi is under evaluation in Africa by a consortium of independent research groups together with the United Nations Children's Fund (UNICEF) and the World Health Organization [1]. Results from already-published trials of IPTi with SP show protective efficacy against malaria episodes that varies from 22% to 59% [2–4]. However, exposure of a pathogen to sub-therapeutic drug levels could result in conditions for the selection of resistance. Given the long half-life of SP [5], there is a need to assess any potential contribution of IPTi with SP to drug resistance [6, 7]. Further, levels of resistance to SP across Africa have increased in the past few years [8], leading most African countries to abandon SP as monotherapy in first-line treatment. There are questions regarding the efficacy of SP in the prevention of malaria in the face of parasite resistance.

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Parasites that have been selected by SP in vitro over time tend to have mutations in the genes encoding the dihydrofolate reductase (*dhfr*) [9] and dihydropteroate synthase (*dhps*) [10] enzymes. The level of resistance to SP has been shown to increase with the accumulation of mutations in codons 108, 51, 59, and 164 in *dhfr* and in codons 437 and 540 in *dhps* [11, 12]. However, studies have demonstrated that intrinsic parasite resistance is not the sole mediator of clinical response and may even be a poor predictor of drug efficacy in areas of high transmission [13, 14].

To evaluate the impact that IPTi with SP has on the selection of drug-resistance–mediating mutations, we compared the frequency of clinical episodes of malaria caused by *P. falciparum* parasites with point mutations in codons 108, 51, 59, and 164 in *dhfr* and in codons 437 and 540 in *dhps* among sick children who received SP or placebo in the context of a randomized, double-blind, placebo-controlled IPTi trial [4]. We also analyzed codon 76 in *P. falciparum* chloroquine resistance transporter (*pfcr1*) [15] and codon 86 in *P. falciparum* multidrug resistance 1 (*pfmdr1*) [16] and assessed the complexity of the infections. (Hereafter, codons will be referred to as the gene name and codon number separated by a hyphen; e.g., “*dhfr*-164.”)

PATIENTS, MATERIALS, AND METHODS

Design. This study was performed in the context of a randomized, double-blind, placebo-controlled trial of IPTi with SP. Doses of SP (body weight <5 kg, one-quarter tablet of Fansidar [Hoffmann-La Roche]; 5–10 kg, one-half tablet; >10 kg, 1 tablet) or placebo were administered according to body weight to infants at 3, 4, and 9 months of age in Manhica, southern Mozambique. Clinical follow-up of study participants has been described in detail elsewhere [4]. In brief, case assessment was performed through a passive surveillance system. Infants in whom fever (axillary temperature, $\geq 37.5^{\circ}\text{C}$) or a history of fever (in the past 24 h) was reported had a thick blood smear sample collected and read by use of high-quality microscopy fully described elsewhere [17]. Capillary blood samples on DNA filter paper (Schleicher & Schuell number 903) were also collected. Episodes of uncomplicated malaria in study infants were treated for 7 days with quinine administered orally, if the IPTi intervention had been administered within the preceding 2 weeks. We analyzed filter paper blood samples available from microscopically confirmed malaria episodes occurring within the 2 months after the third IPTi dose and a random selection of 30% of the episodes occurring between month 2 after the third IPTi dose and the end of the follow-up period at age 24 months. The study was approved by the National Mozambican Ethics Review Committee and the Hospital Clinic of Barcelona Ethics Review Committee.

Assessment of parasite mutations and complexity of infection. DNA was extracted using a standard protocol [15]. *P. falciparum* *dhfr*-51, -59, -108, and -164; *dhps*-437 and -540; *pfcr1*-76; and *pfmdr1*-86 genotypes were assessed by restriction

fragment–length polymorphism analysis of amplicons generated by nested polymerase chain reaction assays and electrophoresis on agarose gels. Primers, amplification conditions, and restriction enzymes are described elsewhere [11]. Each infection was characterized as “wild type,” “mutant,” or “mixed” (when both the wild-type and the mutant codon were found in the same infection) with respect to a given codon. Infections involving some parasites with all 3 mutations in *dhfr* were categorized as “triple mixed,” and they were characterized as “triple pure” if all 3 mutations were detected and there was no evidence of any wild-type parasite. The categories “double mixed” and “double pure” for *dhps* and “quintuple mixed” and “quintuple pure” for *dhfr/dhps* were similarly defined. Genetically different *P. falciparum* populations in each infection were discriminated on the basis of the fragment size of the amplified loci *msp1* and *msp2*, using an approach described elsewhere [18]. Multiplicity of infection (MOI) was defined as the number of distinct *msp1/msp2* amplicons detected in the sample. Molecular analysis was blinded to treatment group allocation.

Data analysis. Statistical analysis was performed using STATA software (version 8.2; Stata Corporation). To estimate the protective efficacy of IPTi during the 35 days after the third dose, the original study data [4] were reanalyzed at the individual level, using more-stringent criteria to include children in both cohorts who (1) received 3 doses of IPTi, (2) received the last IPTi dose between 9 and 10 months of age, and (3) had at least 1 month of follow-up after the last IPTi dose. Protective efficacy was calculated as described elsewhere [4]. The analysis of molecular data was done separately for parasites collected within 2 months after receiving the third IPTi dose and those collected between month 2 after the third IPTi dose and the end of the follow-up period. The proportions of patients in each intervention arm with wild-type, mixed, or pure mutant infections for each genotype were compared using the χ^2 or Fisher’s exact test. For parasites collected between month 2 after last IPTi dose and the end of the follow-up period, the strength of association was evaluated using odds ratios (ORs). MOIs were compared using Fisher’s exact test. Geometric mean values are presented for parasite densities. Density distributions were compared using the Wilcoxon rank sum test. $P < .05$ was considered to be statistically significant.

RESULTS

The protective efficacy of IPTi within the 35 days after the third SP dose was calculated for the 1285 children who received all 3 IPTi doses (634 in the placebo group and 651 in the SP group). During this period, there were 33 episodes of clinical malaria in the placebo group and 10 in the SP group. The estimated protective efficacy of SP for this period after the third IPTi dose was 70.8% (95% confidence interval [CI], 40.7%–85.6%; $P < .001$).

During the entire follow-up period, 593 blood samples on filter paper were collected from the 1503 infants participating in the IPTi study who had microscopically confirmed clinical episodes of asexual falciparum malaria. For the molecular analysis, we selected 195 filter paper samples from children with a malaria episode after the third IPTi dose. Nine filter paper samples were subsequently not found. We successfully analyzed all of the parasite mutations in 174 (93%) of the available filter paper samples. Of these, 27 corresponded to episodes that occurred within the first 2 months after dose 3, and 147 corresponded to randomly selected episodes that occurred from month 2 after dose 3 until the end of the follow-up period. Eighty-six samples were from children who had received placebo, and 88 were from children who had received SP. There were no differences between groups in sex composition (40 male and 46 female in the placebo group vs. 41 male and 47 female in the SP group; $P = .89$), age at first dose (3.38 months in both groups; $P = 1.00$), or body weight (6.10 kg in the placebo group vs. 6.00 kg in the SP group; $P = .23$). Molecular analysis showed that 45 (52%) of the 86 children who received placebo and 29 (33%) of the 88 children who received SP harbored quintuple-pure mutant parasites during the follow-up period ($P = .001$).

Within the first 2 months after the third IPTi dose, the 2 treatment groups were similar with regard to geometric mean \pm SD parasite density ($33,914.1 \pm 35,248.5$ parasites/ μ L in the placebo group vs. $32,101.8 \pm 65,095.2$ parasites/ μ L in the SP group; $P = .490$) and arithmetic mean \pm SD MOI (2.31 ± 0.87 in the placebo group vs. 2.27 ± 1.01 in the SP group; $P = .917$). No mutation in *dhfr*-164 was detected in any isolate. The prevalence of children harboring parasites with mutations in *dhfr* codons was similar in the 2 intervention arms (table 1). A trend toward an increase in carriage of parasites with mutations in codons *dhps*-437 and *dhps*-540 and of *dhps* double-pure mutants was found among the infants in the SP group, compared with those in the placebo group (table 1). A similar trend was found for quintuple-pure parasites. Carriage of parasites with mutations in codons *pfcr*-76 and *pfmdr*-86 was not associated with the intervention (table 1).

Between month 2 after the third IPTi dose and the end of the follow-up period, malaria episodes in children in the SP and placebo groups were characterized by similar geometric mean \pm SD parasite densities ($26,045.2 \pm 44,635.6$ parasites/ μ L in the placebo group vs. $19,743.7 \pm 38,314.8$ parasites/ μ L in the SP group; $P = .344$) and arithmetic mean \pm SD MOIs (1.91 ± 0.93 in the placebo group vs. 2.00 ± 1.03 in the SP group; $P = .718$). All of the isolates carried the wild-type *dhfr*-164 codon. The prevalence of isolates consisting of codon *dhps*-437 mixed infections was higher among children who had received SP than among those who had received placebo (table 2). A similar trend was found for codon *dhfr*-51 (table 2). SP recipients had a lower risk of carrying *dhps* double-pure parasites, and a similar trend was found for *dhfr* triple-pure and *dhfr*/

Table 1. Prevalence of infection with *Plasmodium falciparum* with different molecular markers of resistance, during the first 2 months after the third dose of intermittent preventive treatment.

Codon, genotype	No. (%) of patients in treatment arm		P^a
	Placebo ($n = 16$)	SP ($n = 11$)	
<i>dhfr</i> -51			.624
Wild type	3 (19)	1 (9)	
Mutant	13 (81)	10 (91)	
<i>dhfr</i> -59			.499
Wild type	2 (13)	0 (0)	
Mutant	14 (88)	11 (100)	
<i>dhfr</i> -108			1.000
Wild type	1 (6)	0 (0)	
Mutant	15 (94)	11 (100)	
<i>dhfr</i> haplotype			.624
<3 mutations	3 (19)	1 (9)	
Triple mixed	0 (0)	0 (0)	
Triple pure	13 (81)	10 (91)	
<i>dhps</i> -437			.090
Wild type	7 (44)	1 (9)	
Mutant	9 (56)	10 (91)	
<i>dhps</i> -540			.090
Wild type	7 (44)	1 (9)	
Mutant	9 (56)	10 (91)	
<i>dhps</i> haplotype			.090
<2 mutations	7 (44)	1 (9)	
Double mixed	0 (0)	0 (0)	
Double pure	9 (56)	10 (91)	
<i>dhfr/dhps</i> haplotype			.109
<5 mutations	9 (56)	2 (18)	
Quintuple mixed	0 (0)	0 (0)	
Quintuple pure	7 (44)	9 (82)	
<i>pfcr</i> -76			.428
Wild type	7 (44)	2 (18)	
Mutant	7 (44)	7 (64)	
Mixed	2 (13)	2 (18)	
<i>pfmdr</i> -86			.390
Wild type	1 (6)	2 (18)	
Mutant	15 (94)	8 (73)	
Mixed	0 (0)	1 (9)	

NOTE. SP, sulfadoxine-pyrimethamine.

^a Fisher's exact test.

dhps quintuple-pure parasites (table 2). The prevalences of *pfcr*-76 and *pfmdr*-86 genotypes were not associated with the intervention (table 2).

DISCUSSION

The results of the present study suggest that IPTi with SP may have an effect on the composition of *P. falciparum dhfr/dhps* genotypes harbored by children who develop clinical malaria after receiving 3 SP doses. In malaria episodes occurring between

Table 2. Prevalence of infection with *Plasmodium falciparum* with different molecular markers of resistance, between month 2 after the third dose of intermittent preventive treatment and the end of the follow-up period.

Codon, genotype	No. (%) of patients in treatment arm			OR (95% CI)	P ^a
	Placebo (n = 70)	SP (n = 77)			
<i>dhfr-51</i>					.040
Wild type	7 (10)	13 (17)	1		
Mutant	62 (89)	55 (71)	0.48 (0.18–1.28)		
Mixed	1 (1)	9 (12)	4.85 (0.51–46.49)		
<i>dhfr-59</i>					.558
Wild type	3 (4)	5 (6)	1		
Mutant	66 (94)	69 (90)	0.63 (0.14–2.73)		
Mixed	1 (1)	3 (4)	1.80 (0.12–26.20)		
<i>dhfr-108</i>					.830
Wild type	2 (3)	3 (4)	1		
Mutant	67 (96)	72 (94)	0.72 (0.12–4.42)		
Mixed	1 (1)	2 (3)	1.33 (0.07–26.62)		
<i>dhfr</i> haplotype					.086
<3 mutations	9 (13)	17 (22)	1		
Triple mixed	0 (0)	7 (9)	...		
Triple pure	61 (87)	53 (69)	0.46 (0.19–1.12)		
<i>dhps-437</i>					.016
Wild type	26 (37)	32 (42)	1		
Mutant	43 (61)	32 (42)	0.60 (0.30–1.21)		
Mixed	1 (1)	13 (17)	10.56 (1.30–86.14)		
<i>dhps-540</i>					.103
Wild type	22 (31)	29 (38)	1		
Mutant	42 (60)	34 (44)	0.61 (0.3–1.26)		
Mixed	6 (9)	14 (18)	1.77 (0.59–5.35)		
<i>dhps</i> haplotype					.040
<2 mutations	27 (39)	45 (58)	1		
Double mixed	1 (1)	2 (3)	1.2 (0.10–13.87)		
Double pure	42 (60)	30 (39)	0.43 (0.22–0.84)		
<i>dhfr/dhps</i> haplotype					.005
<5 mutations	32 (46)	53 (69)	1		
Quintuple mixed	0	4 (5)	...		
Quintuple pure	38 (54)	20 (26)	0.26 (0.02–3.08)		
<i>pfprt-76</i>					.574
Wild type	2 (3)	5 (6)	1		
Mutant	65 (93)	68 (88)	0.42 (0.08–2.23)		
Mixed	3 (4)	4 (5)	0.53 (0.06–4.91)		
<i>pfmdr1-86</i>					.370
Wild type	20 (29)	19 (25)	1		
Mutant	43 (61)	44 (57)	1.08 (0.51–2.29)		
Mixed	7 (10)	14 (18)	2.11 (0.70–6.35)		

NOTE. CI, confidence interval; OR, odds ratio; SP, sulfadoxine-pyrimethamine.

^a Univariate logistic regression model.

month 2 after the third IPTi dose and the end of the follow-up period, codon *dhps-437* and *dhfr-51* mixed infections were more prevalent among children who received SP than among those who received placebo. It might be argued that IPTi with SP could have impaired the development of specific malaria immunity,

leading to difficulty in controlling the parasite and, thus, to higher prevalences of mixed infections [19]. However, 3 IPTi doses were not associated with differences in parasite densities, in *pfprt* and *pfmdr1* genotypes, or in the complexity of infections, as quantified by genotyping of *msp1* and *msp2*. Furthermore, in

a parallel study of this same cohort of children, immunological responses to *P. falciparum* antigens were not affected by IPTi (D. Quelhas, unpublished data). In a similar study performed in Ifakara, Tanzania, the incidence of clinical malaria episodes was significantly lower (protective effect, 36% [95% CI, 11%–53%]), during the 2 years after the intervention was discontinued, among children who had received the 3 doses of IPTi than among those who received placebo [20]. This evidence suggests that the association of IPTi with an increase in infections that are mixed with regard to parasite mutations in resistance codons is not mediated by an impairment of malaria-specific immunity.

The pharmacodynamics of SP in blood, together with different degrees of parasite chemosensitivity to the drug, may explain our findings. During the period when the drug is still active in the bloodstream (the first ~2 months after the last dose [5]), mutated parasites tended to be more frequent among children in the SP group than among children in the placebo group, which suggests that susceptible wild-type parasites may be more easily eliminated by the drug than mutated parasites. Prompt treatment of these early episodes with an effective antimalarial drug other than SP could minimize the survival of parasites that are highly mutated in the *dhfr* and *dhps* genes. Semiresistant parasites carrying a smaller number of mutations may survive at sub-clinical densities during the active period of SP in blood [11] and recur later, together with newly acquired parasites. Clinical episodes triggered by this mixture of parasites may result in the observed increment of *dhfr* and *dhps* codon mixed infections and, subsequently, in the reduction in *dhps* double-pure mutant infections in children in the SP group, compared with children in the placebo group.

The small proportion of individuals who would receive SP as IPTi (4% of the total population in Manhica are children [A. Nhacolo, personal communication]), compared with all of the individuals receiving SP treatment in the community, together with the reduction in malaria episodes resulting from the preventive intervention, should also be considered when estimating drug pressure through IPTi. Mathematical models predict that intermittent preventive treatment given only to infants is unlikely to shorten the useful life of the drug used [21] or to significantly affect the spread of highly resistant parasites in areas where partial resistance to SP is already established [22], as is currently the case in many African countries. Furthermore, SP alone is no longer a first-line antimalarial drug for case management in most African settings. Artemisinin-based combination therapies are being introduced, and, thus, the contribution of IPTi with SP to drug pressure is likely to be minimal. Notably, similar drug pressure that could result from intermittent preventive treatment in pregnancy has not hindered the recommendation and implementation of this approach to malaria control for pregnant women [23]. To effectively evaluate changes in treatment efficacy and thus provide advance warning of increases in drug resistance, potential implementation of IPTi

should be accompanied by the establishment of drug-resistance monitoring programs.

Our results show that half of the children who received placebo in the IPTi trial harbored quintuple-pure mutant parasites during their clinical episodes. In spite of this highly resistant genetic background of the *P. falciparum* parasite population in Manhica, the protective efficacy of IPTi during the first 35 days after the third dose was remarkably high (70.8%). A similar situation was seen in Senegal, where seasonal IPTi with SP and artesunate showed a high protective efficacy (86%) in spite of the high frequency of *dhfr* triple mutations (67%) and *dhps*-437 mutations (29%) [7]. The frequency of mutations does not seem to be a useful tool to predict the efficacy of SP in the prevention of malaria in African infants. Similar discrepancies between in vivo resistance and molecular markers have already been shown [13, 14, 24–29], highlighting the existence of several other factors, apart from the parasite genotype, that may play a role in the response to SP, such as the complexity of the parasite infection, the rate of drug metabolism, the host's nutritional status, and, in particular, the host's malaria-specific immunological status [30]. Therefore, caution is needed when extrapolating parasite-related genetic findings to clinical efficacy in the treatment of malaria in semi-immune children, and probably more so when attempting to predict efficacy in preventing malaria in infants. Differentiation of these elements will help us to understand how IPTi confers protection to children. The effect of high-coverage IPTi on the drug resistance of the parasite population as a whole may be addressed by conducting large cross-sectional surveys before and after intervention. This will be useful to establish sound evidence-based policy recommendations for the use of IPTi with SP in Africa.

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References

1. Egan A, Crawley J, Schellenberg D. Intermittent preventive treatment for malaria control in infants: moving towards evidence-based policy and public health action. *Trop Med Int Health* **2005**; 10:815–7.
2. Schellenberg D, Menendez C, Kahigwa E, et al. Intermittent treatment for malaria and anaemia control at time of routine vaccinations in Tanzanian infants: a randomised, placebo-controlled trial. *Lancet* **2001**; 357:1471–7.
3. Chandramohan D, Owusu-Agyei S, Carneiro I, et al. Cluster randomised trial of intermittent preventive treatment for malaria in infants in area of high, seasonal transmission in Ghana. *BMJ* **2005**; 331:727–33.
4. Macete E, Aide P, Aponte JJ, et al. Intermittent preventive treatment for malaria control administered at the time of routine vaccinations in Mozambican infants: a randomized, placebo-controlled trial. *J Infect Dis* **2006**; 194:276–85.

5. Watkins WM, Mosobo M. Treatment of *Plasmodium falciparum* malaria with pyrimethamine-sulfadoxine: selective pressure for resistance is a function of long elimination half-life. *Trans R Soc Trop Med Hyg* **1993**; 87:75–8.
6. Marks F, von Kalckreuth V, Kobbe R, et al. Parasitological rebound effect and emergence of pyrimethamine resistance in *Plasmodium falciparum* after single-dose sulfadoxine-pyrimethamine. *J Infect Dis* **2005**; 192:1962–5.
7. Cisse B, Sokhna C, Boulanger D, et al. Seasonal intermittent preventive treatment with artesunate and sulfadoxine-pyrimethamine for prevention of malaria in Senegalese children: a randomised, placebo-controlled, double-blind trial. *Lancet* **2006**; 367:659–67.
8. White NJ. Antimalarial drug resistance. *J Clin Invest* **2004**; 113:1084–92.
9. Peterson DS, Walliker D, Wellems TE. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proc Natl Acad Sci USA* **1988**; 85:9114–8.
10. Triglia T, Menting JG, Wilson C, Cowman AF. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* **1997**; 94:13944–9.
11. Plowe CV, Cortese JF, Djimde A, et al. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *J Infect Dis* **1997**; 176:1590–6.
12. Kublin JG, Witzig RS, Shankar AH, et al. Molecular assays for surveillance of antifolate-resistant malaria. *Lancet* **1998**; 351:1629–30.
13. Mberu EK, Nzila AM, Nduati E, et al. *Plasmodium falciparum*: in vitro activity of sulfadoxine and dapsone in field isolates from Kenya: point mutations in dihydropteroate synthase may not be the only determinants in sulfa resistance. *Exp Parasitol* **2002**; 101:90–6.
14. Aubouy A, Jafari S, Huart V, et al. DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with in vitro activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy. *J Antimicrob Chemother* **2003**; 52:43–9.
15. Djimde A, Doumbo OK, Cortese JF, et al. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* **2001**; 344:257–63.
16. Foote SJ, Kyle DE, Martin RK, et al. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* **1990**; 345:255–8.
17. Alonso PL, Smith T, Schellenberg JR, et al. Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in southern Tanzania. *Lancet* **1994**; 344:1175–81.
18. Robert F, Ntoumi F, Angel G, et al. Extensive genetic diversity of *Plasmodium falciparum* isolates collected from patients with severe malaria in Dakar, Senegal. *Trans R Soc Trop Med Hyg* **1996**; 90:704–11.
19. Smith T, Felger I, Tanner M, Beck HP. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. *Trans R Soc Trop Med Hyg* **1999**; 93:59–64.
20. Schellenberg D, Menendez C, Aponte JJ, et al. Intermittent preventive antimalarial treatment for Tanzanian infants: follow-up to age 2 years of a randomised, placebo-controlled trial. *Lancet* **2005**; 365:1481–3.
21. Alexander N, Sutherland C, Roper C, Cisse B, Schellenberg D. Modelling the impact of intermittent preventive treatment for malaria on selection pressure for drug resistance. *Malar J* **2007**; 6:9.
22. O'Meara WP, Smith DL, McKenzie FE. Potential impact of intermittent preventive treatment (IPT) on spread of drug-resistant malaria. *PLoS Med* **2006**; 3:e141.
23. World Health Organization (WHO). A strategic framework for malaria prevention and control during pregnancy in the African region. AFR/MAL/04/01. Brazzaville, Republic of the Congo: WHO Regional Office for Africa, **2004**.
24. Jelinek T, Ronn AM, Curtis J, et al. High prevalence of mutations in the dihydrofolate reductase gene of *Plasmodium falciparum* in isolates from Tanzania without evidence of an association to clinical sulfadoxine/pyrimethamine resistance. *Trop Med Int Health* **1997**; 2:1075–9.
25. Nzila AM, Mberu EK, Sulo J, et al. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. *Antimicrob Agents Chemother* **2000**; 44:991–6.
26. Ringwald P, Keundjian A, Same Ekobo A, Basco LK. Chemoresistance of *Plasmodium falciparum* in the urban region of Yaounde, Cameroon. Part 2: evaluation of the efficacy of amodiaquine and sulfadoxine-pyrimethamine combination in the treatment of uncomplicated *Plasmodium falciparum* malaria in Yaounde, Cameroon. *Trop Med Int Health* **2000**; 5:620–7.
27. Basco LK, Tahar R, Keundjian A, Ringwald P. Sequence variations in the genes encoding dihydropteroate synthase and dihydrofolate reductase and clinical response to sulfadoxine-pyrimethamine in patients with acute uncomplicated falciparum malaria. *J Infect Dis* **2000**; 182:624–8.
28. Omar SA, Adagu IS, Warhurst DC. Can pretreatment screening for dhps and dhfr point mutations in *Plasmodium falciparum* infections be used to predict sulfadoxine-pyrimethamine treatment failure? *Trans R Soc Trop Med Hyg* **2001**; 95:315–9.
29. Alifrangis M, Enosse S, Khalil IF, et al. Prediction of *Plasmodium falciparum* resistance to sulfadoxine/pyrimethamine in vivo by mutations in the dihydrofolate reductase and dihydropteroate synthetase genes: a comparative study between sites of differing endemicity. *Am J Trop Med Hyg* **2003**; 69:601–6.
30. White NJ. Why is it that antimalarial drug treatments do not always work? *Ann Trop Med Parasitol* **1998**; 92:449–58.