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Five Years of Large-Scale *dhfr* and *dhps* Mutation Surveillance Following the Phased Implementation of Artesunate Plus Sulfadoxine-Pyrimethamine in Maputo Province, Southern Mozambique

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Abstract. Accumulation of mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) is strongly associated with sulfadoxine-pyrimethamine (SP) treatment failure. Routine surveillance for these resistance markers was conducted annually at 26 sentinel sites in Maputo Province, Mozambique, before and after the phased deployment of artesunate plus SP (AS-SP), with 15,758 children sampled between 2004 and 2008. Mean asexual parasite prevalence, polymerase chain reaction (PCR) corrected, decreased from 44.2% in 2004 to 3.8% in 2008 ($P < 0.0001$). Among the 2,012 PCR-confirmed falciparum samples, the *dhfr* triple mutation remained close to fixation, whereas both *dhps* double and *dhfr/dhps* "quintuple" mutations increased from 11.0% in 2004, to 75.0% by 2008 ($P < 0.0001$). Adding artesunate to SP did not retard the spread of SP-resistant parasites. The high "quintuple" mutation prevalence suggests a limited useful therapeutic lifespan of AS-SP for treating uncomplicated malaria, and may curb efficacy of SP-monotherapy for intermittent preventive treatment in Mozambique.

INTRODUCTION

A major factor contributing to the continued public health burden of malaria is the spread of drug-resistant *Plasmodium* parasites.[1,2] In response to the threat posed by antimalarial drug resistance the World Health Organization (WHO) has recommended a shift from antimalarial monotherapy, particularly chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) to combination therapy.[3] It is expected that combining antimalarials with differing modes of action would reduce the probability of a resistant (mutant) parasite surviving treatment.[4] Artemisinin-based combination therapies (ACTs) are preferred to other combination therapies or monotherapies, as they have higher cure rates, more rapid parasite clearance times, and the potential to reduce malaria transmission caused by their gametocidal effect, further limiting the spread of antimalarial resistance.[5–7]

By 2008, 77 countries had adopted an ACT as their first line antimalarial treatment policy.[8] Artesunate plus SP (AS-SP), one of the WHO recommended ACTs,[7] has the operational advantages of lower cost and the full dose of the partner drug being administered as a single dose, but unfortunately is not suitable for manufacture as a fixed dose combination. Separate tablets, even if blister packed, allow patients to choose which drug to take, potentially negating the purported benefits of combination therapy. The widespread use of SP monotherapy has resulted in a high prevalence of SP-resistant *Plasmodium falciparum* isolates in many southern African countries.[9–13] There may be further selection for these resistant parasites through the current large-scale use of SP-monotherapy for intermittent preventive treatment (IPT) of high-risk groups, particularly pregnant women.[14]

Resistance to SP develops because of an accumulation of single nucleotide polymorphisms in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes. The presence of three mutations in the *dhfr* gene (at codons 51, 59, and 108, known as the *dhfr* triple) and two mutations in the *dhps* gene (at codons 437 and 540, known as the *dhps* double), together referred to as the "quintuple mutation," is strongly associated with *in vivo* and *in vitro* SP resistance in East and Southern Africa.[10,11]

Within the case management component of the Lubombo Spatial Development Initiative malaria control program,[15] large-scale deployment of AS-SP as first line treatment of definitely diagnosed uncomplicated malaria commenced in Maputo Province, southern Mozambique, in 2004. This ACT was selected to replace chloroquine following *in vivo* efficacy trials, conducted from 2003 until 2005, which showed AS-SP to be highly effective with an adequate clinical and parasitological response of 98% at 42 days.[16] This study, conducted on uncomplicated malaria patients 1 to 65 years of age, found an over 3-fold increased risk of recrudescence among patients infected with parasites carrying the "quintuple" mutation. Phased implementation began in the Namaacha District in April 2004 and included all districts in Maputo Province by May 2006 (Figure 1). Adequate supplies of AS-SP have been sustained in all public sector facilities since implementation of this policy, with ACT availability extended to the community health post level in 2006. This has ensured high ACT coverage following the deployment of this treatment policy.[17]

Limiting the spread of antimalarial resistance is one of the key rationales motivating ACT deployment. However, surprisingly little research has been conducted on the spread of antimalarial resistance following large-scale ACT deployment, with almost all the research in this area being conducted in Asia.[18,19] Antimalarial resistance is usually monitored through *in vivo* therapeutic efficacy studies, which are costly and may not be the most sensitive tool for detecting resistance given the contribution of partial immunity to clinical and parasitological treatment response.[20] With highly effective malaria control programs, the marked reductions in malaria incidence decrease the feasibility of conducting adequately powered *in vivo* therapeutic efficacy studies. This study reports on

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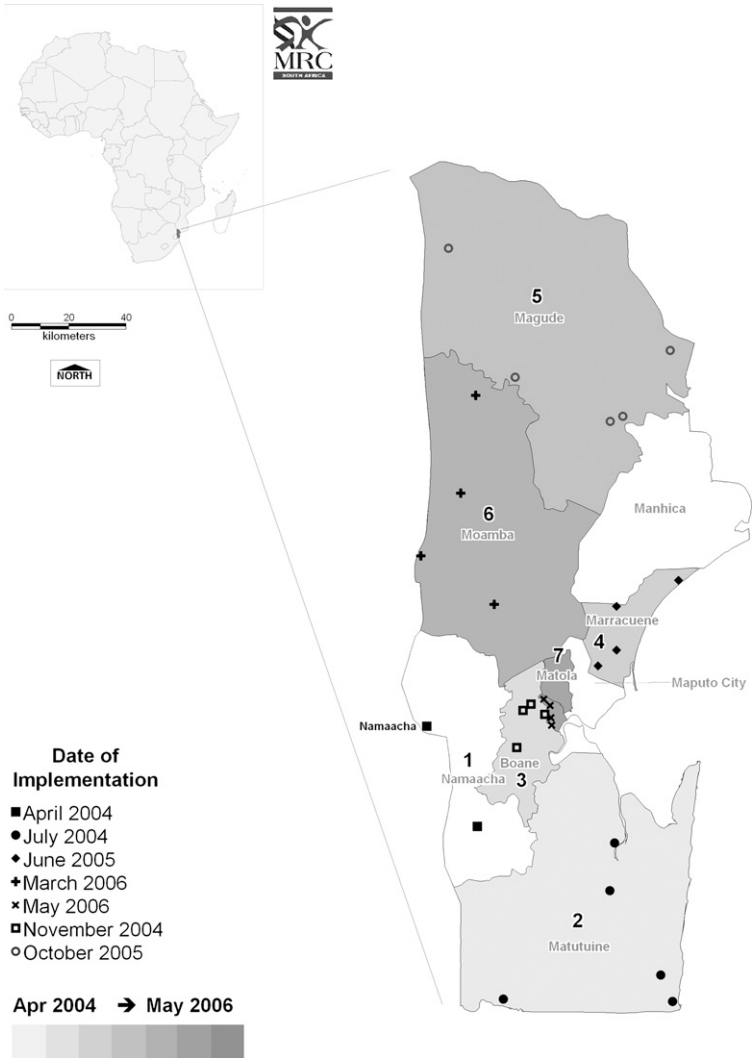

FIGURE 1. Sentinel sites and AS-SP implementation date in Maputo Province, Mozambique.

the large-scale surveillance of *dhfr* and *dhps* mutations over a 5-year period before and after the wide-scale deployment of AS-SP as first line treatment of uncomplicated malaria in Maputo Province, southern Mozambique.

MATERIALS AND METHODS

Study population and blood sample collection. Finger-prick blood from a random sample of children (2 to 15 years of age) were collected during annual cross-sectional surveys of asexual parasite prevalence in 26 sentinel sites in Maputo Province, Mozambique (Figure 1) from 2004 to 2008.[15,21] Areas within each district were identified as sentinel sites based on population size, geographical structure, and proximity to health facilities. Blood samples were collected on filter paper strips (Whatman filter paper no 1; Merck Laboratory Suppliers (Pty) Ltd., Durban, South Africa) from 120 children per sentinel site. The air dried filter paper blood spots were stored individually in zip-lock bags containing desiccant at room temperature until assayed.

Sample preparation and analysis. Parasite DNA, from filter paper blood samples of rapid test (ICT; Global Diagnostics, Cape Town, South Africa) positive individuals, was extracted using the Chelex method.[22] Once a sample was confirmed as *P. falciparum* positive by nested PCR,[23] it was subjected to *dhfr* (codons 51, 59, 108, and 164) and *dhps* (codons 436, 437, 540, and 518) mutational analysis.[24] Digestion products separated on a 2% agarose gel using electrophoresis were visualized and photographed using the MiniBIS documentation system (BioSystematica, Ceredigion Wales, UK). Codons were classified as either pure wild type, pure mutant, or mixed (both wild type and mutant haplotypes present in an individual sample). All genotyping analyses were run in duplicate with a third trial conducted on discordant results. When calculating overall prevalence of *P. falciparum* isolates with mutant codons, codons with mixed genotypes were analyzed together with pure mutant codons.

Statistical analysis. Statistical analysis was performed using Stata 10 (Stata Corp., College Station, TX). The prevalence of *dhfr* and *dhps* mutations were calculated for individual years and odds ratios (ORs), relative to 2004, and the association between "quintuple" mutation prevalence and prospectively defined explanatory variables (time since the introduction of AS-SP, time since deployment of SP-IPTp, age, and parasite prevalence) was assessed using a multilevel mixed effects logistic regression model.[25] Spearman's correlation coefficients

were used to quantify the level of association between year and these more biologically plausible explanatory variables. "Quintuple mutation" prevalence and parasite prevalence were assumed constant within each site for each study year. Sentinel sites were nested within districts, within which time since AS-SP and SP-IPT deployment remained constant. Within site and within district correlations of responses, and the number of children surveyed, were taken into account in the estimation of 95% confidence intervals (CIs).

Ethical considerations. Ethics approval for this study was obtained from the South African Medical Research Council and the Ministry of Health in Mozambique. Blood samples were only taken if full informed consent from a parent/guardian had been obtained. Children testing positive for malaria were referred to the closest health facility for appropriate treatment.

RESULTS

A total of 15,758 samples were collected over the 5-year study period, of which 2,361 (15.0%) were rapid test positive for *P. falciparum*. DNA could be extracted from 2,329 (98.6%) rapid test positive samples of which 2,012 (86.4%) were confirmed as *P. falciparum* positive by PCR. This discrepancy could partially be explained by the rapid test detection of the histidine-rich protein 2 malaria antigens in children in whom all parasites had been cleared 2 to 4 weeks previously.[26] Mean asexual parasite prevalence, based on PCR results, decreased from 44.2% in 2004 to 3.8% in 2008 (rate ratio [RR] 0.09; 95% CI: 0.07–0.11; $P < 0.0001$, Table 1). Mixed genotypes were detected in 1030 (51%) samples.

No mutant alleles were detected in any of the samples tested at *dhfr* codon 164 and *dhps* codon 581. The *dhps* 436 mutation was rare, with a prevalence of 0.003%.

Before the introduction of AS-SP, *dhfr* triple mutation prevalence in Maputo Province was at 91.7%, increasing to 96.3% by 2005, and remaining close to fixation (> 98%) for the remainder of the study period (Figure 2A).

Prevalence of the *dhps* double was below 20% throughout all districts in Maputo Province before the introduction of AS-SP, with a mean prevalence of 11% (Figure 2B). However, as AS-SP implementation progressed through Maputo Province, the *dhps* double mutation prevalence began increasing, reaching high levels (64.5%) in 2007, a year after AS-SP and IPT with SP monotherapy had been deployed nationally (OR = 21.3; 95% CI: 12.6–36.3; $P < 0.0001$, Figure 2B). This increase in prevalence continued, reaching 75.0% by 2008 (OR = 40.4; 95% CI: 20.4–80.3; $P < 0.0001$, Figure 2B).

Because the *dhfr* triple mutation was already at fixation in 2004, the prevalence of parasites carrying the "quintuple" mutation tracked the *dhps* double mutation prevalence. "Quintuple" mutation prevalence had increased markedly by 2006 (OR = 9.4; 95% CI: 6.9–13.0; $P < 0.0001$) and continued to increase in 2007 (OR = 22.1; 95% CI: 13.0–37.6; $P < 0.0001$), reaching 75.0% by 2008 (OR = 42.2; 95% CI: 21.3–83.7; $P < 0.0001$; Figure 2C).

Univariate and multiple logistic regression analysis were used to explore whether prospectively defined variables could explain the observed increase in "quintuple" mutation prevalence over time (Table 2). Univariate analysis showed a positive relationship between "quintuple" mutation prevalence and months since artesunate plus SP deployment (OR = 1.11; 95% CI: 1.10–1.12; $P < 0.0001$), years since deployment of SP IPTp policy (OR = 4.40; 95% CI: 3.43–5.65; $P < 0.0001$), and fever (OR = 2.26; 95% CI: 1.32–3.87; $P = 0.003$). There was a negative association with sentinel site parasite prevalence (OR = 0.95; 95% CI: 0.94–0.95; $P < 0.0001$) and with age (OR = 0.95; 95% CI: 0.93–0.98; $P = 0.003$). There was strong colinearity between calendar year and duration of AS-SP use ($R = 0.83$); a weak negative correlation with parasite prevalence ($R = 0.49$) but none with age ($R = 0.04$) (Figure 3). As SP-IPTp was implemented nationally (and thus in all study sites) in mid 2006 there was perfect correlation of this explanatory variable with the year after 2005. Probably as a result of these correlations with time, only age and fever remained significantly associated with "quintuple" mutation prevalence after adjusting for confounding with study year (Table 2). No association between "quintuple" mutation prevalence and gender ($P = 0.31$) was found.

DISCUSSION

We report the first data documenting the routine surveillance of temporal changes in resistance after large-scale systematic deployment of an ACT in Africa. Following the phased deployment of AS-SP in Maputo Province, southern Mozambique, *dhfr* triple mutation prevalence remained at fixation. More importantly, the prevalence of parasites with the *dhps* double, associated with sulfadoxine resistance, and "quintuple" mutations, associated with SP treatment failure, increased rapidly, both reaching an overall prevalence of 75% by 2008. Our findings suggest that the systematic, large-scale deployment of artesunate plus SP, as first line treatment of uncomplicated malaria since 2004, has not delayed the spread of SP resistance markers and may be contributing to the selection of parasites carrying these resistance markers.

These findings contrast sharply with the observed decrease in mefloquine resistance after the systematic deployment of the artesunate-mefloquine combination on the north-west border of Thailand,[27,28] although falciparum malaria transmission

TABLE 1

Parasite prevalence (%) based on polymerase chain reaction (PCR) results by district and year

District	2004	2005	2006	2007	2008
Namaacha	4.6% (11/239)	3.9% (10/256)	3.8% (10/263)	1.7% (2/118)	1.0% (2/200)
Matutuine	8.5% (45/529)	6.3% (18/286)	3.3% (19/576)	2.4% (9/375)	1.5% (7/467)
Boane	28.0% (87/311)	15.3% (46/301)	12.8% (58/453)	1.4% (4/286)	4.0% (14/350)
Marracuene	29.5% (115/390)	34.4% (97/282)	21.4% (68/318)	4.0% (12/300)	2.9% (10/345)
Magude	62.0% (337/544)	40.7% (190/467)	24.2% (123/508)	11.1% (54/486)	5.2% (18/346)
Moamba	42.5% (16 /393)	18.3% (81/443)	19.3% (59/306)	6.2% (9/145)	2.1% (9/429)
Matola	36.8% (79/215)	47.9% (104/217)	34.8% (71/204)	37.1% (51/137)	5.1% (16/314)
Total	44.2% (841/1903)	33.7% (546/1620)	21.8% (408/1872)	18.6% (141/758)	3.8% (76/2000)

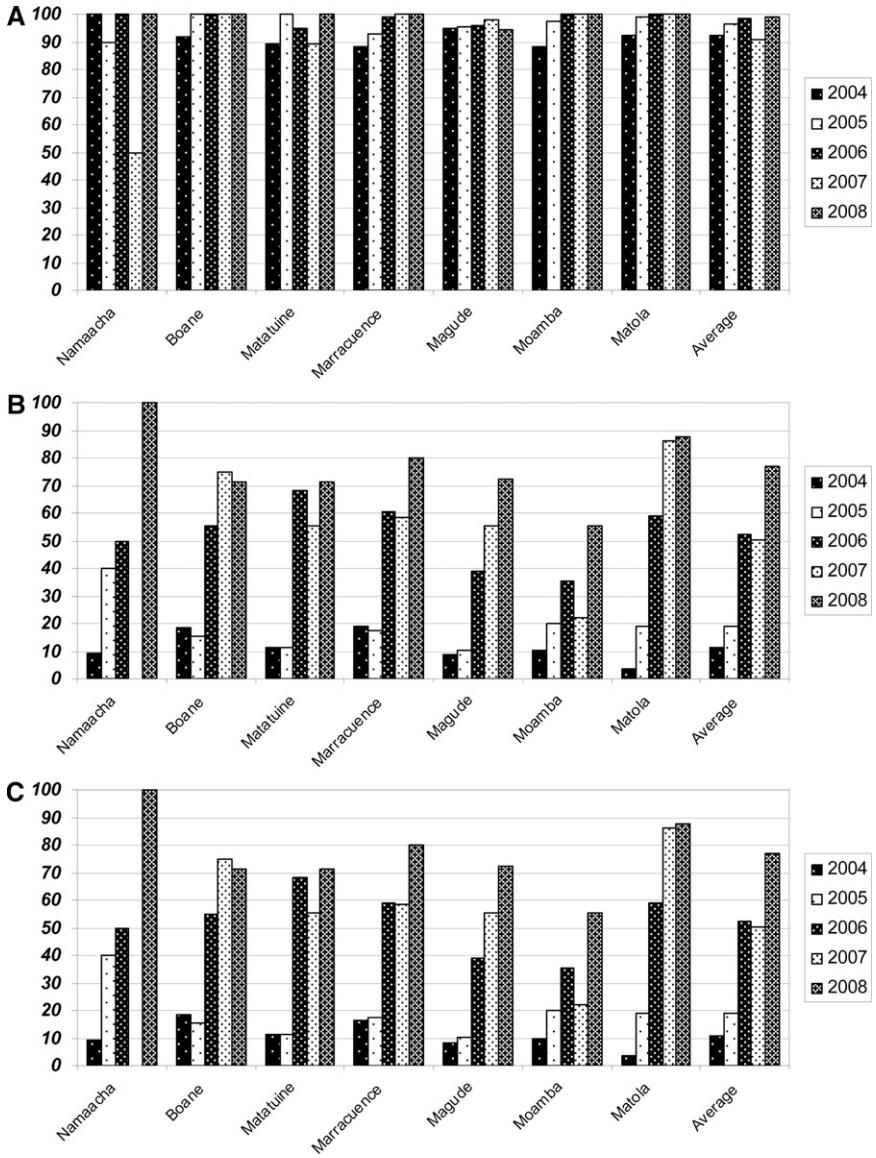

FIGURE 2. Prevalence of the (**A**) *dhfr* triple mutation, (**B**) *dhps* double mutation, and (**C**) "quintuple" mutation by district and year in Maputo Province, Mozambique.

reduced significantly over the study periods in both locations.[15,27] Both mefloquine and SP have a long elimination half-life, which provides a selective filter for resistant parasites acquired elsewhere.[29–32] A plausible explanation for these contrasting results is that on the north-west border of Thailand there is minimal local transmission, with most malarial infections originating in neighboring Burma, where the limited availability of mefloquine has resulted in most falciparum isolates

TABLE 2

Factors associated with "quintuple" mutation prevalence in Maputo Province between 2004 and 2008 (within district and site correlations are taken into account in the estimation of confidence intervals)

	Unadjusted OR	95% CI*	*P* value	Adjusted OR	95% CI*	*P* value
2004	1.0	–	–	1.0	–	–
2005	1.56	1.12–2.18	0.009	1.50	1.05–2.14	0.026
2006	9.44	6.86–13.00	< 0.0001	9.30	5.48–15.80	< 0.0001
2007	22.14	13.03–37.60	< 0.0001	21.47	8.48–54.34	< 0.0001
2008	42.24	21.32–83.67	< 0.0001	37.04	9.45–145.19	< 0.0001
Duration AS-SP use (months)	1.11	1.10–1.12	< 0.0001	0.99	0.95–1.02	0.416
Duration SP-IPTp use (years)	4.40	3.43–5.65	< 0.0001	†	†	†
Age (years)	0.95	0.93–0.99	0.003	0.92	0.89–0.96	< 0.0001
Parasite prevalence (%)	0.95	0.94–0.95	< 0.0001	0.99	0.98–1.00	0.290
Gender	0.90	0.74–1.11	0.311	0.84	0.66–1.07	0.152
Fever	2.26	1.32–3.87	0.003	2.35	1.29–4.29	0.005

*Within site and within district correlations of mutation frequency were taken into account in the estimation of 95% confidence intervals (CI).
†The adjusted odds ratio (OR) for the effect of intermittent preventive treatment in pregnancy (IPTp) could not be evaluated because of perfect correlation with the year after 2005.

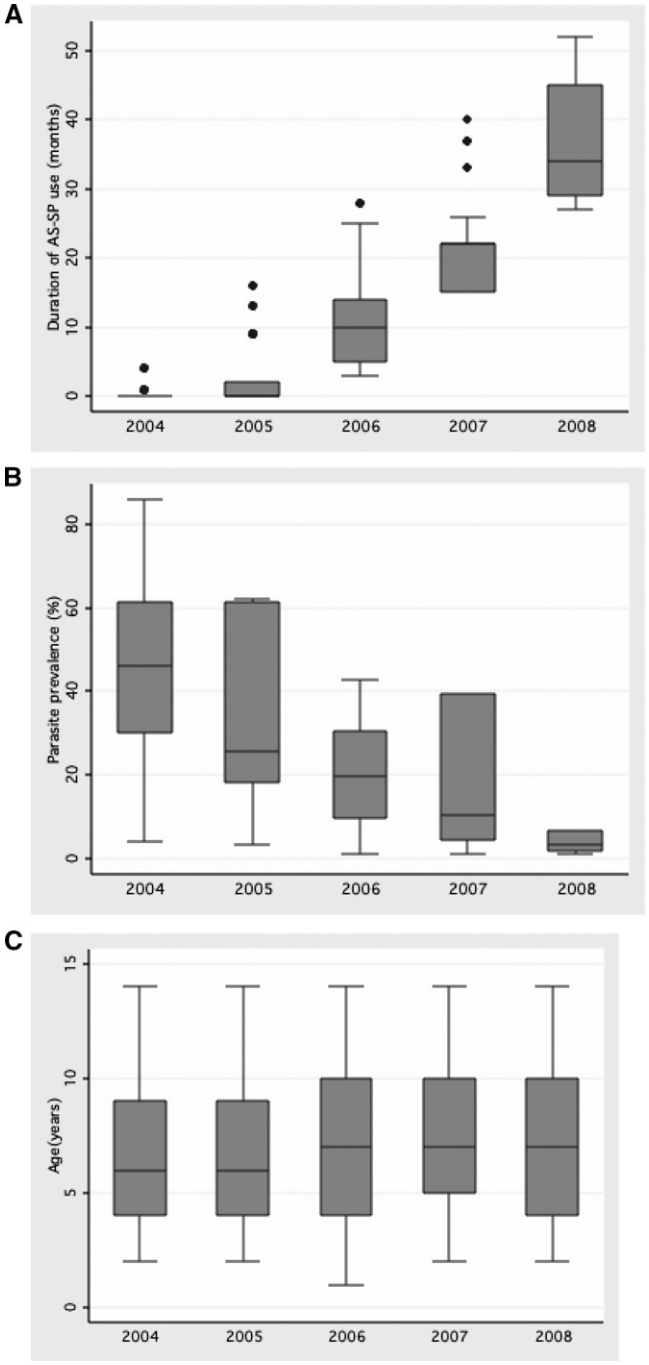

FIGURE 3. Correlation between study year and (**A**) duration of AS-SP use ($R = 0.84$), (**B**) parasite prevalence ($R = -0.49$), and (**C**) age ($R = 0.04$).

being mefloquine sensitive. This is not the case in Mozambique where most neighboring countries show a high prevalence of parasites carrying the *dhfr* and *dhps* mutations.[11–13,21]

There are a number of local factors other than the region wide increase in *dhfr* and *dhps* mutations that may have contributed to the alarmingly rapid spread of SP-resistant parasites in Maputo Province after the systematic large-scale deployment of AS-SP. These include the fixation of the *dhfr* triple mutation before the commencement of AS-SP deployment in 2004,[21] and the use of SP monotherapy for IPT of pregnant women since 2006. People with resistant parasites tend to have enhanced gametocyte carriage and are more infectious to mosquitoes than individuals with wild parasites, even during the primary infection.[33,34] Although gametocyte carriage is substantially reduced after the addition of AS to SP,[35] gametocyte transmission to mosquitoes is not completely halted[36] and data are needed to determine whether this ACT alters the ratio of resistant to sensitive infections seen after SP monotherapy.

In addition to the factors listed previously that could have contributed to the rapid selection and spread of SP-resistant falciparum parasites in southern Mozambique, pharmacokinetic studies have shown that children less than 5 years of age have been systematically under-dosed with the currently recommended SP dose.[37] Sub-therapeutic drug concentrations together with lack of acquired immunity could explain the significantly increased risk of mutations found in parasites infecting young children in Maputo Province.[16]

For the benefits of ACTs to be realized, it is essential that the individual component drugs are effective in their own right. This is most critical now that artemisinin resistance has been confirmed in South East Asia[38,39] In infections with concomitant resistance to the longer-acting partner drug, ACTs provide selective pressure for artemisinin resistance. It is imperative that resistance in partner drugs is closely monitored, to ensure that national treatment policies remain effective. This is of particular importance in countries where malaria control interventions have been successful, markedly reducing malaria incidence and thereby limiting the feasibility of *in vivo* therapeutic efficacy studies.

Although the negative association between parasite prevalence and "quintuple" mutation prevalence was not confirmed in our multivariable analysis, the primary sources of antimalarial drug resistance historically have been low intensity transmission areas. Because of the lack of immunity in these populations, most infections are symptomatic, resulting in increased treatment seeking behavior and drug pressure. This is becoming increasingly pertinent in Africa in light of the recent successes in drastically reducing the malaria burden in countries with previously high transmission intensities, including Kenya, Rwanda, Tanzania, Zanzibar, The Gambia, Eritrea, Equatorial Guinea, and southern Mozambique.[40]

The role of SP monotherapy for IPT may need to be reconsidered in light of our data showing that the "quintuple" mutation is nearing fixation in southern Mozambique, and the potential contribution of SP-IPTp to the spread of SP resistance.[14] Although a relationship between SP resistance and the benefits provided by SP-IPTp must exist, the SP resistance threshold at which IPTp ceases to be effective has not yet been determined.[41]

African countries with high pre-existing levels of SP resistance have achieved poor cure rates using AS-SP.[42,43] Although AS-SP was shown to be highly efficacious at the start of ACT roll out,[16] the high pre-existing *dhfr* triple mutation prevalence at the time of AS-SP implementation, together with the rather sharp increase in both the *dhps* double and "quintuple" mutations following ACT implementation, is cause for concern. Allen and colleagues[16] showed the presence of the "quintuple" mutation resulted in a 3-fold increased risk of treatment failure in Maputo Province, after adjusting for treatment arm, age, and temperature. Despite the marked increase in the "quintuple" mutation over the study period, the *dhfr* 164 mutation,

associated with highly pyrimethamine-resistant parasites was not detected.

The dramatic reduction in asexual parasite prevalence in Maputo Province can be attributed to a combination of intensive indoor residual spraying[15] and effective case management following the AS-SP deployment. For this impressive malaria control program to be sustained it is essential that effective insecticides and antimalarials continue to be used. The steep rise in "quintuple" mutations found in this study points to a reduced useful therapeutic lifespan of AS-SP. The low asexual parasite prevalence and high "quintuple" mutation prevalence found in our study, combined with the ongoing use of SP monotherapy for IPTp, could provide favorable conditions for artesunate resistance emergence. Additional concerns were that SP dosing is probably sub-optimal in young children and that AS-SP could not be provided as a fixed dose combination. On these grounds a change in antimalarial treatment policy to artemether-lumefantrine is currently being implemented by the Mozambican Ministry of Health.

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