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Increased prevalence of *dhfr* and *dhps* mutants at delivery in Malawian pregnant women receiving intermittent preventive treatment for malaria

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Summary

In the context of an Intermittent preventive treatment (IPTp) trial for pregnant women in Malawi, *P. falci-parum* samples from 85 women at enrollment and 35 women at delivery were genotyped for mutations associated with sulfadoxine-pyrimethamine resistance. The prevalence of the highly resistant haplotype with mutations at codons 51 and 108 of dihydrofolate reductase (*dhfr)* and codons 437 and 540 of dihydropteroate synthase (*dhps)* increased from 81% at enrollment to 100% at delivery (p=0.01). Pregnant women who were smear-positive at enrollment were more likely to have *P. falciparum* parasitemia at delivery. These results lend support to concerns that IPTp use may lead to increased drug resistance in pregnant women during pregnancy and emphasize the importance of screening pregnant women for malaria parasites in areas with prevalent SP resistance even when they are already on IPTp.

Keywords

malaria; Intermittent preventive treatment; pregnancy; *dhfr*; *dhps*; Malawi

Introduction

Pregnancy-associated malaria is the most important preventable cause of poor birth outcomes in sub-Saharan Africa (Umbers et al. 2011). Intermittent preventive treatment in pregnancy (IPTp) with 2–3 doses of sulfadoxine-pyrimethamine (SP) during the second and third trimesters reduces low birth weight infants, preterm births, maternal anemia, and neonatal mortality (Desai et al. 2007, Eisele et al. 2012). This has prompted adoption of IPTp policies in many African countries over the last decade as recommended by the WHO. However, there is increasing concern that this strategy is losing effectiveness in areas of existing and worsening high-grade SP resistance (Harrington et al. 2011, ter Kuile et al.

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2007). Also, it is uncertain if IPTp itself may be selecting for resistant parasites during pregnancy.

Malawi was the first African country to adopt IPTp with SP in 1993. It is also among the countries with the highest prevalence of SP-resistance associated mutations of *P. falciparum* in Africa, with high rates of *dhfr* (dihydrofolate reductase) triple mutants and *dhps* (dihydropteroate synthetase) double mutants since the early 2000s (Sridaran et al. 2010, Taylor et al. 2012). We genotyped samples from a large clinical trial of Malawian pregnant women receiving IPTp with SP, either alone or together with azithromycin, to examine whether progressive mutations developed from the time of enrollment to delivery. We also examined the relationship of enrollment genotypes to malaria parasitemia at delivery.

Methods

Between 2003 and 2006, 1320 pregnant women presenting to Lungwena Health Center in southern Malawi between 14–26 gestational weeks were randomized to one of three IPTp regimens: standard IPTp with 2 doses of SP, or intensive IPTp with monthly SP or monthly SP plus two doses of azithromycin (AZI-SP) (Luntamo et al. 2010). As previously reported, 117/1319 (8.9%) women were smear-positive at enrollment for *P. falciparum* or *P. malariae*. 475 women who delivered at healthcare facilities had blood samples available for analysis. Of these, 11 (2.3%) were smear-positive for *P. falciparum* at delivery; with real-time PCR detection, the number of women with evidence of malaria at delivery increased to 51/475 (10.7%) (Rantala et al. 2010). Microscopic examination of placental and cord blood yielded an additional 4 cases with evidence of *P. falciparum* parasitemia at delivery despite negative smear results from the peripheral blood. The intensive IPTp regimens were protective against PCR-positive parasitemia in women at delivery (Luntamo et al. 2012). Though a similar trend was seen for smear positivity at delivery, it was not significant probably due to low numbers. Women in the AZI-SP arm also had lower rates of preterm delivery and low birth weight compared to those who received standard IPTp, but the benefit observed in the monthly SP group was not statistically significant (Luntamo et al. 2010).

We genotyped the 117 smear-positive enrollment samples and 51 PCR-positive delivery samples at codons 51, 59, 108, and 164 in *dhfr* and codons 437, 540, and 581 in *dhps* using real-time PCR assays (Alker et al. 2004, 2005). Mixed alleles were categorized as mutants. All isolates that amplified at *dhfr*108 showed the Asn/Thr mutant allele, while the *dhfr*164 codon was universally wild type in amplified samples. Successful amplification at 4 other loci – *dhfr*51 and *dhps* codons 437, 540, and 581 - was achieved for 88 (75%) of the enrollment and 35 (69%) of the delivery samples. None of the samples amplified at *dhfr*59, but prior studies in Malawi have shown that this single nucleotide polymorphism was fixed in the population by 2003 (Taylor et al. 2012).

Results

At enrollment the genotype dhfr 51^{Ile}/dhps 437^{Gly} 540^{Glu}, consistent with the resistant quintuple *dhfr/dhps* mutant, comprised 81% of isolates (Table 1). Only one isolate out of 88 (1.1%) was mutant at *dhps* codon 581, considered the final mutation on the path to increasing SP resistance. The remaining 18% of enrollment isolates showed a less resistant genotype. In contrast, at delivery, 100% of isolates harbored resistance mutations at *dhfr* 51 and *dhps* 437/540, a significant difference (p=0.01 by Fisher's exact test). No isolates were mutant at *dhps* codon 581.

Discussion

It is unclear how the increase in resistant genotypes at delivery impacted the potential added efficacy of monthly ITPp over standard 2 dose SP in reducing preterm delivery and low birth weight infants. Although in the study sample the difference in treatment effect between the monthly and stardard regimens was not statistically significant, the observed effect sizes for duration of pregnancy and low birth weight suggested a sizable benefit in favor of the monthly regimen. It is likely that in areas of high SP resistance, the efficacy of 2 dose SP is partially compromised because of the shortening duration of post-treatment prophylaxis, and monthly dosing compensates for this by increasing the overall duration of protection relative to the 2 dose regimen. Indeed, based upon multiple studies showing that monthly SP is better at reducing the risk of low birth weight even in areas where a high proportion of parasites carry quintuple *dhfr/dhps* mutants, an evidence review group convened by the WHO has recommended SP administration at every antenatal visit (WHO 2012).

Importantly, the accumulation of more resistant alleles at delivery suggests increased selection of SP-resistant mutants during pregnancy, possibly as a result of IPTp. Other studies have shown conflicting results in this regard. For example, in a 2006 sampling of pregnant women in Ghana by Mockenhaupt et al. (2008), 73% (69/95) harbored *dhfr* triple mutants in early gestation, almost none of whom had received IPTp. This percentage remained constant among delivering woman, of whom the majority had taken IPTp with SP at least once, with 73% (55/75) of placental PCR genotypes revealing triple *dhfr* mutants. Thus, the administration of IPTp in pregnancy in that cohort did not seem to affect the prevalence of the *dhfr* triple mutant genotype at delivery. In another study in Tanzania, the proportion of pregnant women who harbored resistant alleles at *dhps* codon 581 at delivery was significantly higher in those who had received any IPTp vs. no IPTp (Harrington et al. 2009). Among all the isolates in our study, only one parasitemia out of 149 (0.7%) harbored the *dhps* 581 mutation. The paucity of this mutation is consistent with other studies of pregnant women in Malawi despite heavy IPTp-SP use (Taylor et al. 2012). Thus, selection pressures on *dhfr* and *dhps* may vary from location to location depending on the level of resistance in the parasite population.

Of the 35 women with quintuple mutants at delivery, 24(69%) had received standard IPTp with two doses of SP, while 6(17%) had received SP monthly, and 5(14%) had received SP monthly plus two doses of azithromycin as intensive IPTp regimens. This ratio is reflective of the frequency of PCR-positive malaria in the 3 groups [33/159(21%), 10/150(6.7%), and 8/166(4.8%) in the standard, monthly SP and AZI-SP groups, respectively]. Thus, by reducing the number of women with parasitemia, presumably as a result of more successfully killing of partially resistant parasites, intensive regimens have the potential to reduce selection pressure in the population. However, no inferences can be made about whether the combination regimen (AZI-SP) protects against the development of resistance.

When parasitemia at delivery and enrollment in the same women were compared, those who were smear-positive at enrollment maintained a higher risk of *P. falciparum* parasitemia at delivery (Table 2). This increased risk -- approximately two-fold for PCR outcomes and greater for smear positivity -- existed despite dosing of SP or SP and azithromycin in 95% (1251/1320) of study participants between 28–34 weeks gestation. Thirty-six women were smear positive at this third trimester visit. Of the16/36 women who had delivery samples available, only 10/16 (63%) were successfully treated with no evidence of malaria at delivery. 3/16 were smear-positive at delivery and 6/36 were PCR positive. Five of these parasitemic women were in the standard IPTp group. This is not to suggest that these women were parasitemic throughout pregnancy, as the majority of women (101/106 or 95%) who were smear-positive at enrollment showed evidence of clearance at their 28–34 week

visit. Rather, these recurrent infections at delivery could be due to recrudescences; alternatively, the women may have had multiple infections as a result of residing in local transmission “hot spots” (Bousema et al. 2010).

There are several limitations to this study. The fixation of *dhfr* and *dhps* mutants in the delivering women does not allow us to detect an influence of the different treatment arms on the prevalence of SP resistance alleles at delivery. Since the trial did not include women who did not receive any ITPp, we also cannot rule out the possibility that the increased prevalence of drug resistant mutants at delivery was due to a general trend of increasing drug resistance over time. Finally, the limited number of malaria positive delivery samples also precludes an assessment of whether enrollment genotype influenced malaria outcome at delivery. As yet, dhfr alleles have not been correlated to SP effectiveness in pregnant women. Among other factors, the contribution of immunity to parasite clearance likely influences the association of treatment outcomes with genotypic status.

In conclusion, within a cohort of pregnant women on IPTp, we detected an increase in the prevalence of alleles associated with SP resistance from enrollment to delivery. However, few or no high-level resistance mutations (*dhps*581 and *dhfr*164) were seen. We also found that despite intensive IPTp regimens, in this area of high prevalence of SP resistant parasites, one-fifth of smear-positive pregnant women at enrollment had evidence of malaria parasites also at delivery. Our findings are consistent with a moderate level of selection of SP resistance in women receiving SP IPTp in Malawi.

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Table 1

Prevalence of SP-resistance associated mutations in malaria-positive pregnant women at enrollment and delivery

Mutated codons						No. of positive isolates (%)		
DHFR			DHPS			Enrollment (n=88)	Delivery (n=35)	p-value
108*	51	164**	437	540	581			
+	−	−	−	−	−	1 (1.1%)	0	
+	−	−	+	−	−	1 (1.1%)		
+	−	−	+	+	−	4 (4.5%)	0	
+	+	−	−	−	−	7 (8.0%)	0	
+	+	−	+	−	−	3 (3.4%)	0	
+	+	−	+	+	−	**71 (81%)**	**35 (100%)**	**0.01**
+	+	−	+	+	+	1 (1.1%)	0	

*All isolates amplified at *dhfr* 108 were mutant

**All isolates amplified at *dhps* 164 were wild type

Table 2

Malaria prevalence at delivery based on enrollment malaria status

	Smear-positive at enrollment (n=117)	Smear-negative at enrollment (n=1202)	total	p-value
No. with delivery samples available	43 (37%)	432 (36%)	475	
PCR+ at delivery	8/43 (19%)	43/432 (10%)	51	0.08
Smear+ at delivery *	6/43 (14%)	9/432 (2.1%)	15	<0.001
Malaria+ at delivery **	10/43 (23%)	45/432 (10%)	55	0.01

* smear-positive for *P. falciparum* in peripheral, placental, or cord blood

** combined outcome of smear or PCR positivity for *P. falciparum* in peripheral blood

P-value compares proportion with malaria outcome at delivery in those who were smear-positive vs. smear-negative at enrollment.