

A Community-Randomized Evaluation of the Effect of Intermittent Preventive Treatment in Infants on Antimalarial Drug Resistance in Southern Tanzania

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Background. Intermittent preventive treatment in infants (IPTi) is the administration of sulfadoxine-pyrimethamine (SP) at 2, 3, and 9 months of age to prevent malaria. We investigated the influence of IPTi on drug resistance.

Methods. Twenty-four areas were randomly assigned to receive or not receive IPTi. Blood collected during representative household surveys at baseline and 15 and 27 months after implementation was tested for SP and resistance markers.

Results. The frequency of SP in blood was similar in the IPTi and comparison areas at baseline and at 15 months. *dhfr* and *dhps* mutations were also similar at baseline and then increased similarly in both arms after 15 months of SP-IPTi. First-line treatment was switched from SP to artemether-lumefantrine before the final survey, when SP positivity fell among infants in comparison areas but increased in IPTi areas. This was accompanied by an increase in *dhfr* but not *dhps* mutations in IPTi areas ($P = .004$ and $P = .18$, respectively).

Conclusions. IPTi did not increase drug pressure or the selection on *dhfr* and *dhps* mutants, when SP was the first-line malaria treatment. Introduction of artemether-lumefantrine was followed by an increase in *dhfr* mutations, consistent with weak selection attributable to SP-IPTi, but not by an increase in *dhps* mutations, suggesting a fitness cost of this mutation.

Keywords. Malaria; IPTi; sulfadoxine; pyrimethamine; *dhfr*; *dhps*; mutations.

Intermittent preventive treatment in infants (IPTi) consists of treatment doses of an antimalarial at the time of routine vaccinations in the first year of life. IPTi can reduce malaria and anemia in young children in malaria-endemic countries [1], but concerns remain about its effect on drug resistance.

IPTi has been associated with increases in resistant parasites among recipients [2–4], although this is not

a consistent finding [5]. More important, however, is the effect of IPTi on the spread of resistance through the whole population. Models suggest only a small effect at this level [6, 7], but this has not been evaluated in the field. We measured the impact of IPTi with sulfadoxine-pyrimethamine (SP) on population-wide resistance in a setting of high malaria endemicity in southern Tanzania.

There is no established method for the evaluation of the effect of public health interventions on the spread of resistance. It is important to test the effects over as many replicates as possible, to reduce the likelihood of confounding due to an imbalance between intervention and comparison arms. The optimal size of each replicate is not known: they should be large enough to ensure that contaminating gene flow between intervention and comparison areas does not obscure

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selection of resistant parasites but not so large that the evaluation becomes unfeasible or the parasite populations in different replicates are so unrelated they are incomparable. This suggests a need for baseline studies of genetic exchange between replicates, which we did using neutral microsatellite markers.

The drug recommended for IPTi is SP, a coformulation of competitive inhibitors of the folate biosynthesis enzymes dihydropteroate synthase (DHPS; inhibited by sulfadoxine) and dihydrofolate reductase (DHFR; inhibited by pyrimethamine). Resistance to SP is conferred by substitutions within the active site of target enzymes, as inferred by *in vitro* studies [8–12], by the predictive association of particular mutations with treatment failure [13–15], and from overrepresentation of mutations in recrudescing infections after treatment [16–26]. In both enzymes, increasing numbers of substitutions confer increasing levels of insensitivity to drug, with single- or double-mutant *dhfr* alleles being mildly resistant while the triple-mutant *dhfr* allele is highly resistant. The combination of the *dhfr* triple-mutant N51I + C59R + S108N (CIRN) and the *dhps* double-mutant A437G + K540E (SGE) predicts failure of SP treatment [13–15]. Drug pressure arising from IPTi-SP can be assessed in a number of ways. First, blood specimens from a representative sample of people living in intervention and comparison areas can be tested for sulfadoxine; the proportion of positive blood spots reflects drug pressure in the different populations.

Second, *dhfr* and *dhps* resistance mutations can be tracked in intervention and comparison areas. If gene frequencies measured at baseline are similar in the 2 arms, then direct comparison of frequencies at follow-up indicates the differing degrees of selection. Estimation of the selection coefficient of key resistance alleles in intervention and comparison areas provides a measure of the rate of change of gene frequencies.

The third approach is to estimate the linkage disequilibrium between different *dhfr* and *dhps* alleles, in particular the *dhfr* triple-mutant CIRN and the *dhps* double-mutant SGE. These 2 alleles independently segregate on 2 different chromosomes but can be maintained in linkage disequilibrium by drug selection despite recombination, because of the improved survival of the combined CIRN +SGE genotype.

By using these approaches, we report a community-randomized study that evaluated the extent to which IPTi increased drug pressure and the selection of resistant parasites.

METHODS

Study Area

The study was conducted in 5 districts of southern Tanzania [27], with a total population of approximately 900 000 individuals, including approximately 23 400 infants. The districts are subdivided into 24 administrative areas, called divisions. IPTi was randomly assigned to 12 divisions [28], leaving 12

divisions as controls, and unblinded IPTi implementation started in all 62 vaccinating health facilities in intervention divisions in April 2005. Tablets of SP were offered to children attending facilities for routine vaccinations with diphtheria-tetanus toxoid-acellular pertussis, hepatitis B virus, and oral poliovirus vaccine, at approximately 2 and 3 months of age, and a third dose was offered when attending for measles vaccination, at about 9 months of age. Children weighing <5 kg received a quarter tablet of Fansidar, and those weighing ≥5 kg received a half tablet of Fansidar; 1 tablet contains 500 mg of sulfadoxine and 25 mg pyrimethamine. In 2006, a minimum estimate of 47% of neonates aged 6–11 months had received at least 2 doses of IPTi, with a maximum of 76% having received at least 1 dose in IPTi areas [28].

At study start, first-line treatment for malaria was SP, but this changed in 2006 to artemether-lumefantrine (ALu) which became available after the 2006 survey. Diagnostic facilities were only available at approximately 10% of health facilities, so the majority of malarial diagnoses were made presumptively.

Household Surveys

Cross-sectional surveys in a representative cluster sample of households from the 24 divisions were conducted in July–August 2004 (baseline) and July–August 2006 (follow-up). A third survey was conducted during July–November 2007. The baseline [27] and 2006 [28] surveys are described in detail elsewhere. In brief, all residents in 8 clusters of 30 households were sampled from each division for blood sampling. Each household in a division had an equal chance of inclusion, and no substitute household was included if residents were repeatedly absent or did not wish to participate. A modular questionnaire was administered using personal digital assistants (PDAs) [29]. Participants of all ages were invited to attend a measuring station located in the middle of the village, where a sample of blood was collected by finger prick and tested for *Plasmodium falciparum* malaria parasitemia, using the HRP-II-based Paracheck rapid diagnostic test (Orchid Biomedical Systems, Goa, India). A drop of blood was collected onto Whatman III filter paper. After air drying, filter papers were stored in ziplock plastic bags and shipped to the molecular laboratory for analysis. Sample sizes were amended for the 2006 and 2007 surveys, based on the prevalence of parasitemia and intracluster correlation coefficient of parasitemia in the previous survey. In 2006, this resulted in the random selection of 10 households per cluster for molecular studies. In 2007, sampling was stratified by age on the basis of the prevalence of parasitemia, such that all children aged 2–11 months, 12% of children aged 1–4 years, and 3% of children aged ≥5 years were sampled. This approach was expected to yield similar numbers of positive samples in each age group, and overall estimates of prevalence were weighted during the analysis

according to the sampling fraction. Each survey was powered to estimate the prevalence of *dhfr* triple-mutant and *dhps* double-mutant alleles to within 15% of the true absolute prevalence in each trial arm. The 2006 and 2007 surveys each had 80% power to detect a change of ≥ 10 percentage points in the frequency of *dhps* double-mutant alleles from baseline.

High-Performance Liquid Chromatography (HPLC) Analyses

The quality of a representative number of tablets of Fansidar was assessed using HPLC as described previously [30]. A simple random sample of filter papers with adsorbed blood samples were analyzed for SP, using HPLC with photodiode array detection. Antimalarial drugs were extracted from the filter paper into methanol, and 20 μ L was injected directly onto the HPLC column with gradient elution from 100% solvent A (20 mM ammonium formate; pH 2.7) to 100% solvent B (acetonitrile) over 15–25 minutes at a flow rate of 1.35 mL/minutes and a detector setting of 259 nm. Peak identity was confirmed by measuring the retention time, spiking the sample with commercially available standards, and determining the absorbance spectra by using the photodiode array detector. HPLC equipment and software were from Dionex (Camberley, United Kingdom). The sampling procedure was modified in the 2007 survey to account for the age stratification, with a simple random sample of 200 individuals per age group selected without considering the treatment arm from which the samples came.

SNP Genotyping

Blood spots from infected individuals were selected for analysis, with a maximum of 1 infected individual sampled at random per household. Parasite DNA was extracted using the chelex method [31], and polymerase chain reaction (PCR)-based sequence-specific oligonucleotide probing (SSOP) at codons 436, 437, and 540 of *dhps* and codons 50, 51, 59 and 108 of *dhfr* was performed using methods described previously [31]. Samples taken during 2007 were sequenced using Big Dye V3.1 dye terminator sequencing and ABi 3730 DNA analyzer (Applied Biosystems, Foster City, CA). The same primers were used to preamplify *dhfr* for SSOP and direct sequence typing [31]. Two additional primers were used in sequencing *dhps* [32]. A sample was considered to have a single haplotype when only 1 sequence variant was found at each locus. In mixed genotype infections, majority genotypes were recorded if their hybridization signal was twice that of the minority sequence. One haplotype only was counted from each infection, and mixed infections in which haplotypes could not be resolved were omitted from the frequency calculations.

Microsatellite Analysis

To describe the extent of gene flow between divisions, 6 microsatellite markers, PFG377, PFPk2, PolyA, ARA, TA109, and

TA87, were amplified in baseline survey samples following a protocol previously described [33]. Amplification products were diluted a ratio of 1:100 into Hi Di formamide, run with LIZ-500 size standard on the ABi 3730 DNA analyzer (Applied Biosystems), and analyzed using Genemapper software (Applied Biosystems).

Multiplicity of infection was determined by counting the number of peaks observed at PolyA, PFPk2, and TA109 and calculating the average number of peaks across the 3 loci. This is a more conservative estimator of multiplicity of infection than taking the largest of the 3 values.

Molecular and HPLC laboratory analyses were conducted with laboratory workers blind to whether samples came from IPTi or comparison areas.

Data Processing and Analytical Methods

Palm m130 PDAs with 8 Mb RAM and running Pendragon Forms 4.0 software were used to enter data in the field. Automated range, consistency, and completeness checks were performed at the time of data entry. Particular attention was paid to the accuracy of dates because age is prone to recall errors.

Molecular and HPLC data analyses were performed in the statistical package R [34]. Analytical methods were those appropriate for cluster-randomized trials with a small number of clusters [35], with statistical testing based on the *t* test, using a summary measure (eg, mean frequency) of the data from each of the 12 intervention and 12 comparison divisions. This adjusts both for the survey and study designs. Weighted *t* tests were used for comparisons across age strata of the HPLC data, using Bland's method [36].

Population structure at the unlinked microsatellites for all pairwise comparisons of divisions was determined using the fixation index F_{ST} , calculated in Arlequin [37], and Nei's standard genetic distance [38], calculated in Phylip [39]. F_{ST} calculates the portion of expected heterozygosity that is attributable to population subdivision, whereas Nei's distance is based on allele frequency differences between populations. Both indices range between 0 and 1, with 0 indicating that populations are identical.

Ethics

The trial (clinical trials registration NCT00152204) was approved by local and national institutional review boards (the Ifakara Health Institute [formerly the IHRDC] and the National Tanzania Medical Research Co-coordinating Committee). Ethics and research clearance were also obtained from LSHTM and from the Ethics Commission of the Cantons of Basel-Stadt and Basel-Land, Switzerland. During field work, information sheets in Swahili were provided, and written informed consent was sought from the heads of all households.

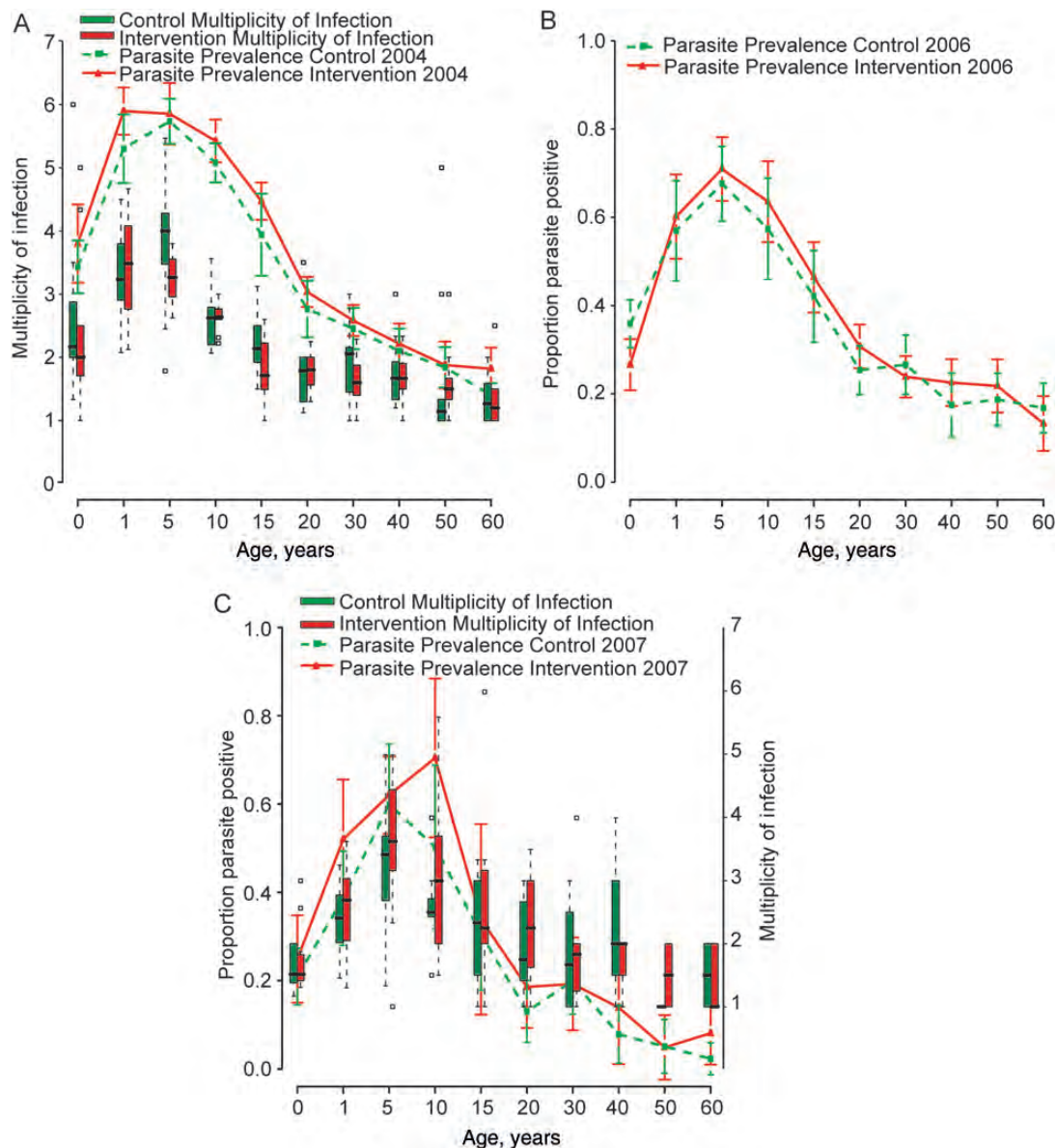


Figure 1. Parasite prevalence among age groups, with 95% confidence intervals, for the baseline 2004 survey (A) and the postintervention surveys in 2006 (B) and 2007 (C). The comparison arm is shown by a dashed green line, and the intervention arm is denoted by a solid red line. The multiplicity of infection was measured in 2004 and 2007 and is shown by the box plots (comparison, green; intervention, red), which specify the median among divisions in each arm, with interquartile ranges and the upper and lower extremes of the distribution. Statistical outliers are indicated by small squares.

RESULTS

Figure 1 shows that parasite prevalence, multiplicity of infection, and the age-pattern of each was comparable in intervention and comparison divisions at each survey point. Parasite prevalence fell to a similar extent in both arms during the trial. Heterozygosity, a measure of genetic diversity, was high across the 24 divisions, with mean values ranging from 0.771 to 0.831. Pairwise F_{ST} values ranged from <0 to 0.07 (only 5 comparisons were significantly >0), and Nei's standard genetic distance ranged from 0.031 to 0.139 (data available on

request), indicating a high degree of genetic similarity among divisions.

SP Detection

Figure 2 shows the proportion of SP-positive blood spots in comparison and intervention populations, by age. At both baseline and in 2006, there was no evidence of any difference in SP positivity between trial arms in the group aged <1 year (2004, $P = .251$; 2006, $P = .186$) or the group aged ≥ 1 year (2004, $P = .34$; 2006, $P = .38$). In 2007, the proportion of SP-positive blood spots was lower in the comparison arm than in

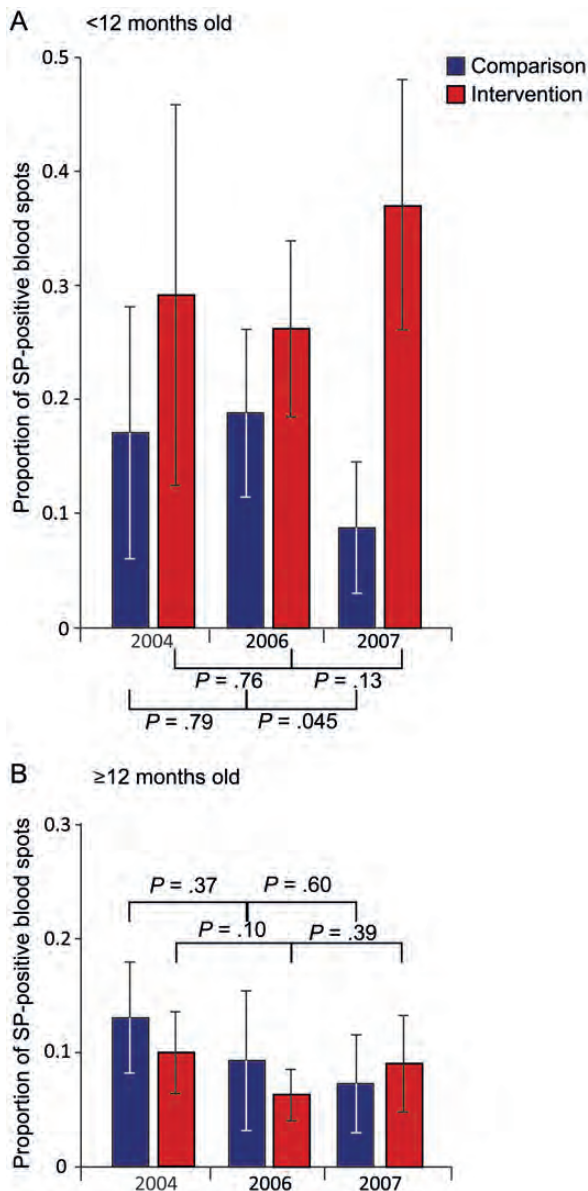


Figure 2. Proportion of sulfadoxine-pyrimethamine (SP)-positive blood spots in control (blue) and intervention (red) arms. SP positivity among children aged <12 months (A) and children aged ≥ 12 months (B) are shown for 3 surveys, conducted in 2004, 2006, and 2007. Whiskers denote 95% confidence intervals, and the significance values arising from comparisons of SP positivity rates within the arms of the trial between consecutive time points are indicated.

the intervention arm in the <1 year group (8.8% [n = 117] vs 37.1% [n = 79]; $P = .0003$). There was no significant difference in the ≥ 1 year group (7.3% [n = 218] vs 9.1% [n = 180]; $P = .57$).

Resistance Alleles and Selection Coefficients

Table 1 shows the number of samples typed and included in subsequent analyses and the frequency of the various alleles

identified. Mutations were found at codons 51, 59, and 108 but not codon 164 of *dhfr* and at codons 436, 437, and 540 of *dhps*. In 2007, the 581 *dhps* mutation was found in 0.5% of samples from the comparison arm but was absent from the intervention arm. A previously unidentified S436H substitution was found among 12 of the 394 samples sequenced for *dhps* in 2007.

The frequencies of the *dhfr* triple-mutant CIRN allele and the *dhps* double-mutant SGE haplotypes in each division are shown in Figure 3. The general trend was for *dhfr* mutant frequencies to increase over time. The CIRN allele frequency was not different in the 2 study arms in 2004 ($P = .89$) or 2006 ($P = .93$), and SGE frequencies were also comparable between arms at these times (2004, $P = .52$; 2006, $P = .54$). However, in 2007, the CIRN allele frequency was higher in the intervention than in the comparison arm ($P = .004$). The frequency of the SGE allele was (nonsignificantly) higher in the intervention arm than in the comparison arm ($P = .18$).

The corresponding selection coefficients were similar in the 2 arms of the trial between 2004 and 2006 (Table 2). However, the 2006–2007 SGE selection coefficient became negative, most markedly in the comparison arm.

To assess the specific impact of IPTi among infants, we compared resistance allele frequencies in subjects aged <1 year with those among subjects aged > 1 year. There was some evidence that the frequency of resistance alleles was higher among those aged <1 year than in older age groups across both arms at all time points (Table 3). There was no discernible effect of IPTi on the frequency of resistance among subjects aged <1 year in the intervention arm. Among subjects aged ≥ 1 year, the only evidence of a difference in resistance allele frequencies was in 2007, when the CIRN allele frequency was 13 percentage points higher in the intervention arm ($P = .034$).

Linkage Disequilibrium

There was strong evidence of linkage disequilibrium between the *dhfr* triple-mutant CIRN and the *dhps* double-mutant SGE (Table 4), with a statistically significant association in both arms at all 3 time points, except for the intervention arm in 2007.

DISCUSSION

We have described the impact of IPTi on the spread of resistance to SP at the population level. We monitored SP drug pressure, *dhfr* and *dhps* mutation frequencies, selection coefficients, and linkage disequilibrium in the context of a pilot implementation of IPTi in a total population of approximately 1 million in southern Tanzania. Representative cross sectional household surveys were performed in 2004, before the introduction of IPTi, and again in 2006 and 2007, after 15 and 27

Table 1. Number of Samples Typed and Included in the Analysis of Drug Resistance Alleles and the Frequencies of All *dhfr* and *dhps* Alleles Detected Over the 3 Surveys

Survey	2004		2006		2007	
DHFR						
No. samples	2144		2441		850	
Typed at all SNPs	1234		1539		513	
Mixed	411		424		104	
	Comparison (n = 415)	Intervention (n = 408)	Comparison (n = 524)	Intervention (n = 591)	Comparison (n = 202)	Intervention (n = 207)
CIRN	0.435	0.444	0.512	0.517	0.600	0.743
CNRN	0.295	0.284	0.340	0.350	0.196	0.138
CNCS	0.131	0.148	0.052	0.059	0.047	0.036
CICN	0.058	0.063	0.069	0.053	0.15	0.08
CNCN	0.035	0.029	0.006	0.007	0.008	0.006
CICS	0.005	(...)	0.004	(...)	(...)	(...)
CIRS	0.016	(...)	(...)	0.002	(...)	(...)
CNRS	0.025	0.032	0.016	0.012	(...)	(...)
DHPS						
No. samples	2144		2441		850	
Typed at all SNPs	1199		1458		529	
Mixed	197		379		135	
	Comparison (n = 499)	Intervention (n = 503)	Comparison (n = 509)	Intervention (n = 570)	Comparison (n = 194)	Intervention (n = 200)
SGE	0.352	0.383	0.529	0.564	0.467	0.570
SAK	0.488	0.487	0.320	0.322	0.402	0.330
SGK	0.021	0.026	0.015	0.014	0.006	0.005
AAK	0.075	0.052	0.045	0.029	0.038	0.012
AAE	0.002	0.004	0.002	0.003	(...)	(...)
AGE	(...)	(...)	0.017	0.004	0.012	0.003
CAE	(...)	(...)	(...)	0.002	(...)	(...)
CAK	0.010	0.008	0.005	0.008	0.013	0.021
FAE	(...)	0.003	(...)	(...)	(...)	(...)
FAK	0.028	0.024	0.019	0.017	0.011	0.018
FGK	(...)	0.002	(...)	(...)	(...)	(...)
SAE	0.025	0.010	0.048	0.036	(...)	(...)
HAKAA	na	na	na	na	0.046	0.041
SGEGA	na	na	na	na	0.005	(...)

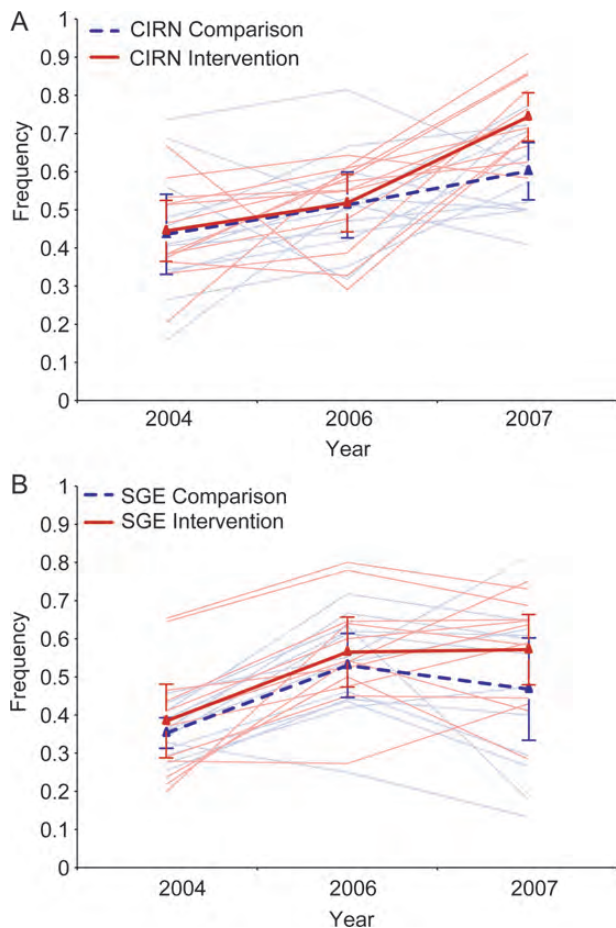


Figure 3. Division-specific frequencies of the *dhfr* triple-mutant CIRC allele (A) and the *dhps* double-mutant SGE allele (B) in the comparison (blue) and intervention (red) replicate divisions over time. The bold lines indicate the mean frequency in each treatment arm.

months of IPTi implementation. Malaria transmission, reflected by parasite prevalence, multiplicity of infection, and the age-pattern of each, was comparable in IPTi and comparison areas throughout the study. There was considerable genetic diversity among parasites across the study area, and gene flow

studies found no evidence of population subdivision between divisions. At baseline, the genetic determinants of SP resistance were present at similar frequencies in each study arm.

Between 2004 and 2006, while SP was the first-line treatment for malaria infections and the drug used for IPTi, the frequency of the CIRC and SGE alleles increased at a comparable rate in both study arms. The overall prevalence of SP was 11%–13% and not different between the comparison and intervention arms. Thus, IPTi had no measurable effect on SP drug pressure over that generated by its widespread use for treatment, suggesting that the number SP doses used for IPTi was balanced by the number of SP treatments for illness episodes in comparison areas. IPTi may therefore be of greater value in areas where access to vaccination-based preventive services is better than access to drugs through curative services.

In late 2006, the national treatment policy with ALu was rolled out, and SP use decreased. However, the proportion of people aged >12 months who were positive for SP had changed little by the time of the 2007 survey, with SP detected in all age strata. There was a statistically significant decrease in detectable SP among individuals aged <12 months living in comparison areas, presumably because these infants were increasingly treated with ALu. In contrast, SP increased in infants where IPTi-SP continued.

The change in SP drug pressure in infants living in IPTi areas was associated with an overall increase in the frequency of the *dhfr* triple mutation and a corresponding 3-fold difference in the selection coefficient for the CIRC allele. This suggests a population-wide effect on *P. falciparum* genotypes of an intervention targeting children aged 2–9 months. In contrast, the *dhps* double-mutant frequencies stabilized in both comparison and intervention divisions following withdrawal of SP as first-line therapy, with a suggestion of a decrease in the comparison arm, in keeping with the notion that the SGE double mutant carries a fitness cost [40].

The study has a number of limitations. We were limited to 12 intervention and 12 comparator divisions. Therefore,

Table 2. Selection Coefficients and Mean Change in CIRC and SGE Allele Frequencies Across Divisions Within Trial Arms Between Consecutive Time Points

Allele, Trial Arm	Mean Difference in Frequency									
	2004–2006	<i>P</i>	<i>s</i>	2006–2007	<i>P</i>	<i>s</i>	2004–2007	<i>P</i>	<i>s</i>	<i>s</i> , 2004–2006–2007
CIRC										
Comparison	0.077	.955	0.090	0.089	.057	0.044	0.166	.152	0.075	0.077
Intervention	0.073		0.089	0.226		0.146	0.299		0.108	0.105
SGE										
Comparison	0.177	.938	0.069	–0.062	.278	–0.060	0.115	.316	0.026	0.033
Intervention	0.181		0.067	0.006		–0.002	0.187		0.044	0.047

Abbreviation: *s*, selection coefficient.

Table 3. Allele Frequencies in People Under 1 Year Old and in People Older Than 1 Year

	2004						2006							
	Within Age Groups			Between Age Groups			Within Age Groups			Between Age Groups				
	Control ^a	95% CI	Intervention ^a	95% CI	<i>P</i> ^b	Control <i>P</i> ^b	Intervention <i>P</i> ^b	Control ^a	95% CI	Intervention ^a	95% CI	<i>P</i> ^b	Control <i>P</i> ^b	Intervention <i>P</i> ^b
<i>dhfr</i> age <1 y														
CNCS	1/21 (0.05)	-.56 (.147)	0/20 (0)341			2/41 (0.05)	-.024 (.122)	1/52 (0.02)	-.020 (.053)	.4021		
CNRN	7/21 (0.33)	.073 (.594)	4/20 (0.2)	-.22 (.455)	.468			10/41 (0.24)	.115 (.404)	21/52 (0.4)	.166 (.626)	.2845		
CIRN	12/21 (0.57)	.334 (.848)	16/20 (0.8)	.545 (1.022)	.234			22/41 (0.54)	.341 (.756)	29/52 (0.56)	.355 (.787)	.8718		
<i>dhps</i> age ≥1 y														
CNCS	59/394 (0.15)	.035 (.236)	65/388 (0.17)	.06 (.249)	.767	.178	.004	23/483 (0.05)	.025 (.079)	33/539 (0.06)	.032 (.143)	.221	.928	.030
CNRN	113/394 (0.29)	.217 (.373)	107/388 (0.28)	.186 (.391)	.909	.760	.546	169/483 (0.35)	.275 (.418)	189/539 (0.35)	.240 (.402)	.6046	.253	.510
CIRN	166/394 (0.42)	.321 (.531)	169/388 (0.44)	.337 (.508)	.950	.209	.008	250/483 (0.52)	.421 (.607)	275/539 (0.51)	.342 (.585)	.4784	.741	.354
<i>dhps</i> age <1 y														
SAK	4/26 (0.15)	-.56 (.456)	6/23 (0.26)	.038 (.38)	.931			12/46 (0.26)	.127 (.533)	12/52 (0.23)	.147 (.532)	.9402		
AAK	1/26 (0.04)	-.42 (.109)	0/23 (0)343			1/46 (0.02)	-.025 (.067)	0/52 (0)3388		
SGE	20/26 (0.77)	.496 (.987)	14/23 (0.61)	.445 (.919)	.698			27/46 (0.59)	.355 (.716)	37/52 (0.71)	.452 (.787)	.4603		
<i>dhps</i> age ≥1 y														
SAK	242/473 (0.51)	.436 (.580)	237/480 (0.49)	.412 (.585)	.840	.025	.005	146/463 (0.32)	.246 (.400)	155/518 (0.30)	.237 (.411)	.9814	.946	.876
AAK	36/473 (0.08)	.055 (.097)	28/480 (0.06)	.033 (.076)	.126	.246	.000	21/463 (0.05)	.017 (.078)	17/518 (0.03)	.015 (.048)	.3329	.298	.001
SGE	152/473 (0.32)	.290 (.366)	180/480 (0.38)	.274 (.470)	.373	.004	.018	247/463 (0.53)	.439 (.613)	303/518 (0.58)	.460 (.649)	.6246	.918	.468

Table 3 continued.

	2007					2006 vs 2007			
	Within Age Groups					Between Age Groups		Within Age Groups	
	Control ^a	95% CI	Intervention ^a	95% CI	<i>P</i> ^b	Control <i>P</i> ^b	Intervention <i>P</i> ^b	Control <i>P</i> ^b	Intervention <i>P</i> ^b
<i>dhfr</i> age <1 y									
CNCS	1/47 (0.013)	−0.16 (.042)	1/46 (0.023)	−0.28 (.073)	.715			.334	.832
CNRN	11/47 (0.18)	.051 (−.311)	5/46 (0.10)	−0.034 (.239)	.363			.383	.027
CIRN	28/47 (0.66)	.472 (−.840)	37/46 (0.80)	.632 (−.963)	.217			.402	.081
<i>dhfr</i> age ≥1 y									
CNCS	9/155 (0.06)	.021 (−.089)	7/161 (0.04)	.008 (−.072)	.479	.049	.532	.870	.119
CNRN	30/155 (0.19)	.110 (−.265)	23/161 (0.15)	.068 (−.223)	.406	.930	.553	.003	.002
CIRN	95/155 (0.60)	.513 (−.681)	119/161 (0.73)	.633 (−.821)	.034	.528	.423	.160	.001
<i>dhps</i> age <1 y									
SAK	12/40 (0.31)	.036 (−.574)	11/42 (0.3)	.082 (−.518)	.974			.871	.766
AAK	2/40 (0.036)	.023 (−.095)	1/42 (0.03)	−0.037 (.098)	.886			.654	.341
SGE	25/40 (0.634)	.381 (−.887)	25/42 (0.55)	.297 (−.798)	.592			.487	.602
<i>dhps</i> age ≥1 y									
SAK	62/154 (0.424)	.301 (−.548)	48/158 (0.33)	.238 (−.416)	.175	.380	.801	.142	.960
AAK	6/154 (0.035)	.006 (−.063)	2/158 (0.01)	−0.006 (.027)	.122	.964	.540	.504	.057
SGE	69/154 (0.443)	.314 (−.572)	96/158 (0.59)	.488 (−.686)	.066	.154	.749	.257	.608

^a Data are proportion (%) of subjects.

^b Calculated by the *t* test.

Table 4. Linkage Disequilibrium Between CIRN and SGE Alleles Across the 3 Time Points

Trial Arm	2004				2006				2007			
	D'	P	SGE-CIRN	n	D'	P	SGE-CIRN	n	D'	P	SGE-CIRN	n
Comparison	0.241	.001	54	269	0.138	.026	111	312	0.286	.004	52	144
Intervention	0.232	.001	69	257	0.135	.028	128	362	0.143	.159	65	139

Abbreviations: n, total number of samples genotyped; SGE-CIRN, number of samples with the SGE-CIRN genotype.

despite restricted randomization, there is still potential for unrecognized confounders to render the study arms different in important respects. Because the study arms were comparable in terms of transmission intensity, baseline SP resistance markers, and SP drug pressure, and because care was taken to select representative samples of households during the surveys, there should be increased confidence in the validity of the results. We used counting of haplotypes among single and majority infections to generate simple estimates of haplotype frequency. While there are computationally sophisticated methods of incorporating mixed infections, these require thresholds of detection of minor alleles to be standard across assays for different loci, if systematic bias is to be avoided. We did not use these measures because this could not be guaranteed across the large number of replicate assays performed during the course of this study. Second, a large number of significance tests were carried out and some of the differences may have arisen by chance. Nevertheless, adjustment of *P* values using the Bonferroni correction does not materially affect our results or conclusions. Third, follow-up continued for <2 years after the introduction of IPTi, and estimates of IPTi coverage were between 47% and 76% [28]. More pronounced effects may have been documented with longer follow-up or higher levels of coverage. Fourth, the use of HRP-II-based rapid diagnostic tests means that, because the HRP-II antigen persists after treatment, an individual may have been identified as parasite positive for >2 weeks after the infection had been effectively treated [41]. This resulted in a proportion of RDT-positive samples being PCR negative, reducing the number of samples contributing to analyses and the statistical power of the evaluation. However, both the geographical scale and the significant differences in selection pressures and haplotype frequencies between the study arms add plausibility to the evaluation. These results and conclusions are specific to SP in eastern Africa. The fitness costs of the SGE allele may be different in western Africa, where an alternative *dhps* allele is found [42]. In addition, the increase in frequency of resistance mutations is not linear, and this study was conducted when the baseline level of resistance was already high. The results may be different in settings with lower baseline resistance.

This study was conceived to evaluate the effect of IPTi-SP on the spread of SP resistance and the risk that IPTi-SP might undermine SP treatment efficacy. We have shown that IPTi-SP did not exacerbate the spread of resistance when delivered alongside SP for first-line malaria treatment. However, malaria treatment in many settings now depends on ACTs. When ACTs were introduced into the study area, the frequency of the *dhfr* triple mutant continued to climb, but the frequency of the *dhps* double mutant appeared to decrease. As treatment failure occurs primarily when *dhfr* triple is found in combination with *dhps* double, we conclude that IPTi-SP is unlikely to undermine SP treatment efficacy.

The World Health Organization has recommended that IPTi-SP be considered in areas of moderate or intense malaria transmission [43], but not if the frequency of the 540 mutation—a marker of the quintuple mutation—is $\geq 50\%$. Many countries fulfilling the intensity criterion fail the resistance criterion [44]. Our study suggests that quintuple-mutation frequencies may fall once ACTs are deployed, and it draws attention to the need to monitor molecular markers of SP resistance where IPTi-SP may otherwise be useful for malaria control.

Notes

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Potential conflicts of interest. M. T. is a member of the scientific advisory board of the Novartis Institute for Tropical Diseases, Singapore, and a member of the external board of UBS Optimus Foundation, Switzerland. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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