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Prevalence of molecular markers of resistance to sulfadoxine–pyrimethamine before and after community delivery of intermittent preventive treatment of malaria in pregnancy in sub-Saharan Africa: a multi-country evaluation

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Summary

Background The effectiveness of community delivery of intermittent preventive treatment (C-IPT) of malaria in pregnancy (IPTp) with sulfadoxine–pyrimethamine has been evaluated in selected areas of the Democratic Republic of the Congo, Madagascar, Mozambique, and Nigeria. We aimed to assess the effect of C-IPTp on the potential development of *Plasmodium falciparum* resistance to sulfadoxine–pyrimethamine, since it could threaten the effectiveness of this strategy.

Methods Health facility-based cross-sectional surveys were conducted at baseline and 3 years after C-IPTp implementation in two neighbouring areas per country, one with C-IPTp intervention, and one without, in the four project countries. Dried blood spots from children under five years of age with clinical malaria were collected. Sulfadoxine–pyrimethamine resistance-associated mutations of the *P falciparum dhfr* (Asn51Ile/Cys59Arg/Ser108Asn/Ile164Leu) and *dhps* (Ile431Val/Ser436Ala/Ala437Gly/Lys540Glu/Ala581Gly/Ala613Ser) genes were analysed.

Findings 2536 children were recruited between June 19 and Oct 10, 2018, during baseline surveys. Endline surveys were conducted among 2447 children between July 26 and Nov 30, 2021. In the Democratic Republic of the Congo, the *dhfr/dhps* IRNI/ISGEAA inferred haplotype remained lower than 10%, from 2% (5 of 296) at baseline to 8% (24 of 292) at endline, and from 3% (9 of 300) at baseline to 6% (18 of 309) at endline surveys in intervention and non-intervention areas respectively with no significant difference in the change between the areas. In Mozambique, the prevalence of this haplotype remained stable at over 60% (194 [64%] of 302 at baseline to 194 [64%] of 303 at endline, and 187 [61%] of 306 at baseline to 183 [61%] of 301 in endline surveys, in non-intervention and intervention areas respectively). No isolates harbouring the *dhps* ISGEAA genotype were found in Nigeria. In Madagascar, only five isolates with this haplotype were found in the non-intervention area (2 [$>1\%$] of 300 at baseline and 3 [1%] of 300 at endline surveys). No isolates were found carrying the *dhps* ISGEA genotype.

Interpretation C-IPTp did not increase the prevalence of molecular markers associated with sulfadoxine–pyrimethamine resistance after three years of programme implementation. These findings reinforce C-IPTp as a strategy to optimise the control of malaria during pregnancy, and support the WHO guidelines for prevention of malaria in pregnancy.

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Introduction

Malaria in pregnancy has deleterious effects in maternal and neonatal health.¹ It is estimated that 13·3 million (32%) of pregnancies were exposed to malaria in the WHO African region and 961 000 children were born with low birthweight as a consequence of malaria in pregnancy in 2021.² In addition, malaria in pregnancy is responsible for 20% of stillbirths and 11% of all newborn deaths in sub-Saharan Africa.²

Since 1998, WHO has recommended intermittent preventive treatment of malaria in pregnancy (IPTp) with sulfadoxine–pyrimethamine to control infection in

pregnant women living in moderate-to-high malaria transmission areas in sub-Saharan Africa. As of 2012, IPTp is administered at each scheduled antenatal care clinic visit from the second trimester of gestation onwards.³ The intervention has been shown to reduce maternal and placental infection,^{4,5} as well as low birthweight, maternal and fetal anaemia, and maternal and neonatal mortality.^{5–7}

The efficacy of sulfadoxine–pyrimethamine as IPTp is determined by multiple factors, such as the multiplicity of infection, the host's naturally acquired immunity to malaria, nutritional status, the rate of drug metabolism,

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See [Comment](#) page e1676

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Research in context

Evidence before this study

WHO recommends intermittent preventive treatment for malaria in pregnancy with sulfadoxine-pyrimethamine to avoid adverse pregnancy outcomes due to malaria infection. However, the efficacy of this strategy may be threatened by resistances to sulfadoxine pyrimethamine. We searched for studies reporting the prevalence of molecular markers associated with antimalarial drug resistance following the implementation of intermittent preventive treatment of malaria in pregnancy (IPTp) or other chemoprevention strategies at the community level in sub-Saharan Africa. We searched MEDLINE (via PubMed) for articles published from database inception up to May 23, 2023, using the search terms ("mass drug administration" OR "seasonal malaria chemoprevention" OR "intermittent preventive treatment" OR "perennial malaria chemoprevention" OR "post-discharge malaria chemoprevention") AND ("Plasmodium falciparum" OR "malaria") AND "drug resistance". No date or language restrictions were applied. The search did not yield any study assessing molecular markers of sulfadoxine-pyrimethamine resistance following community delivery of IPTp.

23 articles assessing the prevalence of molecular markers associated to sulfadoxine-pyrimethamine resistance across different locations in sub-Saharan Africa were retrieved. The prevalence of these molecular markers has increased since IPTp with sulfadoxine-pyrimethamine implementation, with different patterns across African countries. The *dhfr* IRNI triple mutant is above 50% in most of the countries where genomic studies were conducted, while the *dhps* ISGEEA double mutant is present in southern countries of the continent, such as Mozambique, and only present in very low frequencies in countries from West Africa, where other molecular markers such as the *dhps*-431 point mutation are present in higher incidence.

We identified two studies assessing the effect of mass drug distribution on the prevalence of molecular markers of drug resistance. One study carried out in Mozambique reported no statistically significant difference in the proportion of isolates with multicopy *pfpm2*, *pfprt* polymorphisms, and *pfmdr1* when comparing samples collected before and after mass drug

distribution with dihydroartemisinin-piperazine. Another study, performed in Grande Comoro Island, reported no evidence for selection of *pfk13* Kelch-propeller mutation after the last of three rounds of mass drug distribution with primaquine.

Additionally, we identified four studies that were undertaken in the Sahel region assessing the prevalence of molecular markers associated with drug resistance following seasonal malaria chemoprevention (SMC). The first of these studies was carried out in seven countries in the region and found no significant changes in the proportion of parasites carrying molecular markers associated to sulfadoxine-pyrimethamine and amodiaquine resistance between SMC implementation and two years later. Similarly, two additional studies performed in Mali and Senegal found that the frequencies of these molecular markers remained similar, except for *pfmdr1* Asn86Tyr in Mali and Senegal, whose frequency increased significantly. The last study, performed in Burkina Faso, found similar occurrences of molecular markers associated with sulfadoxine-pyrimethamine and amodiaquine resistance between SMC-eligible and SMC-uneligible children.

Added value of this study

Previous evaluation of the effectiveness of community delivery of intermittent preventive treatment in pregnancy (C-IPTp) has been carried out in Tanzania, Uganda, Malawi, Nigeria, and Burkina Faso, however the effect of the intervention on the prevalence of molecular markers associated to sulfadoxine-pyrimethamine resistance was not reported in any of these studies. This is, to our knowledge, the first study assessing the impact of C-IPTp on the development of parasite resistance to sulfadoxine-pyrimethamine. This information is crucial for policy makers' decisions regarding the implementation of the intervention in their countries.

Implications of all the available evidence

The study findings indicate that C-IPTp might not increase the prevalence of molecular markers associated with sulfadoxine-pyrimethamine resistance. These results provide reassurance to policy makers that C-IPTp increases IPTp coverage without threatening its efficacy.

and the development of parasite resistance to the drugs.^{8,9} In turn, parasite drug resistance is influenced by the parasite mutation rate, the fitness costs associated to the mutation, the overall parasite load, and treatment compliance.¹⁰

Parasite resistance to sulfadoxine-pyrimethamine is mediated by single nucleotide polymorphisms (SNPs) in codons 51, 59, 108, and 164 of the *Plasmodium falciparum* dihydrofolate-reductase (*dhfr*) gene, and in codons 437, 540, 581, and 613 of the *P falciparum* dihydropteroate-synthetase (*dhps*) gene. Mutations in both genes tend to accumulate in a progressive, step-wise fashion, with resistance increasing as these mutations successively

accumulate.¹¹ Some studies suggest that the protective efficacy of IPTp-sulfadoxine-pyrimethamine might be reduced in *P falciparum* carrying quintuple mutations associated with sulfadoxine-pyrimethamine resistance (the accumulation of mutations in codons 51, 59, and 108 of the *dhfr* gene; and codons 437 and 540 of *dhps* gene).¹² In addition, sextuple mutations (quintuple mutants with an additional mutation in codon 581 of the *dhps* gene) have been associated with clinical failure and loss of protective efficacy of sulfadoxine-pyrimethamine as part of IPTp when this prevalence is over 10%.¹³⁻¹⁶ Moreover, the evidence suggests that other concerning mutations are emerging, particularly the mutation in the *dhps*

431 codon originating from Nigeria.¹⁷ However, studies suggest that while the use of IPTp-sulfadoxine-pyrimethamine often results in both the selection for and increased prevalence of genetic mutants associated with resistance, this does not necessarily lead to a decrease in malaria prevention efficacy.⁹ In this regard, a meta-analysis including seven clinical trials concluded that IPTp-sulfadoxine-pyrimethamine reduced placental malaria, low birthweight, and anemia among HIV-negative pregnant women in areas where sulfadoxine-pyrimethamine had lost treatment efficacy in children.¹⁸

The Transforming Intermittent Preventive Treatment for Optimal Pregnancy (TIPTOP) project implemented a community-based program of IPTp-sulfadoxine-pyrimethamine delivery through community health workers in addition to antenatal care clinic-based delivery, in selected areas of four sub-Saharan countries—the Democratic Republic of the Congo, Madagascar, Mozambique, and Nigeria—with the aim of expanding coverage of IPTp-sulfadoxine-pyrimethamine. The project results showed that community delivery of IPTp (C-IPTp) was associated with an increase in IPTp uptake without reducing antenatal care attendance.¹⁹

Providing evidence on the effect of C-IPTp on sulfadoxine-pyrimethamine resistance is important for decision making as to whether C-IPTp can be recommended as an additional channel for IPTp delivery. We aimed to determine the effect of C-IPTp in the prevalence of the sextuple and quintuple *dhps/dhfr* mutations associated with sulfadoxine-pyrimethamine resistance.

Methods

Study design and participants

The TIPTOP project was an implementation project that trained community health workers to identify pregnant women in the community, screen them for IPTp eligibility, provide eligible people the sulfadoxine-pyrimethamine doses under direct observation, and then refer them to the health facility for comprehensive antenatal care. In collaboration with the National Malaria Control Programs, project countries were purposely selected based on specific criteria (ie, Ministry of Health [MoH] commitment to implement C-IPTp if shown to be effective, an existing community health worker's program within the MoH, and geographical and language diversity). Per country, areas that were similar in size were purposely selected for implementation, in collaboration with the national malaria control programmes. At project inception, levels of sulfadoxine-pyrimethamine resistance in the study countries varied greatly, being highest in Mozambique, and lowest in Nigeria (table 1).

This is a descriptive study where health facility-based, cross-sectional surveys were conducted at baseline, and 3 years after the implementation of C-IPTp (endline). Surveys were performed in an area (Phase 1 area) per

country where C-IPTp was implemented (intervention area),¹⁹ and in a neighbouring area with similar epidemiologic characteristics but where IPTp was delivered through antenatal care only (non-intervention area) in the four selected sub-Saharan countries. Surveys were conducted in four purposely selected health facilities in each area.

To assess whether C-IPTp increased the selection of parasites with molecular markers associated with sulfadoxine-pyrimethamine resistance, we recruited children aged younger than 5 years as the study population, with no lower age limit at baseline, but with an age limit of 6 months or older at endline to facilitate blood sampling. Monitoring the prevalence of alleles associated with resistance to drugs was done by collecting samples from symptomatic children with evidence of malaria infection. The rationale for this approach is that any overuse or misuse of the study drug in any subpopulation (including pregnant women) could select parasites strains resistant to sulfadoxine-pyrimethamine, and that those strains would then be transferred by mosquitoes to the general population, and most easily detected in children. Children aged younger than 5 years, attending the selected health facilities with clinical signs or symptoms suggestive of malaria (fever, defined as an axillary temperature $\geq 37.5^{\circ}\text{C}$, or parent or guardian reported history of fever in the previous 24 h) were eligible for a malaria rapid diagnostic test. Children were recruited if they were residents in the project areas at the time of the survey, had a positive rapid diagnostic test result, and their parent or guardian gave written informed consent. Children with signs or symptoms of severe malaria (per WHO guidelines) were excluded.

The protocol and informed consent were reviewed and approved by the WHO Ethics Review Committee (Geneva, Switzerland), the Hospital Clinic Research Ethics Committee (Barcelona, Spain), and the National Ethics Committees of the Democratic Republic of the Congo, Nigeria, Madagascar, and Mozambique (appendix 1 pp 2–3)

See Online for appendix 1

Procedures

Written informed consent was obtained from the parent or the guardian of each participant. The informed consent form was available in English, French, and Portuguese, and was translated into local languages where necessary. A questionnaire was administered to the parents or guardians to record the participants' sociodemographic characteristics. Biological sex of the child was reported as per their health card (options of male or female). Finger-prick dried blood samples were collected on filter papers. Samples were identified with a unique participant number and barcode, and were stored at 4°C at local headquarters until they were shipped for analysis to the laboratory in charge of the analysis (Institut Pasteur, Paris, France).

For DNA extraction, dried blood samples were incubated in a shaking incubator set to $60\text{--}65^{\circ}\text{C}$ and 250 revolutions

	Province or state	Intervention area	Non-intervention area	Year	Prevalence of malaria parasitaemia U5 (%)	Year	IPTp coverage (%)	Year	Prevalence of mutations related to SP resistance (%)		
									<i>dhfr</i> triple	<i>dhps</i> double	<i>dhfr/dhps</i> inferred quintuple
Democratic Republic of the Congo ^{20,21}	Kwango	Kenge	Boko	2013–14	30.9%	2013	21.0%	2004	46.2%	45.2%	27.1%
Madagascar ^{22,23}	Fianarantsoa	Manajary	Manakara and Ifanadiana	2016	7.7%	2016	12.2%	2008	55.7%	0	NI
Nigeria ^{24,25}	Ebonyi	Ohaukwu	Ezza South	2015	51.1%	2015	41.0%	2003–07	46.0%	38.5%	29.0%
Mozambique ^{26,27}	Sofala	Nhamatanda	Dondo	2015	31.5%	2015	36.1%	2015	NI%	88.4%	NI

dhfr=dihydrofolate reductase. *dhps*=dihydropteroate synthase. IPTp=intermittent preventive treatment in pregnancy. SP=sulfadoxine-pyrimethamine. U5=under 5 years of age. NI=no information. *IPTp coverage: three doses except for DRC: two doses or more and at least one antenatal care visit.

Table 1: Prevalence of malaria parasitaemia and molecular markers related to sulfadoxine-pyrimethamine resistance by study area and year of data collection

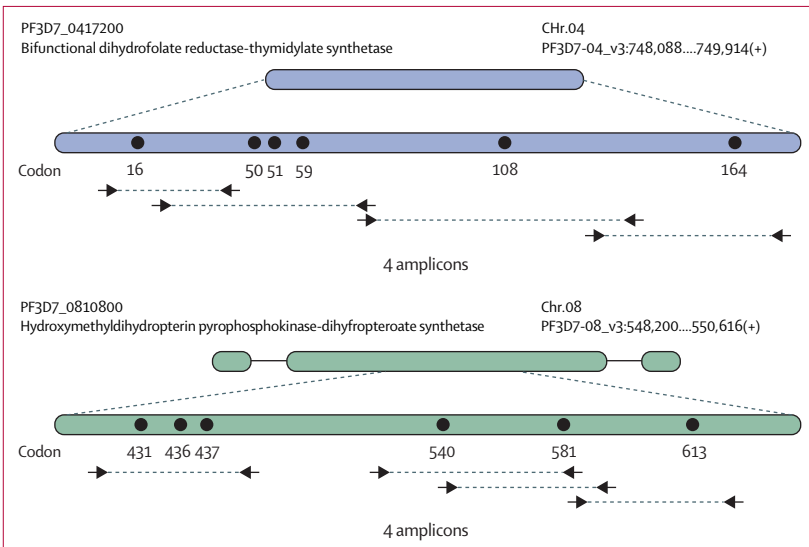


Figure 1: Schematic representation of the primers used to detect *dhfr* mutations located at codons 16, 50, 51, 59, 108, and 164, and *dhps* mutations located at codons 431, 436, 437, 540, 581, and 613
dhfr=*Plasmodium falciparum* dihydrofolate-reductase gene. *dhps*=*P falciparum* dihydropteroate-synthetase gene.

per min to assist in reconstituting the dried blood samples with lysis buffer. The lysate was transferred in 96-well DNA filter plates used for nucleic acid binding. After two washes, DNA was transferred into a new 96-well plate. The eluted DNA was quantified by fluorometric quantification (Qubit, Thermo Fisher, France), and adjusted to 20 ng/μL.²⁸ The design of the primers is detailed in figure 1. Indexed primers contained specific barcodes consisting of individual eight-base indices, specific to the sample and adapter sequences, allowing the final PCR product to bind to the sequencing MiSeq flow cell (Illumina, Evry, France). Sequencing reactions were carried out with an Illumina MiSeq instrument (Illumina, Evry, France) using paired-end 2×300 bp reads (MiSeq Reagent Nano Kit v2–300 cycles, Illumina, Evry, France) and a MiSeq flow cell, according to the manufacturer’s recommendation. The amino acid sequences were

compared with a custom database consisting of the 3D7 reference sequence. The full description of DNA extraction and sequencing is provided in appendix 1 (p 2). Parasites with mixed alleles (in which both wild-type and mutant alleles were present) were considered mutants.

Outcomes

The primary outcome was the change in the prevalence of *dhfr/dhps* sextuple mutant (ie, *dhfr* substitutions Asn51Ile, Cys59Arg, and Ser108Asn, and *dhps* substitutions Ala437Gly, Lys540Glu, and Ala581Gly, henceforth known as IRNI/ISGEGA) between the non-intervention and intervention areas over the period before the onset of the intervention to three years after baseline. Secondary outcomes were the change in the prevalence of other molecular markers associated to sulfadoxine-pyrimethamine resistance between the non-intervention and intervention areas from baseline to three years after the onset of the intervention, comprising *dhfr/dhps* quintuple mutant (ie, *dhfr* substitutions Asn51Ile, Cys59Arg, and Ser108Asn, and *dhps* substitutions Ala437Gly and Lys540Glu, henceforth known as IRNI/ISGEAA); *dhfr* triple mutant IRNI *dhps* double mutant ISGEAA; and *dhps* triple mutant ISGEGA.

Statistical analysis

To calculate our sample size, it was assumed that the prevalence of *dhfr/dhps* sextuple mutants was 0% in the intervention areas of the four countries, and that a sample size of 242 children per survey per country would provide 90% power to detect an increase of 5% or higher in the prevalence of molecular markers between baseline and endline surveys using a two-sided significance test with alpha 0.05. Assuming 10% loss of samples or uninterpretable analysis, the sample size was rounded up to 300 samples per area (intervention and non-intervention) per survey per country.

Participants’ sociodemographic characteristics were described using summary statistics. Continuous variables were summarised using means and standard

	Democratic Republic of the Congo				Madagascar			
	Baseline		Endline		Baseline		Endline	
	Non-int (n=296)	Int (n=300)	Non-int (n=292)	Int (n=309)	Non-int (n=300)	Int (n=299)	Non-int (n=300)	Int (n=300)
Age in months, mean, SD	31.2 (16.5)	31.4 (17.3)	32.4 (16.3)	28.9 (15.3)* [308]	39.9 (15.6)	35.2 (18.4)*	40.7 (15.4)	43.2 (15.5)*
Female	155 (52%)	151/298 (51%)†	132 (45%)	155/308 (50%)†	156 (52%)	139 (46%)	140 (47%)	141 (47%)
Male	141 (48%)	147/298 (49%)	160 (55%)	153/308 (50%)†	144 (48%)	160 (54%)	160 (53%)	159 (53%)
Fever episodes in the previous month, mean (SD)	2.3 (1.8)	1.2 (0.8) [291]*	2.4 (1.4) [263]	1.9 (1.0) [280]*	0.6 (0.8) [299]	1.7 (1.2) [295]*	1.2 (0.5)	1.7 (0.7)*
Axillary temperature at recruitment, mean (SD)	37.9 (0.7)	38.2 (0.9)*	37.6 (0.7)	38.0 (1.0) [308]*	38.1 (1.1)	38.0 (0.8)	37.9 (1.0)	38.2 (0.8)*
Slept under a bednet (previous night)	205 (69%)	219 (73%)	135 (46%)	155/308 (50%)	227 (76%)*	281 (94%)*	274 (91%)	282 (94%)
Took antimalarials (previous 24 h)	26 (9%)	105 (35%)*	37 (13%)	34/308 (11%)	38 (13%)	17/298 (6%)*	0	2 (1%)
On antiretroviral drugs	0	1 (<1%)	0	0/308	NI	NI	NI	NI

Data are n (%) with available data unless indicated otherwise. C-IPTp=community delivery of intermittent preventive treatment of malaria in pregnancy. Int=intervention. Non-int=non-intervention. NI=no information. *p<0.05 between non-intervention and intervention area. †Some sex data not recorded in case report forms by field workers at the time of interview.

Table 2: Sociodemographic characteristics of participating children by survey for Democratic Republic of the Congo and Madagascar

deviations, and categorical variables were described using frequencies, percentages, and 95% CIs. χ^2 or Fisher exact tests were used to assess differences between participants from baseline and endline surveys in both the intervention and non-intervention areas. For each mutation, genotype, and inferred haplotype (based on the most dominant *dhfr* and *dhps* genotypes) in each country and area, prevalence was calculated using percentages. Logistic multivariate regression was used to estimate the change of the prevalence of molecular markers between intervention and non-intervention at baseline to 3 years after the onset of the intervention through an interaction term (area#timepoint; appendix 1 p 4). The analysis was adjusted by age and sex. Statistical significance was considered reached when the p value test was less than 0.05. The analysis was performed with STATA version 17.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Baseline surveys were conducted between June 18 and Oct 10, 2018, before C-IPTp implementation, which started directly after the baseline surveys had been performed. Endline surveys were carried out after 3 years of C-IPTp implementation, between July 26 and Nov 30, 2021. Exact dates of data collection per country are provided in appendix 1 (p 3). 11951 children attending the health facility were screened for eligibility. 4983 (38.5%) children were enrolled. 4930 (98.9%) blood samples, with a maximum of one per child, were collected on filter paper. DNA was successfully extracted from 2505 dried blood samples collected at baseline

(1259 [50.3%] from children from intervention areas and 1246 [49.7%] from non-intervention areas), and 2406 dried blood samples at endline (1210 [50.3%] from children from intervention areas and 1196 [49.7%] from children from non-intervention areas). Participants' sociodemographic characteristics are shown in tables 2, 3. No mutations were observed at codons 16 and 50, so these codons were excluded from any further analysis. The survey profile is presented in figure 2.

Prevalences of the *dhfr* IRNI genotype, *dhps* ISGEAA genotype, and the union of both (IRNI/ISGEAA inferred haplotype) by country, area and timepoint are shown in table 4. In the Democratic Republic of the Congo, the prevalence of the *dhfr* IRNI genotype increased significantly, from 64% (188 of 296) to 77% (226 of 292) in the non-intervention area, and from 69% (206 of 300) to 83% (256 of 309) in the intervention area (odds ratio [OR] 1.93 [95% CI 1.34–2.77] and 2.18 [1.48–3.21], respectively; $p_{\text{interaction}}=0.627$) during the study period. The prevalence of the *dhps* ISGEAA genotype was low at baseline, and increased significantly from 2% (seven of 296) to 8% (24 of 292; 3.86 [1.63–9.16]) in the non-intervention area, but the increase was not significant in the intervention area, with prevalences of 5% (15 of 300) at baseline and 7% (21 of 309) at endline (1.34 [0.67–2.68]). The changes in *dhps* double mutant were mirrored by the *dhfr/dhps* IRNI/ISGEAA inferred haplotype, with a significant increase in the non-intervention area, from 2% (five of 296) to 8% (24 of 292) (5.46 [2.04–14.57]), and a non-significant increase in the intervention area (nine [3%] of 300 at baseline and 18 [6%] of 309 at endline; 1.90 [0.83–4.35]), indicating that the increase found in the intervention area was not significantly higher than the increase found in the non-intervention area ($p_{\text{interaction}}=0.118$). In addition, one sample was also found containing the *dhfr/dhps* IRNI/VAGKGS

	Mozambique				Nigeria			
	Baseline		Endline		Baseline		Endline	
	Non-int (n=302)	Int (n=306)	Non-int (n=303)	Int (n=301)	Non-int (n=348)	Int (n=354)	Non-int (n=300)	Int (n=300)
Age in months, mean, SD	27.1 (13.8) [301]	26.3 (14.4) [303]	31.8 (13.7)	26.0 (12.8)*	35.8 (15.6) [347]	31.4 (15.6)*	34.5 (15.5)	35.4 (14.9)
Female	146/301 (49%)†	146/302 (48%)†	162 (54%)	130/300 (43%)*†	161/347 (46%)†	191 (54%)	142 (47%)	141 (47%)
Male	155/301 (51%)†	156/302 (52%)†	139 (46%)	170/300 (57%)†	186/347 (54%)†	163 (46%)	158 (53%)	159 (53%)
Fever episodes in the previous month, mean (SD)	0.3 (2.4) [301]	0.4 (0.7) [303]*	0.4 (0.7)	0.3 (0.6) [300]	1.5 (1.4) [338]	1.2 (1.0) [350]	2.6 (1.3)	2.9 (1.4)*
Axillary temperature at recruitment, mean (SD)	37.7 (1.1) [298]	37.9 (1.0) [301]	38.1 (1.2) [302]	37.8 (1.3) [297]*	37.9 (0.8) [339]	37.9 (1.1)	37.5 (0.9)	37.7 (0.9)*
Slept under a bednet (previous night)	286/301 (9%)	289/303 (95%)	291 (96%)	276 (92%)	273 (79%)	208 (59%)*	245 (82%)	230 (77%)
Took antimalarials (previous 24 h)	3/299 (1)	1/303 (0.3%)	18 (6%)	6/299 (2%)*	70/346 (20%)	120 (34%)*	49/300 (16%)	102/300 (34%)*
On antiretroviral drugs	3/301 (1)	1/303 (<1%)	5/302 (2%)	9/301 (3%)	1/347 (<1%)	1 (<1%)	1 (<1%)	0

Data are n (%) with available data unless indicated otherwise. C-IPTp=community delivery of intermittent preventive treatment of malaria in pregnancy. Int=intervention. Non-int=non-intervention. NI=no information. *p<0.05 between non-intervention and intervention area. †Some sex data not recorded in case report forms by field workers at the time of interview.

Table 3: Sociodemographic characteristics of participating children by survey for Mozambique and Nigeria

inferred haplotype (combination of *IRNI* genotype with *dhps* substitutions Ile431Val, Ser436Ala Ala437Gly, Ala581Gly, and Ala631Ser) in the Democratic Republic of the Congo at the endline survey in the non-intervention area (appendix 1 p 7).

In Mozambique, a significant decrease in the prevalence of the *dhfr IRNI* genotype was observed, from 96% (289 of 302) to 78% (237 of 303; OR 0.15 [95% CI 0.08–0.29]) in the non-intervention area, and from 93% (284 of 306) to 74% (222 of 301; 0.22 [0.13–0.37]) and intervention areas, respectively ($p_{\text{interaction}}=0.422$). By contrast, the highest proportion of the *dhps* ISGEAA genotype was found in Mozambique, with prevalence increasing significantly from 67% (202 of 302) to 83% (250 of 303; 2.31 [1.57–3.41]) in the non-intervention area, and from 66% (202 of 306) to 83% (251 of 301) in the intervention area (2.57 [1.75–3.81], $p_{\text{interaction}}=0.774$). The changes in prevalence of these genotypes did not have an effect on the prevalence of the *dhfr/dhps IRNI/ISGEAA* inferred quintuple mutant, which remained stable between timepoints in both areas from baseline to endline in the non-intervention and intervention areas (194 [64%] of 302 to 194 [64%] of 303; 0.96 [0.68–1.35] and 187 [61%] of 306 to 183 [61%] of 301; 0.98 [0.71–1.36] respectively), clearly indicating that the intervention area did not have a statistically significant increase in the prevalence of the quintuple mutant in comparison to the non-intervention area ($p_{\text{interaction}}=0.947$).

In Nigeria, the prevalence of the *dhfr IRNI* genotype raised significantly in both areas, from 73% (255 of 348) to 97% (290 of 300; OR 10.38 [95% CI 5.29–20.37]) in the non-intervention area, and from 81% (286 of 354) to 94% (282 of 300) in the intervention area (3.58 [2.07–6.20]), being the overall increase in the likelihood of carrying the *dhfr* genotype *IRNI* less marked in the intervention area (OR_{interaction} 0.36 [95% CI 0.15–0.85], $p_{\text{interaction}}=0.021$). The

pattern of *dhps* point mutations and the corresponding genotypes of malaria parasites found in Nigeria were different to those observed in Mozambique and the Democratic Republic of the Congo. While no isolates harbouring the *dhps* ISGEAA genotype were found in any area or timepoint in Nigeria, malaria parasites presented the highest prevalence of the *dhps* mutation in codon 431. This mutated codon increased significantly in the non-intervention area (OR=2.47 [95% CI 1.78–3.44]) and in the intervention area (1.87 [1.32–2.64], $p_{\text{interaction}}=0.249$), so the increase was similar between intervention and non-intervention areas (appendix 1 p 5). The *dhps*-431 mutation was commonly found in association with *dhps* mutations in codons 436, 437, 581, or 613. In this sense, 14 genotypes were found where the mutation in codon 431 was present, including six samples with the *dhfr/dhps IRNI/VAGKGS* inferred octuple mutant at the endline survey (appendix 1 p 10).

In Madagascar, the prevalence of the *dhfr* triple mutant *IRNI* decreased from 82% (247 of 300) to 72% (217 of 300; OR 0.57 [95% CI 0.38–0.84]), and from 77% (229 of 299) to 65% (194 of 300; 0.59 [0.41–0.84], $p_{\text{interaction}}=0.216$) in the non-intervention and intervention areas, respectively. Only five isolates with the ISGEAA genotype were observed, all of them in the non-intervention area. Like in Nigeria, the patterns of drug resistance in Madagascar were different to those in observed in the Democratic Republic of the Congo and Mozambique. The highest prevalence of the *dhfr* mutation in codon 164 was observed in Madagascar, ranging from 4% (11 of 300) in the non-intervention area and 3% (eight of 299) in the intervention area at the baseline survey respectively, to 1% (four of 300) at endline in both areas (appendix 1 p 6). However, this mutation was found alone, except for three isolates containing the concerning *dhfr IRNL* quadruple mutant (appendix 1 p 8).

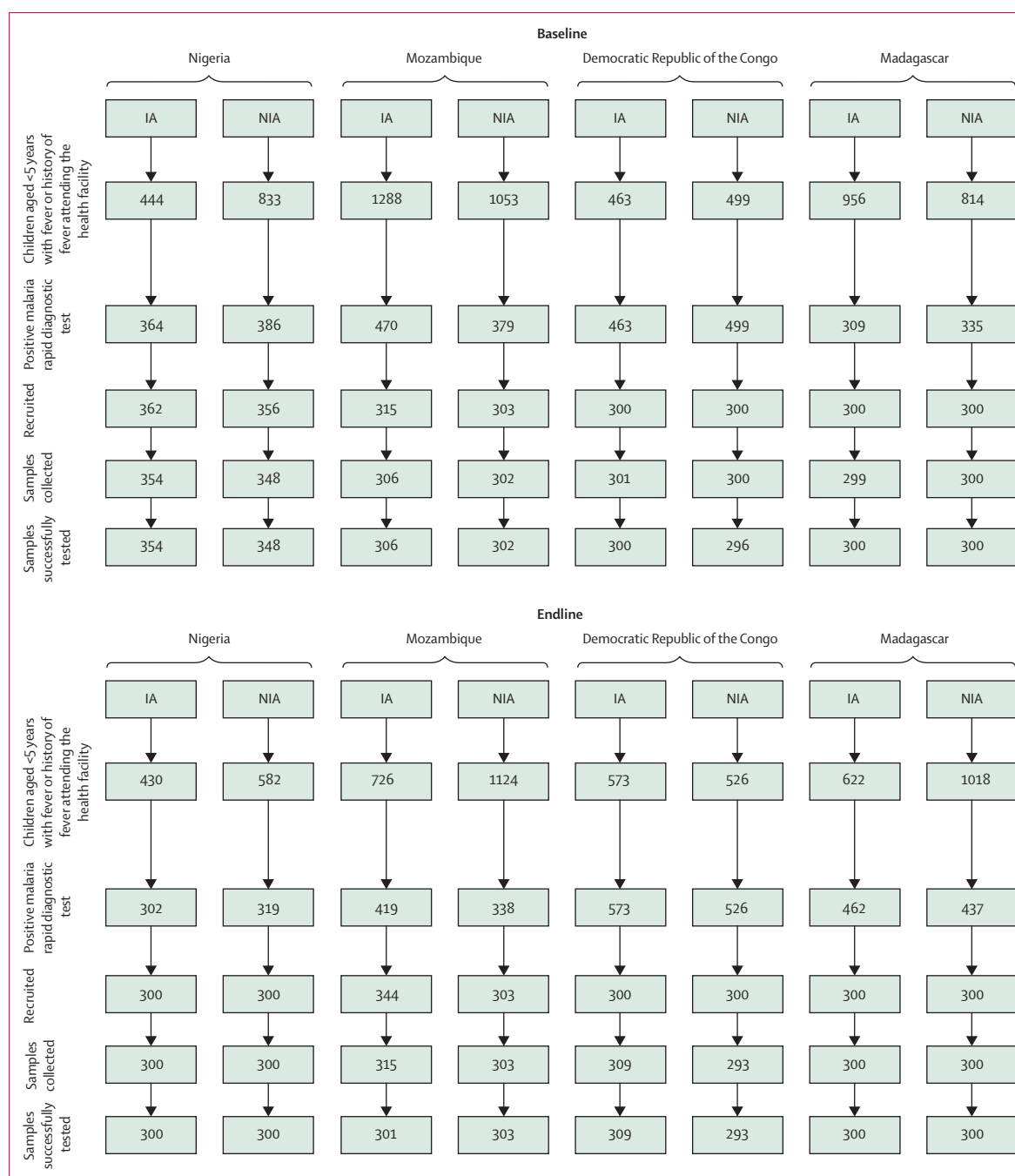


Figure 2: Survey profile

IA=intervention area. NIA=non-intervention area.

Prevalence of other point mutations did not differ significantly from baseline surveys to endline surveys in any project area, with the exception of the *dhps* mutation in codon 437, whose prevalence increased significantly from 39% (116 of 300) at baseline to 56% (169 of 300) at endline (OR 2.06 [95% CI 1.49–2.86]) in the non-intervention area. This increase did not translate into an increased prevalence of the *dhfr/dhps* IRNI/ISGKAA

inferred quadruple mutant, which was 32% (96 of 300) at baseline and remained stable at the endline survey (appendix 1 pp 6, 11). A significant increase in the prevalence of the *dhps* mutation in codon 437 from 34% (101 of 299) to 50% (149 of 300; 1.88 [1.34–2.63]) was found in the intervention area. The prevalence of the mutation in *dhps* codon 436 was 13% (38 of 300) and 5% (15 of 299) at baseline in the non-intervention and the

Genotype or inferred haplotype	Non-intervention area			Intervention area			Adjusted OR between areas* (95% CI)	Adjusted p-value between areas*
	Baseline n (%)	Endline n (%)	Adjusted OR (95% CI)	Baseline n (%)	Endline n (%)	Adjusted OR (95% CI)		
Democratic Republic of the Congo								
<i>dhfr</i> IRNI	188/296 (64%)	226/292 (77%)	1.93 (1.34–2.77)	206/300 (69%)	256/309 (83%)	2.18 (1.48–3.21)	1.14 (0.67–1.94)	0.627
<i>dhps</i> ISGEAA	7/296 (2%)	24/292 (8%)	3.86 (1.63–9.16)	15/300 (5%)	21/309 (7%)	1.34 (0.67–2.68)	0.35 (0.12–1.07)	0.065
<i>dhfr/dhps</i> IRNI/ISGEAA	5/296 (2%)	24/292 (8%)	5.46 (2.04–14.57)	9/300 (3%)	18/309 (6%)	1.90 (0.83–4.35)	0.36 (0.10–1.30)	0.118
Nigeria								
<i>dhfr</i> IRNI	255/348 (73%)	290/300 (97%)	10.38 (5.29–20.37)	286/354 (81%)	282/300 (94%)	3.58 (2.07–6.20)	0.36 (0.15–0.85)	0.021
<i>dhps</i> ISGEAA	0/348	0/300	..	0/354	0/300
<i>dhfr/dhps</i> IRNI/ISGEAA	0/348	0/300	..	0/354	0/300
Mozambique								
<i>dhfr</i> IRNI	289/302 (96%)	237/303 (78%)	0.15 (0.08–0.29)	284/306 (93%)	222/301 (74%)	0.22 (0.13–0.37)	1.39 (0.62–3.09)	0.422
<i>dhps</i> ISGEAA	202/302 (67%)	250/303 (83%)	2.31 (1.57–3.41)	202/306 (66%)	251/301 (83%)	2.57 (1.75–3.81)	1.08 (0.63–1.87)	0.774
<i>dhfr/dhps</i> IRNI/ISGEAA	194/302 (64%)	194/303 (64%)	0.96 (0.68–1.35)	187/306 (61%)	183/301 (61%)	0.98 (0.71–1.36)	0.99 (0.62–1.58)	0.947
Madagascar								
<i>dhfr</i> IRNI	247/300 (82%)	217/300 (72%)	0.57 (0.38–0.84)	229/299 (77%)	194/300 (65%)	0.59 (0.41–0.84)	0.68 (0.37–1.25)	0.216
<i>dhps</i> ISGEAA	2/300 (1%)	3/300 (1%)	1.53 (0.25–9.34)	0/299	0/300
<i>dhfr/dhps</i> IRNI/ISGEAA	2/300 (1%)	3/300 (1%)	1.53 (0.25–9.34)	0/299	0/300

C-IPTp=community delivery of intermittent preventive treatment of malaria in pregnancy. *dhfr*=dihydrofolate reductase. *dhps*=dihydropteroate synthase. OR=odds ratio. *Interaction term intervention area#endline timepoint.

Table 4: Prevalence of *dhfr* IRNI, *dhps* ISGEAA and inferred *dhfr/dhps* IRNI/ISGEAA by C-IPTp intervention and non-intervention area and survey.

intervention area respectively, and it did not differ significantly between timepoints (0.96 [0.60–1.56] and 1.01 [0.48–2.12] at non-intervention and non-intervention area respectively. Similarly, the prevalence of the *dhps* mutation in codon 613 was of 4% (13 of 300) and 2% (five of 299) in the non-intervention and intervention areas, and it did not differ significantly at the endline survey either at non-intervention area (1.12 [0.52–2.44] or at the intervention area (0.49 [0.11–2.11]).

No isolates were found to carry the *dhps* triple mutant ISGEGA genotype (combination of mutations in codons 437, 540, and 581) in any of the four countries.

Discussion

In this study we report the prevalence of molecular markers associated with sulfadoxine–pyrimethamine resistance before and after 3 years of the implementation of C-IPTp, using areas where C-IPTp was implemented and neighbouring areas with no C-IPTp programmes in four sub-Saharan countries. The molecular analysis included over 4900 *P. falciparum* isolates in blood samples collected from 4983 children younger than 5 years with confirmed malaria infection attending the health facilities in the selected project areas. The results show that parasites carrying the IRNI/ISGEAA inferred quintuple mutant haplotype, which is associated with clinical and parasitological failure, were either absent in both intervention and non-intervention areas at both measured timepoints (in Nigeria and Madagascar), or were present

at the same frequencies in both areas over time (in the Democratic Republic of the Congo and Mozambique). These findings suggest that C-IPTp had no significant effect on the selection of parasite resistance to sulfadoxine–pyrimethamine, at least in the short term.

In line with prevalence of the IRNI/ISGEAA inferred haplotype found in the Democratic Republic of the Congo in this study, a previous report from 2014–2015 in the country found a prevalence of 9.7% of samples with this haplotype among symptomatic patients of all ages attending outpatient clinics, suggesting that the prevalence of this haplotype has fluctuated over the past few years, but has remained at low frequency.²⁹

In Nigeria, frequent self-medication with sulfadoxine–pyrimethamine might explain the drug pressure, which could have led to the significant increase in the prevalence of *dhfr* IRNI genotype found in this study, particularly in the non-intervention area.³⁰ It has been hypothesised that the mutation in codon 431, which significantly increased in both the intervention and non-intervention study areas and is present in Nigeria and neighbouring countries,³¹ confers a selective advantage in the presence of sulfadoxine–pyrimethamine drug pressure, displacing more sensitive mutations such as those in codon 540.³¹ However, it remains unclear how mutations in codon 431 might affect sulfadoxine–pyrimethamine resistance.

High prevalence of the *dhps* ISGEAA genotype has been reported in Mozambique. A study conducted in 2015 among symptomatic children in several locations

in the country found a prevalence of 88.4% for the *dhps* ISGEAA genotype in Dondo (the non-intervention area of this Article), which is higher than the prevalence we found at baseline in both study areas.²⁷ The prevalence of this genotype rose from baseline to endline surveys in both study areas, reaching a prevalence similar to those found in the 2015 study. This increase was not accompanied by an increase in the prevalence of the *dhfr* IRNI genotype, suggesting that drug pressure might result from the widespread use of other sulfa drugs, such as cotrimoxazole, recommended to prevent opportunistic infections in individuals with HIV.³²

Of the four project countries, the lowest prevalence of the *dhps* molecular markers associated with sulfadoxine–pyrimethamine resistance at both timepoints was found in Madagascar, with only five samples containing the double *dhps* mutation, and no other mutations of concern in the country study areas. This is in line with results from other studies undertaken in Madagascar in 2006, 2008, and 2010.^{23,33} In this study, the *dhfr* IRNI genotype decreased significantly in both study areas between timepoints, suggesting that sulfadoxine–pyrimethamine drug pressure might also have decreased.

Caution must be taken when attempting to associate the frequency of *dhfr/dhps* mutations with the effectiveness of chemopreventive strategies such as IPTp. While the use of sulfadoxine–pyrimethamine often results in an increased prevalence of mutations, this does not necessarily lead to meaningful increases in sulfadoxine–pyrimethamine resistance.⁹ Drug resistance is only one of many factors that determine the efficacy of sulfadoxine–pyrimethamine for IPTp, such as the multiplicity of infection, the rate of drug metabolism, and the individual's naturally acquired immunity to malaria, and current nutritional status.⁸ A systematic review and meta-analysis that included 57 studies in sub-Saharan Africa concluded that the effectiveness of sulfadoxine–pyrimethamine as IPTp was reduced only when the prevalence of *P falciparum* carrying the *dhps*-540 mutation exceeded 90%, although sulfadoxine–pyrimethamine still had a modest positive effect on birthweight.³⁴ The scarcity of evidence regarding the relationship of the presence of molecular markers associated to sulfadoxine–pyrimethamine resistance with lower effectiveness of preventive interventions such as IPTp, and intermittent preventive treatment in infants (IPTi), which are both based on the administration of a stat dose of sulfadoxine–pyrimethamine to mostly healthy individuals, has led WHO to continue recommending IPTp with sulfadoxine–pyrimethamine as the most cost-effective malaria preventive intervention in pregnancy.³⁵

Limitations of the study include the scarcity of available data on the prevalence of molecular markers associated with sulfadoxine–pyrimethamine resistance in the study areas in the years before the intervention, thus we assumed that the areas had similar trends in the prevalence of molecular markers before the start of C-IPTp. Moreover, the geographical proximity of the non-intervention and

intervention areas in each country might have decreased the magnitude of our study effect estimates, due to contamination of population in the bordering areas. However, this was done in order to guarantee that both areas presented similar epidemiological characteristics (eg, seasonality, rainfall, temperature, mosquito population) as well as socio-economic and cultural characteristics. In addition, the fact that health facilities were purposively selected might have introduced potential bias. Finally, we inferred *dhfr/dhps* haplotypes from the union of the most dominant *dhfr* and *dhps* genotypes, as we did not have an estimate of complexity of infection, and we did not test parasites for amplification of the *GPT-cyclohydrolase 1* gene (*pfpgch1*), which has been associated with pyrimethamine resistance.³⁶

In conclusion, this study provides evidence that C-IPTp might not increase the prevalence of molecular markers associated with sulfadoxine–pyrimethamine resistance in project areas after 3 years of programme implementation. These findings reinforce C-IPTp as a strategy to optimise control of malaria during pregnancy, and supports WHO guidelines for prevention of malaria in pregnancy.

Contributors

The study was conceived and designed by CMe, FP, RG, CMA and ER. CPD, RG, AM, and ML wrote the study protocol. IA, AN, EL, RR, AFR, ML, FP, and CPD coordinated and participated in data collection. DB, CS, MM, and AR were the country principal investigators of the study and supported project implementation. Laboratory analysis was done by LM, CDL, and DM. Data analysis was done by AFR and SS. AFR and SS had access to and verified the underlying data. All authors had full access to all the data in the study. CMe, AFR, RG, AM, and DM led the interpretation of the results. AFR drafted the manuscript and all authors participated in critical revision of the manuscript for important intellectual content. All authors read and approved the final version of the manuscript and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

Deidentified individual data and the data dictionary will be made available to others upon reasonable requests to the corresponding author. A data transfer agreement will be signed between the TIPTOP consortium and the requesting institution before data sharing.

Equitable partnership declaration

The authors of this paper have submitted an equitable partnership declaration (appendix 2). This statement allows researchers to describe how their work engages with researchers, communities, and environments in the countries of study. This statement is part of *The Lancet* journals' broader goal to decolonise global health.

See Online for appendix 2

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