RESEARCH ARTICLE

Seasonal Malaria Chemoprevention with Sulphadoxine-Pyrimethamine and Amodiaquine Selects \textit{Pfdhfr-dhps} Quintuple Mutant Genotype in Mali

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Abstract

Background

Seasonal malaria chemoprevention (SMC) with sulphadoxine-pyrimethamine (SP) plus amodiaquine (AQ) is being scaled up in Sahelian countries of West Africa. However, the potential development of \textit{Plasmodium falciparum} resistance to the respective component drugs is a major concern.

Methods

Two cross-sectional surveys were conducted before (August 2012) and after (June 2014) a pilot implementation of SMC in Koutiala, Mali. Children aged 3–59 months received 7 rounds of curative doses of SP plus AQ over two malaria seasons. Genotypes of \textit{P. falciparum} \textit{Pfdhfr} codons 51, 59 and 108; \textit{Pfdhps} codons 437 and 540, \textit{Pfcrt} codon 76 and \textit{Pfmdr1} codon 86 were analyzed by PCR on DNA from samples collected before and after SMC, and in non-SMC patient population as controls (November 2014).

Results

In the SMC population 191/662 (28.9\%) and 85/670 (12.7\%) of children were \textit{P. falciparum} positive by microscopy and were included in the molecular analysis before (2012) and after SMC implementation (2014), respectively. The prevalence of \textit{P. falciparum} \textit{Pfdhfr-dhps} quintuple mutant genotype, which was 1.6\% before but 7.1\% after SMC (p = 0.02). The prevalence of \textit{Pfmdr1}-86Y significantly decreased from 26.7\% to 15.3\% (p = 0.04) while no...
significant change was seen for Pfcrt 76T. In 2014, prevalence of all molecular markers of SP resistance were significantly higher among SMC children compared to the non-SMC population patient (p < 0.01). No Pfdhfr-164 mutation was found neither at baseline nor post SMC.

**Conclusion**

SMC increased the prevalence of molecular markers of *P. falciparum* resistance to SP in the treated children. However, there was no significant increase of these markers of resistance in the general parasite population after 2 years and 7 rounds of SMC.

**Introduction**

Administering a full curative course of an antimalarial drug at set times regardless of parasitemia has been used as a malaria prevention tool known as Intermittent Preventive Treatment in infants [IPTi] and children [IPTc] [1]. Landmark studies demonstrated the protective values of both IPTi with sulphadoxine-pyrimethamine [SP] [2] and IPTc with SP and amodiaquine [AQ], which was renamed as Seasonal Malaria Chemoprevention [SMC] and recommended as a new policy by World Health Organization [WHO] [3]. However, both SP and AQ are known to select molecular markers of resistance, which may lead to increased in vivo resistance [3]. Amodiaquine resistance is associated with the molecular determinants of chloroquine resistance, the *Plasmodium falciparum* chloroquine resistance transporter [Pfcrt] and the *P. falciparum* multidrug resistance gene 1 [Pfmdr1] [4]. Resistance to sulphadoxine and pyrimethamine are due to point mutations in *P. falciparum* dihydropteroate synthase [Pfdhps] and *P. falciparum* dihydrofolate reductase [Pfdhfr], respectively [5]. Unlike East and Southern Africa where SP and AQ resistance are widespread, SP plus AQ remain efficacious in West Africa [6]. The quintuple mutant genotype which include Pfdhfr [51I, 59R and 108N] and Pfdhps [437G and 540E] is known to be the most associated with in vivo SP resistance [7] while the mutations Pfcrt 76T and Pfmdr1 86Y are associated with AQ resistance [1]. IPTc with SP plus artemisate [AS] was shown to select the quadruple mutant genotype [Pfdhfr 51, 59 and 108 plus Pfdhps 437] in Senegal [7]. However, no such selection was found in similar interventions in Burkina Faso and Ghana [8]. The two most deleterious mutations for sulphadoxine resistance (Pfdhps 540) and pyrimethamine [Pfdhfr 164] were found during SMC studies neither in Senegal [9] nor in Burkina Faso [10]. Similar studies showed a higher prevalence of Pfdhfr and Pfdhps mutations in infections from children under SMC with SP plus AQ in Burkina Faso and Mali [11–13]. Most of the previous studies assessed the impact of SMC with SP plus AQ either immediately after the intervention or after just one season of SMC. The current study aims to assess the impact of SMC on the selection and spread of *Plasmodium* drug resistant parasites after several rounds of SMC.

**Methods**

**Study site**

This study was conducted in the health district of Koutiala, which has approximately 575,000 inhabitants and is located at 420 km south of Bamako, the capital city of Mali. Twenty rural health centers, all located within a radius of 15 km where randomly selected. Malaria transmission is hyper-endemic in the region with a sharp peak in transmission during the rainy season
April to November. More than 80% of malaria cases occur between August and November (our unpublished data).

Study design and participants

SMC population. Two cross-sectional surveys were conducted, one in August 2012 a few days before the start of the pilot SMC implementation and a second one in June 2014 i.e. 8 months after the last dose of SMC drugs were distributed. A cluster randomization was used to select the children. In each randomly selected cluster, households were also randomly selected to enroll at least 32 children per cluster [in the 2012 study] or 64 children per cluster [in the 2014 study] in the target age group. After identification of the children, informed consent was obtained from their parents prior to their interview and inclusion. Children were eligible to participate to the study if they were aged 3–59 months at the time of enrolment and residents of the study area with no intention to leave during the study period. Children with the following criteria were excluded: presence of a severe or chronic illness, such as severe malnutrition or Human immunodeficiency virus infection and acquired immunodeficiency syndrome [HIV/AIDS], and a history of a significant adverse reaction to SP or AQ. For all participants a thick blood smear and blood spot was collected by finger prick. In the 2012 malaria transmission season, three cycles of SMC with SP plus AQ were provided in August, September and October. In the malaria transmission season of 2013 four cycles of SMC with SP plus AQ were provided in July, August, September and October. SMC population included both symptomatic and asymptomatic children as long as they were within the age bracket of 3–59 months during the survey. The symptomatic cases were diagnosed with a Rapid diagnostic tests [RDT] [SD BIOLINE Malaria Ag Pf®, Suwon City, South Korea]; if found positive, they received artemether-lumefantrine [Coartem®] as per National Malaria Treatment Guidelines [NMTG].

Non-SMC patient population. In November 2014, to assess the potential flow of resistant genes in the neighboring general population we enrolled patients who had never received any round of SMC with SP plus AQ. Rapid diagnostic tests [RDT, SD BIOLINE Malaria Ag Pf®, Suwon City, South Korea] were dispatched by the study team to the health centers serving the clusters included in the SMC study. Patients visiting these health centers that were aged 7 years or above were offered free RDTs if they had any malaria symptoms. Those found to be malaria positive by RDT were treated immediately with artemether-lumefantrine [Coartem®] or artesunate-amodiaquine according to national guidelines. The used positive RDTs were collected and used to extract DNA for molecular analyses and included in the non-SMC patient population.

SMC delivery. SMC drugs were delivered by Médecins Sans Frontières, Bamako [MSF] in collaboration with the National Malaria Control Program of Mali. The delivery teams used a combination of fixed post [for small villages] and door-to-door strategy [for large villages]. The drug resistance study team was not involved in the delivery of SMC drugs.

Assessment of molecular markers of P. falciparum resistance to sulphadoxine, pyrimethamine and amodiaquine

DNA was extracted from selected dried blood spots [DBS] on filter papers (3MM Whatman) as described [14]. Assessment of drug resistance markers was performed by nested PCR followed by restriction digestion [14–16]. Drug resistance markers tested for this study were Pfδhfr N51I, Pfδhfr C59R, Pfδhfr S108N, Pfδhfr I164L for pyrimethamine, Pfδhps A437G and Pfδhps K540E for sulphadoxine, Pfcr K76T and Pfmdr1 N86Y for amodiaquine. Results were classified as wild type, mutant or mix [when both alleles were present]. Cases of mix infection were categorized as mutants.
Sample size

Based on previous studies conducted in Mali, the prevalence of quintuple mutation was zero before SMC [Prevalence (P1) = 0.0%] [12]. We estimate a prevalence of 10% after two years of SMC implementation [P2 = 10.0%] in 2014. To estimate our sample size we set alpha at 5 with a confidence interval of 95% and beta at 10% with a power of 90%. We define $Z_{\alpha}$ as the z value for risk of error $\alpha$ [$\alpha = 0.05, z = 1.96$ two-tailed test], $Z_{\beta}$ as the z value for risk of $\beta$ error [1-$\beta = 0.90, z = 1.282$] and $\Delta$ detection to be 10%. Based on these criteria and using a Poisson approximation, to detect a change from 0.0% to 10.0% in prevalence of quintuple mutation genotype with a power of 90%, the sample size was estimated to 105 subjects per time period [total 210 subjects]. With an estimated 10% of excluded samples, we will need 234 subjects [117 per time period]. Also considering that the prevalence of malaria is 50% in Koutiala, we expect to have 1 out of 2 samples to be positive for malaria. Therefore the total number of volunteers was estimated at 468. Since 10% of dried blood spots may not be usable, the sample size was set at 515 volunteers.

Data management and analysis

Data were collected on case report forms, double entered, and analyzed with MS Access and/or Stata [Stata Corp 11]. Prevalence of single mutations and of various genotypes including the double mutant [Pfcrt 76 + Pfmdr1 86I], triple mutant [Pfdhfr 51 + 59 + 108I], quadruple mutant [triple Pfdhfr mutant + dhps 437], quintuple mutant [quadruple mutant + dhps 540], six mutation genotype with Pfct [Pfdhfr-dhps quintuple + Pfcrt-76T] or Pfmdr1 [Pfdhfr-dhps quintuple + Pfmdr1-86Y] and seven mutations genotype [Pfdhfr-dhps quintuple + Pfcrt-76T + Pfmdr1-86Y] were calculated with 95% Confidence interval [CI]. Chi-square or Fisher exact probability tests were used for comparisons as appropriate with statistical significance set at $P$ value < 0.05.

Ethics

The study protocol was approved by the Ethical Committee of the Faculty of Medicine and Odonto-stomatology (FMOS) and Faculty of Pharmacy (FAPH)/ University of Sciences, Techniques and Technologies of Bamako [USTTB]. Community permission was obtained from each locality prior to the study. Individual, written, informed consent was obtained from parents or guardians of each child prior to screening.

Results

Demographic characteristics

The trial profile is summarized in Table 1. From 4 to 9 August 2012 and 20 to 30 June 2014, 662 and 670 children aged from 3 to 59 months were enrolled in the study, respectively. The children median age and sex ratio of female proportion were 2 years and 54.5% in baseline 2012 and 3 years and 49.7% in post intervention 2014, respectively. The proportion of fever, anaemia and $P. falciparum$ presence was 6.5%, 67.5% and 28.9% in baseline and 2.4%, 48.5% and 12.7% in post intervention, respectively. In the non-SMC patient population with 502 patients, the median age was 20 years with 60.6% for sex ratio of female and the proportion of history of fever and fever were 79.9% and 50%, respectively [S1, S3 and S5 Files].

Molecular markers of antimalarial drug resistance

In the SMC population 191/662 [28.9%] and 85/670 [12.7%] of children were $P. falciparum$ positive by microscopy and were included in the molecular analysis before [2012] and after SMC implementation [2014], respectively. Among the 502 volunteers recruited in 2014 in the
non-SMC patient population, 310/502 [71.5%] had a positive RDT. Upon nested PCR, 220/310 [71.6%] of these RDT positive samples yielded DNA amplification Table 1.

Table 2 shows the prevalence of molecular markers associated with resistance to sulphadoxine, pyrimethamine and amodiaquine at baseline and after SMC intervention in the SMC children. The prevalence of Pfdhfr 51I, 59R and 108N [92.1% to 100%, p = 0.01] and Pfdhps 540E [3.1% to 10.6%, p = 0.01] were significantly higher in children after SMC intervention compared to the baseline. There was a non-significant increase in Pfdhps 437G from baseline to post intervention [61.3% to 70.6%, p = 0.14]. The prevalence of Pfdhfr triple, Pfdhfr-dhps quadruple and Pfdhfr-dhps quintuple mutants at baseline versus after intervention were 92.6% versus 100% [p = 0.01], 57.9% vs 75.3% OR = 1.77 [95% confidence interval (CI) 0.99–3.20] [p = 0.04] and 1.6% vs. 7.1% OR = 4.76 [95% CI 0.98–29.94] [p = 0.02], respectively. Prevalence of Pfmdr1-86Y, Pfcrt-76T and double mutant Pfmdr1-86Y + Pfcrt-76T before vs. after intervention were 26.7% vs. 15.3% [p = 0.04]; 68.1% vs. 75.3% [p = 0.22] and 18.8% vs. 10.6% [p = 0.09],

<table>
<thead>
<tr>
<th>Genes</th>
<th>Baseline 2012</th>
<th>Post intervention 2014</th>
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<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>% Mutant</td>
</tr>
<tr>
<td>Pfdhfr 51</td>
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<td>92.1</td>
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<td>176/191</td>
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<tr>
<td>Pfdhfr 108</td>
<td>176/191</td>
<td>92.1</td>
</tr>
<tr>
<td>Triple Pfdhfr mutants</td>
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<td>92.1</td>
</tr>
<tr>
<td>Pfdhps 437</td>
<td>117/191</td>
<td>61.3</td>
</tr>
<tr>
<td>Pfdhps 540</td>
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<td>3.1</td>
</tr>
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<td>1.6</td>
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<td>Pfcrt 76</td>
<td>130/191</td>
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<td>Pfmdr1- 86</td>
<td>51/191</td>
<td>26.7</td>
</tr>
<tr>
<td>Double mutants (Pfcrt 76 + Pfmdr1- 86)</td>
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<td>18.8</td>
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n, number with mutant; N, number of participants with parasitaemia at blood smear tested.

doi:10.1371/journal.pone.0162718.t002
respectively. No mutation at codon Pfdhfr 164 was found neither at baseline nor post SMC intervention (S2 and S4 Files).

When focusing on 2014, the prevalence of the following mutations were higher in the SMC population than in non-SMC patient population: Pfdhfr 51, 100% vs. 81.7%; Pfdhfr 59R, 100% vs. 87.0%; Pfdhfr 108N, 100% vs. 81.4%; and Pfdhps -540, 10.6% vs. 4.1% [p < 0.05 for each comparison] Table 3. Similarly, the prevalence of Pfdhfr-triple [100% vs. 64.9%], Pfdhfr-dhps quadruple [75.3% vs. 43.6%] and Pfdhfr-dhps quintuple mutant genotypes [7.1% to 0.46%] were significantly higher in the SMC population as compared to non-SMC patient population [p < 0.01 for each comparison]. There were no statistically significant differences with the remaining genotypes and Pfdhfr 164 mutation was not detected in the study (S4 and S6 Files).

The prevalence of molecular markers in the baseline children and the non-SMC patient population [7 years of age or older] is shown in Table 4. Prevalence of Pfdhfr 51I [92.1% to 81.7%] and Pfdhfr 59R [92.1% to 81.4%], [p = 0.001] were higher at baseline and post-intervention in the SMC population, respectively. The prevalence of triple and quadruple mutants were significantly higher in the SMC children at baseline and post-intervention than in the non-SMC patient population with 92.6% vs. 64.9% [p < 0.001] and 57.9% vs. 40.1% [p = 0.009], respectively. There were no statistically significant differences with the remaining codons. Mutation at codon 164 of Pfdhfr was not detected (S2 and S6 Files).

The prevalence of six-mutation genotype with Pfmdr1 [Pfdhfr-dhps quintuple + Pfmdr1-86Y] was 1.2%, six-mutation genotype with Pfcrt [Pfdhfr-dhps quintuple + Pfcrt-76T] was 7.1% and seven-mutation genotype [Pfdhfr-dhps quintuple + Pfmdr1-86Y + Pfcrt-76T] was 1.2% among SMC-children post-intervention in 2014. No six-mutation and seven-mutation genotypes were observed neither among SMC children at baseline nor in the control non-SMC patient population [data not shown].

**Discussion**

We show for the first time the presence of the Pfdhfr and Pfmdhps quintuple mutant genotypes in a population that received SMC with sulphadoxine-pyrimethamine plus amodiaquine over...
two malaria seasons in West Africa. Furthermore, there was a four-fold increase in this quintuple mutant genotype after seven rounds of SMC in this setting. This is particularly worrisome since Pfdhfr-dhps quintuple mutant genotype was shown to be the most associated with in vivo failure of sulphadoxine-pyrimethamine in the treatment of uncomplicated falciparum malaria [17]. Nevertheless, the nearly 10% of quintuple mutant genotype found in our post-SMC population is still significantly lower than rates of this genotype in other malaria endemic regions of Africa [18–20]. Similar to previous studies in West Africa, we found a significant selection of individual Pfdhfr [codons 51, 59, 108] and Pfdhps [codons 437, 540] mutations in children enrolled in the SMC program. As a consequence the frequencies of the Pfdhfr triple mutant genotype and the Pfdhfr-dhps quadruple mutant genotype were significantly higher in the post-SMC. In addition, the post-SMC prevalence found in this study was higher than post-SMC prevalence of Pfdhfr [codons 51, 59, 108] and Pfdhps [codons 437, 540] mutations found in other settings of Mali such as Djoliba, Siby and Ouelessebougou [12] as well as in neighboring countries of Senegal and Burkina Faso [7, 10, 13]. However, the significant post-SMC selection of the Pfdhfr triple mutation and the Pfdhfr-dhps quadruple mutation observed in this study is consistent with results observed after two years of implementation of intermittent preventive treatment [IPTi] with SP alone in infants in Gabon, Cameroun and Senegal [21–23]. Compared to other studies in Mali and in West Africa, this study did show high baseline levels of individual and triple Pfdhfr mutant genotypes of 92.6% [14–16, 24]. This may reflect the long history of usage of sulpha drugs including SP in our study site. Although the Pfdhfr triple mutation was prevalent in more than 90% of infections, it is well established that this genotype does not correlate with in vivo SP failure in West Africa [16, 24]. The prevalence of Pfdhfr-dhps quadruple mutant genotype was higher than the prevalence of that genotype in several West African sites [16, 24–26].

We found Pfdhps 540E in 3% of baseline infections. There is an upward trend of the prevalence of Pfdhps 540E in Mali, as this mutation was not detected at baseline in a study conducted in 2008 Mali [2]. Our rates were similar to findings from Ghana [27] but lower than those of a refugee camp in Guinea [28].

<table>
<thead>
<tr>
<th>Genes</th>
<th>Baseline children 2012</th>
<th>Non-SMC patient population 2014</th>
<th>n/N</th>
<th>% Mutant</th>
<th>Odds Ratio 95%CI</th>
<th>P-value</th>
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<td></td>
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<td>176/191</td>
<td>92.1</td>
<td>165/202 81.7</td>
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<td>92.1</td>
<td>167/192 87.0</td>
<td>1.88 (0.90–4.05)</td>
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<td>144/177 81.4</td>
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<td>109/168 64.9</td>
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<td>1/216 0.46</td>
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<td>18.8</td>
<td>15/106 14.2</td>
<td>1.41 (0.71–2.93)</td>
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</table>

n, number with mutant; N, number of participants with parasitaemia at blood smear tested
N*, number of participants with positive at Rapid diagnostic test

doi:10.1371/journal.pone.0162718.t004
not detected in a study conducted in three health districts in Senegal with 54 health posts with a gradual introduction of SMC [9].

Pfcrt and Pfmdr1 prevalence were similar to previous reports from Mali [12, 14], Burkina Faso [10] and Senegal [9]. The pre-SMC and post-SMC rates of Pfcrt 76T and Pfcrt 76T + Pfmdr1 86Y were comparable. However, there was a significant decrease of the prevalence of Pfmdr1 86Y in post-SMC patients, which may be due to the counter selection exerted by lumefantrine on this mutation [29–31] The effects of SP plus AQ on molecular markers of resistance to AQ require further investigation. A large body of literature shows that after malaria treatment with regimens that included AQ subsequent infections showed selection of parasites with Pfcrt and Pfmdr1 polymorphisms associated with AQ resistance [6, 14, 32, 33] and with decreased in vitro AQ sensitivity [34]. This appears to not be case when SP plus AQ is used.

We found that 8 months after SMC, children in the SMC age bracket had a higher prevalence of molecular markers of SP resistance than the non-SMC patient population, even though the two populations were living together in the same areas. This observation is indeed intriguing and we have no clear explanation for it. However, previous reports showed in vitro that parasites exhibiting pyrimethamine-resistant phenotype appear throughout the 54-day period following SP treatment [35]. We also showed seventeen years ago that following a single dose of sulphadoxine-pyrimethamine oral treatment, PfΔhfr mutations were selected up to 53 days later [36]. Given that these studies were conducted when SP was nearly fully susceptible in vivo as shown by the low prevalence of PfΔhfr and PfΔhps mutations, one could speculate that this selective pressure could last several months when the starting parasite population has much higher rates of these mutations as is the case now. In addition, the seven rounds of SMC with SP plus AQ given in this study could lead to drug accumulation in the treated population, further prolonging the elimination half-lives of the respective drugs and resulting in selection of molecular markers 8 months after the last round of SMC. Additional parasites genetic studies and drug pharmacokinetics studies will be required to clarify these issues.

To assess the potential spread of drug resistance genes in the general parasite population from the SMC areas, we measured the prevalence of molecular markers of resistance to the SMC drugs in contemporaneous populations that were not eligible for SMC because they were older than 5 years of age. Both SMC and non-SMC participants were from the same villages of the Health District of Koutiala although the former group was aged between 3 and 59 months while the latter group was aged 7 years and above. Although most of the SMC children were asymptomatic, some [approximately 5%] were symptomatic during the survey period while all of the non-SMC participants were clinical malaria cases. Interestingly, despite the significant selection of molecular markers of P. falciparum resistance to SP in the population that received the SMC regimens, there was no such increase in the general parasite population from the same study areas. This observation is consistent with previous reports from Mali [12] and in the sub-Region [10]. We reckon that no age matching was done because we purposely had to choose older children so as to exclude patients exposed to SMC. However, both the SMC group and the non-SMC patient populations were from the same exact villages and were therefore exposed to the same malaria transmission conditions and to the same pool of malaria parasites. Nevertheless, because of the difference in age between the two groups, differences in immunity could play a role in the prevalence of parasitaemia and/or in the clearance of mutant parasites [37]. Yet, no difference in prevalence of markers of resistance was found between younger and older patients in a study in Benin, neither in symptomatic patients nor in asymptomatic ones [38].

Although SMC appears to have contributed to the increase in frequency of molecular markers of SP resistance the overall efficacy of SP plus AQ may not be compromised at this stage. Indeed, (i) there was no significant increased prevalence of markers of resistance to AQ
resistance in the general population, (ii) there was no spread of the markers of SP resistance in the general parasite population in the same area, and (iii) the rate of Pfdhps 540E at baseline is still at 3%, much lower than the WHO threshold of 50% that is thought to compromise SP efficacy in intermittent preventive treatment. Indeed, a WHO technical advisory group recommended that in countries with a greater than 50% prevalence of the mutation at Pfdhps 540, IPTi with SP should not be initiated or continued [39] and the expected in vivo failure rate of a 10% quintuple mutant would be 5% [17].

Conclusion

SMC with SP plus AQ remains warranted in this setting but, the increasing trend of the prevalence of Pfdhps 540E and the significant increase in the prevalence of PfDHFR-DHPS quintuple mutant genotype in the treated population several months after the last SMC round call for close monitoring of the efficacy of these SMC drugs as well as an acceleration of the development of replacement regimens.

Supporting Information

S1 File. SMC population 2012 Demographic Data. (PDF)
S2 File. SMC population 2012 PCR Data. (PDF)
S3 File. SMC population 2014 Demographic Data. (PDF)
S4 File. SMC population 2014 PCR Data. (PDF)
S5 File. Non-SMC population 2014 Demographic Data. (PDF)
S6 File. Non-SMC population 2014 PCR Data. (PDF)

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Author Contributions

Conceptualization: HM EL IS AD OKD AAD.
Data curation: IS AD OKD AAD.
Formal analysis: IS HN.
Funding acquisition: HM AAD.
Investigation: HM MD IS AB AT SC SB YD ND AT DT FD AH AAD.
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