BLOOD FOLATE CONCENTRATIONS AND IN VIVO
SULFADOXINE-PYRIMETHAMINE FAILURE IN MALAWIAN CHILDREN WITH
UNCOMPPLICATED PLASMODIUM FALCiPARUM MALARIA

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Abstract. Folate antagonizes the antimalarial action of sulfadoxine-pyrimethamine (SP) in vitro, but its role in vivo is not well understood. We measured blood folate concentrations and SP therapeutic outcomes in Malawian children. Children with late treatment failure and those with adequate clinical and parasitologic responses had similar demographic characteristics, prevalence of parasite mutations conferring resistance to SP, and blood concentrations of antimalarial drugs following treatment. However, a higher folate concentration was associated with late treatment failure. Patients from a low malaria transmission site had higher blood folate concentrations than those in a higher transmission site (mean ± SEM = 39 ± 9.3 ng/mL versus 29 ± 10 ng/mL; P < 0.0001), and there was a higher rate of late treatment failure in the low transmission area (54.4% versus 40.2%; P = 0.010). This study also provides the first evidence of the independent role of physiologic folate concentrations in in vivo SP therapeutic efficacy, and the critical role of pyrimethamine concentrations in the therapeutic efficacy of SP when one controls physiologic folate levels and the frequency of critical dihydrofolate reductase/dihydropteroate synthase mutations.

INTRODUCTION

Malaria is a major infectious disease in many parts of the tropics with 90% of the 300–500 million annual cases occurring in tropical Africa.1 A major component of malaria control in sub-Saharan Africa is prompt treatment with effective antimalarial drugs.2 Chloroquine and sulfadoxine-pyrimethamine (SP) are currently the most widely used antimalarials in Africa. Owing to the spread of parasite resistance to chloroquine, the antifolate combination of SP has become the first-line therapy in many African countries.3 However, the increasing resistance to SP is of major concern.

Pyrimethamine is a competitive inhibitor of dihydrofolate reductase (DHFR, EC 1.5.1.3), whereas sulfadoxine inhibits dihydropteroate synthase (DHPS, EC 2.5.1.15). In Plasmodium falciparum, DHFR and DHPS are key enzymes in the biosynthesis of folate, a cofactor required for the transfer of CI units used in the biosynthesis of DNA and protein.4 Protozoa require folate coenzymes in the form of either 5,10-methylene-tetrahydrofolate or 10-formyl-tetrahydrofolate. Folate antagonists including pyrimethamine and sulfadoxine effectively inhibit de novo thymidylate biosynthesis in P. falciparum, thus blocking DNA synthesis and halting cell growth.

An increase in P. falciparum resistance to SP in African countries would hamper malaria control efforts because there are few inexpensive and safe alternative antimalarial drugs available. The molecular basis of antifolate resistance in P. falciparum has been reviewed.5 Mutations in the genes encoding DHFR and DHPS, are associated with in vivo P. falciparum resistance to these antifolates in various field studies.6,7 A reliable marker of SP resistance in the field is the presence of quintuple mutant P. falciparum parasites carrying five mutations: DHFR S108N + C59R + N51I and DHPS A437G + K540E.8 Folate has been shown to aid in vitro malarial growth or survival and to reverse inhibition by sulfonamides and antifolates.4,9 Parasites have the capacity to synthesize folate from both intact and degradation moieties (p-aminobenzoylglutamate and pterine-aldehyde) of exogenous folate added into the growth medium.10,11 This ability of malarial parasites to salvage intact folate or its metabolites to supplement de novo synthesis may contribute to the observed in vivo parasite resistance to antifolate drugs, particularly sulfa drugs.

Humans are unable to synthesize folate and must obtain it through their diet. We hypothesized that differences in blood folate concentrations would influence plasmodial growth in vivo in the presence of SP, with higher folate concentrations being associated with a reduction in antifolate (SP) drug efficacy. Russell and others reported a very wide range (5.0–21.0 ng/mL) for serum folate in Iran, suggesting a wide variation among individuals in different populations representing various socioeconomic classes from rural and urban areas.12 Factors other than intrinsic drug resistance affect antimalarial treatment outcome,13,14 and understanding determinants of antimalarial drug efficacy requires assessing the role of these factors along with the molecular markers of resistance.

Physiologic concentrations (normal blood folate concentrations achieved by diet alone in humans) of folate antagonize SP efficacy in vitro,15,16 but the relationship between folate levels and SP efficacy in vivo has not been studied in patients not taking folate supplementation. Folate supplementation resulted in a significantly higher rate of treatment failure among Gambian children who received SP therapy for uncomplicated malaria,17 but not in parasitemic pregnant women.18 These supplementation studies demonstrated a qualitative in vivo correlation of folate supplementation with SP therapeutic failure but did not measure post-supplementation blood folate concentrations, nor the prevalence of resistance-conferring mutations in the infecting malaria parasites. This precluded these studies determining the effects of folate on parasite susceptibility to SP. To assess the role of physiologic levels of folate on the in vivo efficacy of SP, we prospectively studied Malawian children with symp-
tomatic malaria and collected data that allowed us to adjust for age, sex, site, initial parasite density, hemoglobin concentration, day 3 blood SP concentration, and parasite DHFR/DHPS genotypes.

MATERIALS AND METHODS

Study area and population. The study was undertaken at two sites (Dedza with moderate intensity malaria transmission with pronounced seasonal variation in frequency of malaria illness and Mangochi with higher intensity malaria transmission with moderate seasonal variation in frequency of malaria illness) in central and southern Malawi from August to October 2000 and June to September 2000, respectively. This was during the dry season of low malaria transmission. The study was conducted following the World Health Organization (WHO) protocol for assessing antimalarial efficacy in highly endemic areas, modified to extend follow up to 28 days.19 Children 6 months to 12 years old reporting to the Mangochi or Dedza district hospital outpatient departments with signs and symptoms of malaria, an axillary temperature at presentation ≥ 37.5°C, and a parasite density ≥ 2,000/µL of blood were eligible for study. Only children whose caretakers provided informed consent were enrolled. The ethics committee of the University of Malawi College of Medicine reviewed and approved this study.

Treatment, follow-up of children, and sample collection. After a supervised treatment with a standard SP dose (1/4 tablet per 5 kg of body weight for an age ≤ 12 years; 1 tablet = 25 mg of pyrimethamine and 500 mg of sulfadoxine) on the enrollment day (day 0), each subject was followed again on days 3, 7, 14, 21, and 28. On each of those days finger prick blood samples were collected on 3MM Whatman (Brentford, United Kingdom) filter paper, air-dried, and stored in desiccated resealable plastic bags at room temperature for analysis of DHFR/DHPS mutations. Before treatment (day 0) capillary blood (50 µL) was taken, blotted on filter paper, and stored at 4°C for folate measurement.

Parasite density was determined from thick blood smears using Field stain either by counting parasites against leukocytes for parasite count/mm³ or, when the parasite density was > 200,000 per µL, by counting the percentage of parasitized erythrocytes on thin smears. The hemoglobin concentration was measured using the HemoCue Blood Hemoglobin Photometer (HemoCue Ltd., Angelholm, Sweden).

Capillary blood samples (100 µL) from patients with uncomplicated P. falciparum malaria were collected on 3MM Whatman filter paper before (day 0) and after SP treatment on day 3 for the determination of whole blood pyrimethamine and sulfadoxine concentrations. The sampling for folate, pretreatment drug, and genetic analysis was done at the same time on day 0. Samples for drug and genetic analysis were collected only on day 3.

Definition of treatment outcomes. Therapeutic efficacy was classified according to the World Health Organization protocols.20 Briefly, definitions were as follows. Early treatment failure (ETF) was defined as the development of danger signs or severe malaria after day 3 in the presence of parasitemia without meeting any criteria of early treatment failure; presence of parasitemia and an axillary temperature ≥ 37.5°C on any day from day 4 to day 28, without previously meeting any of the criteria of early treatment failure. Late parasitologic failure (LPF) was defined as the absence of parasitemia on day 28 irrespective of axillary temperature without previously meeting any of the criteria of ETF, LCF, or LPF.

The WHO definitions report LPF and LCF as subcategories of late treatment failure (LTF). In this study, we combined LPF and LCF cases to make the LTF category, which we compared with the ACPR group.

Elution and quantitation of folate. Folate was eluted into an ascorbate-detergent solution provided with the IMx Folate kit (Abbott Laboratories, Abbott Park, IL). Dried blood spots were punched out and the material was placed into a round-bottomed disposable borosilicate tube and covered with eluant (1 mL of folate lysis reagent). The samples were vortex mixed and sonicated (F200 sonicator; Instrolab, Johannesburg, South Africa) for 30 minutes. This extraction of whole dried blood spots into 1,000 µL of lysis reagent provided sufficient eluate for both hemoglobin spectrometry and the folate assay. The hemolyzate was stored at −20°C until analysis within a week.

Dried blood spot eluate (150 µL) was pipetted into the sample well of an IMx ion capture cell (IMx Automated Immunooassay Analyzer; Abbott Laboratories) and the test was initiated within 30 minutes following extraction or thawing. Whole blood (collected into heparinized Vacutainer® tubes; Becton Dickinson, Franklin Lakes, NJ) controls were used to monitor dried blood spot extraction efficiencies. To assess the linearity of the dried blood spot extraction method whole blood from normal individuals was spiked with known concentration of folic acid (BDH Laboratory Suppliers, Johannesburg, South Africa) spanning the range from 0 to 20 ng/mL, and quantitation results were corrected for endogenous blood folate concentrations before generating the calibration curve. The IMx Folate kit folate calibrators and controls were used to ensure accurate quantitation of folate in eluates of either whole blood or dried blood spots.

Drug assays. Whole blood concentrations of pyrimethamine and sulfadoxine were recovered from filter paper blood spots and measured by a modification of a reversed-phase, high-performance liquid chromatography procedure previously described for serum samples.20

Genetic analysis. A previously described nested polymerase chain reaction with mutation-specific restriction enzyme digestion was used to detect resistance-associated mutations at P. falciparum DHFR codons 51, 59, 108, and 164 and DHPS codons 437 and 540. Prevalence rates of the quintuple mutant genotype (S108N + C59R + N51I + A437G + K540E) among outcome groups and at each study site were determined.

Statistical analysis. Data were analyzed using STATA 7.0 (Stata Corporation, College station, TX). The two-sample Wilcoxon rank-sum test with a two-tailed level of significance at P < 0.05 was used in the univariate comparison of variables.
Multivariate analysis. A multivariate regression model analyzed the effect of blood folate levels on treatment outcome and controlled for the covariates blood pyrimethamine and sulfadoxine concentrations on day 3, age, sex, pre-treatment parasite density, and pre-treatment hemoglobin concentration, presence or absence of DHFR triple, DHPS double, or quintuple mutants, and site of study. Results of the multivariate analysis are reported in Table 2. We found that an increase in blood folate levels was independently associated with a higher risk of an LTF therapeutic outcome during the 28-day follow-up after SP therapy (odds ratio = 1.5, 95% CI = 1.08–1.98, \( P = 0.013 \)). Similarly, children < 2.5 years old, pyrimethamine concentration, the presence of a quintuple mutation, residence in the lower intensity transmission study site, and female sex were independently associated with a higher risk of a LTF.

**RESULTS**

Of 217 enrolled subjects, 26 were excluded from the analysis either because consent was withdrawn (n = 4), they were lost to follow-up (n = 21), or because the study protocol was violated (n = 1). One hundred ninety-one completed the study and were eligible for folate analysis. One or more of the following data were missing in 50 patients: no folate results (n = 2); no hemoglobin results (n = 34); no blood SP concentration measurements (n = 13), or incomplete DHFR/DHPS results (n = 20). For those with complete results (n = 141), patient characteristics are summarized in Table 1. There was no difference in geometric mean baseline parasitemia (8.128/μL, 95% confidence interval [CI] = 7.209–9.558/μL versus 8.393/μL, 95% CI = 4.641–9.354/μL, \( P = 0.58 \)), mean age (2.53 years, 95% CI = 2.13–2.92 years versus 2.67 years, 95% CI = 1.30–4.18 years, \( P = 0.64 \)), mean hemoglobin concentration (7.79 g/L, 95% CI = 7.47–8.29 g/L versus 7.49 g/L, 95% CI = 6.53–8.45 g/L, \( P = 0.59 \)), and sex ratio (1:1) between the 141 subjects and the cited exclusions.

**Univariate analysis.** Measured blood folate levels were higher in LTF cases compared with cases with an ACPR treatment outcome (\( P = 0.026 \), Figure 1, n = 141). The geometric mean parasite density at baseline was not different between ACPR and LTF treatment outcomes (7, 832/μL, 95% CI = 6,359–9,645/μL versus 8,360/μL, 95% CI = 7,011–9,968/μL, \( P = 0.57 \)) and parasitemia was not associated with treatment outcome in the univariate analysis. Day 3 post-treatment pyrimethamine and sulfadoxine blood concentrations, sex distribution, mean age, and pre-treatment hemoglobin concentrations were not different between the two treatment outcome groups (Table 1). However, blood folate concentrations tended to be higher in boys than girls, although the difference was not statistically significant (mean ± SD = 34 ± 12 ng/mL versus 30 ± 9.8 ng/mL; \( P = 0.092 \)). Subjects in Dedza had significantly higher blood folate levels than their counterparts in Mangochi (mean ± SD = 39 ± 9.3 ng/mL versus 29 ± 10 ng/mL; \( P < 0.0001 \)) and there was a significantly higher rate of LPF in Dedza compared with Mangochi (54.4% versus 40.2%; \( P = 0.010 \)) despite similar prevalence of the DHFR/DHPS quintuple mutant genotype that is strongly associated with SP treatment failure (Table 1).

**DISCUSSION**

Factors other than infection with SP-resistant *P. falciparum* may contribute to SP resistance, or affect the therapeutic efficacy of SP independent of parasite resistance genotype. These factors include partial immunity, which may contribute to clearance of infections containing resistant genotypes. In this study, we found that older children had a reduced risk of a resistant therapeutic outcome, an observation consistent with what is known about age-dependent acquisition of pre-munition against *P. falciparum*. Since inter-individual variation in pharmacokinetic factors may account for inter-individual variation in the observed SP therapeutic response, we investigated this and found non-significant differences in post-treatment day 3 whole blood pyrimethamine or sulfadoxine concentrations between subjects with an ACPR or LTF outcome. We found that the mean admission blood folate concentration was significantly higher in patients whose outcome was LTF than in those with ACPR. This difference was sustained even after adjusting for age, blood drug concentrations, pre-treatment hemoglobin concentration, pre-treatment parasite density, and parasite DHFR/DHPS genotype. A high blood folate concentration was associated with a 1.5-fold increased risk of LTF.

Our finding of a significant positive association between LTF (based on 28-day follow-up) and high blood folate concentrations in Malawian children who were treated with SP in both univariate and multivariate analysis would be expected because certain *P. falciparum* genotypes are efficient at using

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**Table 1**

Results of a univariate comparison of characteristics between cases of adequate clinical and parasitologic response (ACPR) and cases of late treatment failure (LTF)*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ACPR (n = 60)</th>
<th>LTF (n = 77)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood folate (ng/mL)</td>
<td>30 ± 11 (8.0–58)</td>
<td>34 ± 9.8 (12–58)</td>
<td>0.038†</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>59</td>
<td>42</td>
<td>0.49</td>
</tr>
<tr>
<td>Blood SDX (μg/mL)</td>
<td>80.8 ± 45.8 (23.3–230)</td>
<td>70.8 ± 37.5 (6.44–213)</td>
<td>0.14</td>
</tr>
<tr>
<td>Blood PYR (ng/mL)</td>
<td>264 ± 151 (59.5–668)</td>
<td>226 ± 130 (40.3–513)</td>
<td>0.23</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>2.40 ± 2.17 (0.500–8.33)</td>
<td>2.55 ± 1.74 (0.500–10.0)</td>
<td>0.23</td>
</tr>
<tr>
<td>Prevalence of quintuple mutant (%)</td>
<td>60.0</td>
<td>67.0</td>
<td>0.23</td>
</tr>
<tr>
<td>Day 0 parasite density (parasites/μL)</td>
<td>10,591 ± 8,431 (2.012–35,684)</td>
<td>10,850 ± 8,734 (323.00–57,180)</td>
<td>0.57</td>
</tr>
<tr>
<td>Day 0 Hb level (g/L)</td>
<td>8.04 ± 1.96 (3.20–11.9)</td>
<td>7.93 ± 1.55 (5.20–12.0)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* Values where indicated are the mean ± SD (range). Parasite density is the geometric mean. Reported drug concentrations are on day 3 after treatment. SDX = sulfadoxine; PYR = pyrimethamine; Hb = hemoglobin.

† \( P < 0.05 \)
exogenous folate. Our study provides, to our knowledge, the first published evidence of the independent role of physiological folate concentrations in in vivo SP therapeutic efficacy.

In the multivariate analysis, children in Dedza had a greater risk of LTF compared with children in Mangochi, despite a similar prevalence of the SP-resistant quintuple mutant genotype, and similar mean ± SD blood pyrimethamine (243 ± 167 ng/mL versus 234 ± 138 ng/mL; P = 0.99) and sulfadoxine (70.5 ± 64.2 µg/mL versus 66.8 ± 35.1 µg/mL; P = 0.86) concentrations. Leafy greens such as turnip greens (diet folate equivalent = 65 µg), orange juice (70 µg), peas (105 µg), beans (90 µg), and many other types of fruits and vegetables are rich sources of folate. In Malawi, Dedza is the main producer of these vegetables and fruits, which are more expensive to buy in Mangochi. Consequently, Mangochi residents have a fish/flesh-predominant diet. This difference in dietary pattern might account for the observed difference in blood folate levels between subjects from the two areas and the consequent difference in SP efficacy. However, folate concentrations alone do not fully account for the greater LTF rate seen in Dedza and other differences between Mangochi and Dedza that we have not been able to measure, e.g., level of immunity, may also contribute to the difference in the LTF rate.

Malawian children in this study exhibited a very wide range (8–58 ng/mL) of blood folate levels. Different normal ranges of whole blood folate levels have been reported (mean = 12.0 ng/mL, range = 3.0–20.0 ng/mL; mean = 6.35 ng/mL, range = 1.5–25 ng/mL; and mean = 89 ng/mL, range = 47–149 ng/mL) as determined by different methods. The former two sets of values were quantitated microbiologically using Streptococcus faecalis and Pediococcus cerevisiae, respectively, and the latter set using Lactobacillus casei. The wide variation in whole blood folate levels may be explained by marked differences in dietary patterns between populations since folate is entirely diet-derived in humans. It would therefore be expected that certain populations might have low folate dietary intake, while those that consume folic acid–containing supplements or eat fortified cereals would exhibit higher blood folate levels. Folate results obtained in the Malawian children in our study (the mean ± SD folate concentration for whole blood was 32.4 ± 10.9 ng/mL) are within previously reported ranges for other populations. Significant differences in folate levels in serum and erythrocytes between females and males of the same age have been described. Tsui and others found that boys had significantly higher folate levels in serum and erythrocytes than girls. We also found that blood folate levels tended to be higher in boys than in girls, but this difference was not statistically significant (34 ng/mL versus 31 ng/mL; P = 0.092).

Therapeutic pyrimethamine and sulfadoxine levels were sustained until day 3 post-therapy (Table 1). In the univariate analysis, there was no significant difference in day 3 blood pyrimethamine or sulfadoxine concentrations between ACPR

**TABLE 2**

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Odds ratio (95% CI)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Folate</td>
<td>1.5 (1.08–1.98)</td>
<td>0.013†</td>
</tr>
<tr>
<td>Day 0 hemoglobin</td>
<td>1.6 (0.29–8.51)</td>
<td>0.61</td>
</tr>
<tr>
<td>Parasitemia</td>
<td>1.0 (0.99–1.00)</td>
<td>0.75</td>
</tr>
<tr>
<td>Age &lt; 2.5 years</td>
<td>32 (1.19–855)</td>
<td>0.039†</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>0.99 (0.977–0.997)</td>
<td>0.018†</td>
</tr>
<tr>
<td>Sulfadoxine</td>
<td>0.98 (0.967–1.00)</td>
<td>0.096</td>
</tr>
<tr>
<td>Presence of quintuple mutant</td>
<td>323 (4.310–23,800)</td>
<td>0.0091†</td>
</tr>
<tr>
<td>Presence of DHPS double mutant</td>
<td>1.16 (0.0312–42.9)</td>
<td>0.94</td>
</tr>
<tr>
<td>Presence of DHFR triple mutant</td>
<td>6.337 (0.00268–1.50 × 1010)</td>
<td>0.24</td>
</tr>
<tr>
<td>Being in Dedza study area</td>
<td>6.0 × 10⁶ (73.0–4.96 × 10¹³)</td>
<td>0.010†</td>
</tr>
<tr>
<td>Female</td>
<td>17 (1.68–179)</td>
<td>0.016†</td>
</tr>
</tbody>
</table>

*DHFR/DHPS = dihydrofolate reductase/dihydropteroate synthase; CI = confidence interval.
†P < 0.05
and LPF cases. However, in the multivariate analysis, pyrimethamine, but not sulfadoxine, was associated with SP treatment failure ($P = 0.018$). These data provide the first evidence of the critical role of pyrimethamine in the therapeutic efficacy of SP when one controls for physiologic folate levels and the frequency of critical DHFR/DHPS mutations. These results suggest that reappearance of parasites is due, in part, to the parasite salvaging exogenous folate. The degree of synergy between pyrimethamine and sulfadoxine is highly dependent upon prevailing folate concentrations, suggesting that pyrimethamine acts to block folate uptake and/or use. Recent data suggest there is no significant role for DHPS, the sulfadoxine target enzyme, in folate salvage.

The observations that folate derivatives decrease the in vitro activity of antifolate drugs$^{15-17}$ and the requirement that folate-depleted culture medium be used to enhance antifolate activity strongly suggest that $P$. falciparum folate uptake contributes significantly to antifolate drug efficacy. Thus, our observation of a significantly reduced SP efficacy in children living in an area with comparatively higher blood folate concentrations supports suggestions$^{28}$ that inhibiting folate uptake in $P$. falciparum may be an important therapeutic strategy for potentiating antifolate activity. In a recent study,$^{28}$ the uricosuric agent probenicid increased the sensitivity of a highly resistant isolate to both sulfadoxine and pyrimethamine and showed promise as an adjunct to antifolate-based chemotherapy. Further investigations on the use of uricosuric agents as folate-depleted culture medium be used to enhance antifolate activity strongly suggest that DHPS, the sulfadoxine target enzyme, in folate uptake. Since natural folate occurs in a relatively low density in a wide variety of food, its bioavailability is affected not only by the incomplete removal of the polyglutamate chain by the intestinal conjugase$^{30}$ but also by folate losses during storage and cooking of food. This may lead to daily within-individual fluctuation of folate levels as a function of meal type and method of preparation. Therefore, since folate levels reported in this study were based on a single pre-treatment (baseline) measurement per subject, they could not account for daily fluctuation of blood folate within each subject during follow-up, as well as how that might have influenced the observed difference between the ACPR and LTF groups. The relevance of multiple sampling times for folate is exemplified by the observation of two study subjects who each had the highest measured folate concentration (58 ng/mL), but had an ACPR outcome, and two other subjects who had the lowest folate level (8 ng/mL), but had an LTF outcome. This discordance in the expected antifolate treatment outcome may be explained by the inadequacy of a single measurement of folate level per individual to account for daily fluctuation of folate during follow-up after SP therapy. For instance, it is possible that the individual who had the highest concentration ended up with much lower blood folate concentrations on days 2 and 3–28 because of changing meal types and this might have led to an ACPR outcome. Similarly, the individual with the lowest baseline folate concentration might have attained the high levels of blood folate during follow-up, leading to an LTF outcome. Future studies will have to include multiple blood sampling times during follow-up to tease out the aforesaid discordance.

In addition to the quintuple infection, young age, female sex, study site (considered a surrogate for semi-immunity), baseline folate level, and day 3 pyrimethamine concentrations were the significant risk factors for SP failure in our study. The latter two are potentially amenable to modification by malaria control programs. In the light of our observations, we propose that studies need to be done to qualitatively and quantitatively assess the impact of routine folate acid supplementation in patients with malaria, or pregnant women and infants on SP intermittent presumptive treatment.

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