

Safety and benefits of interventions to increase folate status in malaria-endemic areas

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Summary

For decades, folic acid has routinely been given to prevent or treat anaemia in children, pregnant women and people with sickle cell disease. However, there is no conclusive evidence that folate deficiency anaemia constitutes a public health problem in any of these groups. Industrial flour fortification is recommended and implemented in many countries to combat neural tube defects. Dietary folates or folic acid can antagonise the action of antifolate drugs that play a critical role in the prevention and treatment of malaria. Randomised trials have shown that folic acid supplementation increases the rate of treatment failures with sulfadoxine-pyrimethamine. The efficacy of antifolate drugs against *Plasmodium* is maximized in the absence of exogenous folic acid, suggesting that there is no safe minimum dose of ingested folic acid. We here review the safety and benefits of interventions to increase folate status in malaria-endemic countries. We conclude that formal cost-benefit analyses are required.

Keywords: folic acid, folic acid antagonists, malaria, neural tube defects, sulfadoxine-pyrimethamine.

Introduction

In developing countries, there are concerns that supplementation or food fortification with folic acid may reduce the efficacy of antifolate drugs that play a critical role in the prevention and treatment of malaria. The catalogue of policies to improve folate status nonetheless continues to grow, and now includes supplementation, industrial flour fortification, supplementary feeding and point-of-use fortification (see Supporting information for current guidelines by the World Health Organization, WHO).

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These concerns add to a debate about flour fortification with folic acid (e.g., Hubner *et al*, 2007; Wald & Oakley, 2007). Proponents argue that such fortification should be mandatory because supplementation and dietary modification are largely ineffective in preventing neural tube defects, and an increased intake of folic acid may reduce rates of cardiovascular disease and cognitive decline in older age, whereas opponents contend that health benefits are outweighed by theoretical risks of adverse effects, such as cancer and neuropathies due to masking the diagnosis of vitamin B₁₂ deficiency.

We aim to review the safety and benefits of interventions to improve folate status in malaria-endemic countries. First, we consider folate metabolism, requirements and deficiency from the perspective of the human host. We then review folate metabolism and utilisation in *Plasmodium* parasites, the mechanism of action of antifolate drugs and current policies regarding antifolate drugs against malaria. Finally, we assess the evidence that folic acid interventions and folate status affect treatment efficacy with these drugs. We focus on sub-Saharan Africa because the vast burden of malaria occurs in that region; our conclusions are nonetheless relevant for malaria-endemic areas elsewhere.

Folate functions and metabolism

Folate is present in cells as dihydrofolate or tetrahydrofolate, either in reduced form or as their derivatives with a single carbon unit (Fig 1). The role of reduced tetrahydrofolate, the active form of folate, is to carry one-carbon units, obtained from donor molecules, such as the amino acid serine, to intermediaries in the biosynthesis of a range of compounds including deoxynucleosides, the molecular building-blocks of DNA. These intermediaries also transfer methyl (CH₃) groups for binding to DNA ('methylation'), one of the epigenetic processes that regulate gene expression. Folate deficiency impairs the division of all cells, but disproportionately inhibits the development and propagation of rapidly proliferating cells, such as erythrocyte precursors. As folate depletion progresses further, it reduces proliferation of cells from other

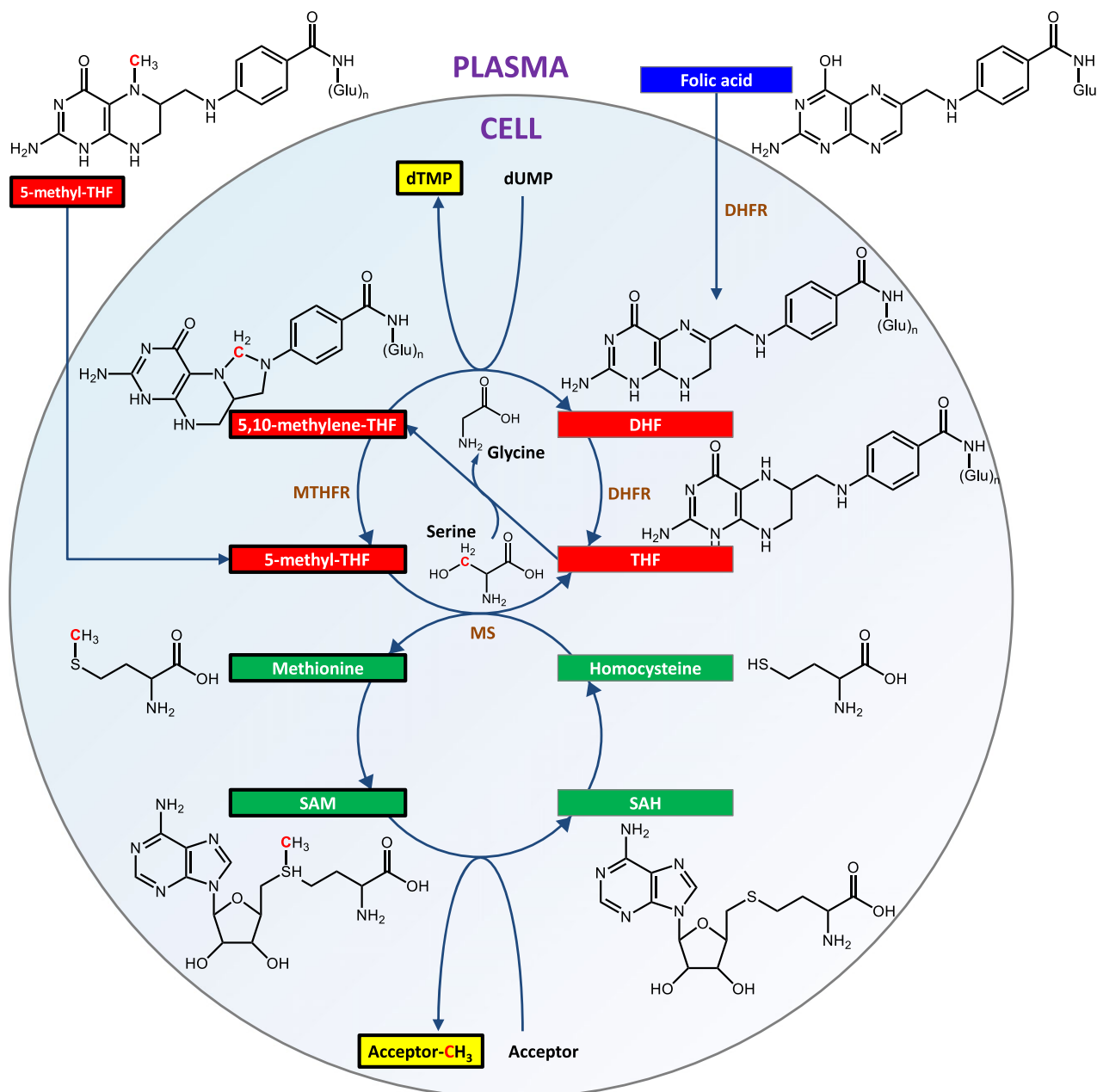


Fig 1. Folate metabolism in human cells. Folates, metabolites involved in the methionine cycle and methylated end products are indicated in red, green and yellow, respectively. Methyl donors and methyl receptors are indicated by thick and thin lines, respectively. Reduced tetrahydrofolate, the active form of folate, serves as a cofactor to carry one-carbon units from donor molecules such as serine to intermediaries in the biosynthesis of a range of compounds. One such compound is thymidine monophosphate (dTMP), a precursor of thymidine that constitutes one of four nucleobases in DNA. In the methionine cycle, a methyl group is transferred from 5-methyl-tetrahydrofolate to S-adenosylmethionine (SAM), which serves as a methyl donor to many acceptor substrates, such as nucleic acids, proteins, lipids and secondary metabolites. Cellular tetrahydrofolate is obtained from plasma mostly in the form of 5-methyl-tetrahydrofolate, but it can also be derived from folic acid, which is taken up and converted by the enzyme dihydrofolate reductase via dihydrofolate to tetrahydrofolate. Several metabolic pathways are not shown. For example, tetrahydrofolate can also react with formic acid to form 10-formyl-tetrahydrofolate, an essential metabolite in the biosynthesis of purines, whilst homocysteine can also be used for the synthesis of the amino acid cysteine. DHF: dihydrofolate; DHFR: dihydrofolate reductase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate (a deoxynucleotide); (Glu)_n: polyglutamate; MS: methionine synthase; MTHFR: 5, 10-methylene-THF reductase; SAH: S-adenosyl homocysteine; SAM: S-adenosyl methionine; THF: tetrahydrofolate.

haematopoietic lineages and those involved in the immunological response to infection (Dhur *et al*, 1991; Koury & Ponka, 2004). Manifestations of folate deficiency include

neural tube defects, haematological abnormalities and impaired immunity. In folate deficiency, erythroblasts undergo increased rates of premature death (ineffective

erythropoiesis) as shown by elevated serum concentrations of bilirubin and lactate dehydrogenase. Although the processes that lead to neural tube defects are poorly understood, there is evidence that the effect of folate deficiency is mediated by a disturbed methylation of DNA, RNA, proteins and lipids (Beaudin & Stover, 2009).

Humans lack several enzymes required to synthesize folate, and are entirely dependent on folate from dietary sources. Dietary folates are mostly conjugated to a polyglutamate chain. Following ingestion, all are converted to 5-methyl-tetrahydrofolate (5-methyl-THF) monoglutamate, mostly in the mucosa of the small intestine and to some degree in the liver, before entering into the peripheral circulation (Fig 2). Absorption of folate polyglutamates is rapid, with blood concentrations of 5-methyl-THF increasing within 15–20 min of ingestion (Pietrzik *et al*, 2010).

Folic acid (pteroylmonoglutamic acid) is the stable, industrially synthesized form of the vitamin that is used for flour fortification or supplementation. It does not normally occur in either dietary sources or the human body. Monoglutamates are absorbed more efficiently than dietary folates. Approximately 90% of a single dose of folate monoglutamates is absorbed, regardless of whether the dose is small (100 µg) or large (15 mg) (Hoffbrand *et al*, 2011). As with dietary folates, most folic acid is converted in the small intestine to 5-methyl-THF. With high oral doses of folic acid, however, this conversion is inefficient, leading to passive diffusion and transitory levels of unmodified folic acid in plasma (Kelly *et al*, 1997; Sweeney *et al*, 2007). The magnitude of this effect is dependent on dose and frequency of intake (Lucock *et al*, 1989; Powers, 2007), and unaltered folic acid persists in plasma for 6 h after ingestion of a single dose (Lucock *et al*, 1989).

Although folic acid is the first choice for supplementation or fortification because of its stability and low cost, several commercially available derivatives from natural folates have been proposed as possible alternatives to folic acid for supplementation (Pietrzik *et al*, 2010). 5-methyl-THF is available as its calcium salt, and has comparable bioavailability, physiological activity and ability to improve folate status at equimolar doses as folic acid (Pietrzik *et al*, 2010). Folinic acid (5-formyl-THF; leucovorin), which has similar vitamin activity, is administered in conjunction with methotrexate, an antifolate used in the therapy of cancer and rheumatoid arthritis. When used against cancer, methotrexate is administered by infusion in high doses that are life threatening. Folinic acid therapy is started sometime after initiation of methotrexate therapy to terminate the toxic effects of methotrexate ('rescue therapy'). As with folic acid, folinic acid is metabolized in the intestinal mucosa and liver to 5-methyl-THF, even at high oral doses (Schalhorn & Kühn, 1992; Stern *et al*, 2000). Not surprisingly, there are concerns that the use of folic acid or folinic acid can lead to reduced efficacy of methotrexate (e.g., Khanna *et al*, 2005; Baggott & Morgan, 2008).

Thus, irrespective of the form or dose of the folate derivative administered, 5-methyl-THF monoglutamate is by far the predominant circulating form of exogenous folate available for cellular uptake. It passes cellular membranes either through the reduced folate carrier or by an endocytotic process mediated by folate receptors (Kamen *et al*, 1988) and acts intracellularly as a methyl donor in the methionine cycle that produces tetrahydrofolate and methionine (Fig 1). Intracellular 5-methyl-THF and many other folate coenzymes are conjugated to a polyglutamate chain, which accounts for their intracellular retention. In addition, the enzymes involved in folate metabolism have higher affinity for folate polyglutamates.

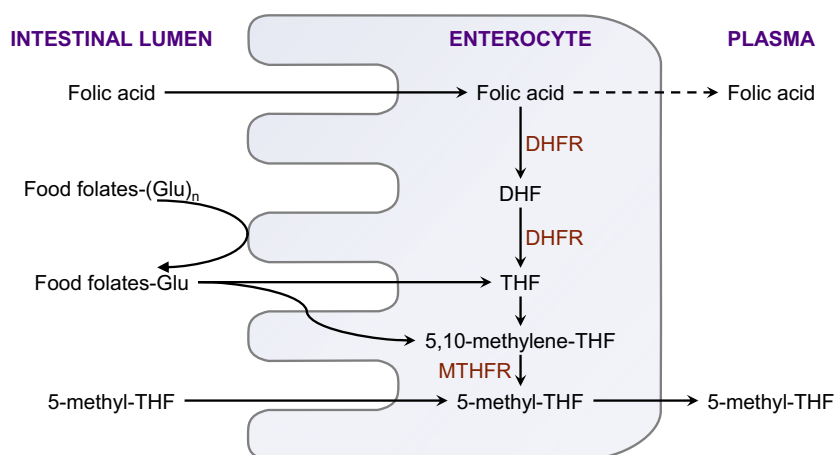


Fig 2. Intestinal absorption of dietary folates, folic acid and 5-methyl-tetrahydrofolic acid. Dietary folates and folic acid at low doses is converted in the small intestine to 5-methyl-THF. At high oral doses, folic acid enters the circulation through passive diffusion in unmodified form (dotted line). DHF: dihydrofolate; DHFR: dihydrofolate reductase; Glu: glutamate; (Glu)_n: polyglutamate; MTHFR: 5, 10-methylene-THF reductase; THF: tetrahydrofolate. Adapted from: *Clinical Pharmacokinetics*, 49, 2010, 535–548, Pietrzik, K., Bailey, L. & Shane, B. Folic acid and L-5-methyltetrahydrofolate: comparison of clinical pharmacokinetics and pharmacodynamics. © 2010. With permission of Springer.

Determinants of folate requirements in Africa

Staple foods commonly consumed in Africa, such as maize, cassava, sorghum, millet and rice, are poor dietary sources of folates. Many vegetables are naturally rich in folates. In rural African populations, however, vegetables are often consumed in soups, stews or sauces, and losses are typically great because folates are degraded by extensive cooking or reheating of foods (Fleming, 1989a).

Pregnant women with malaria are considered to be especially prone to develop folate deficiency (Fleming, 1989a). Folate requirements are particularly high in the second and third trimesters of pregnancy because of the needs imposed by fetal growth and by accelerated folate breakdown, which reflects metabolic turnover of folate (WHO/Food and Agriculture Organization of the United Nations [FAO], 2004). Plasma folate concentrations in breast milk are relatively unaffected by maternal intake, even when the mother is deficient in the vitamin, and maternal supplementation benefits the mother rather than the infant (Allen, 2012).

Folate requirements are associated with the rate of erythropoiesis. Malaria-induced haemolysis may lead to accelerated or ineffective erythropoiesis and thus increased folate requirements. *Plasmodium* infection, however, both during illness episodes and in symptomless carriers, can also present with suppressed erythropoiesis (Verhoef *et al*, 2002), which would reduce folate requirements. In individuals with high levels of acquired immunity, the effect of *Plasmodium* on haemolysis and thus the increase in folate requirements is limited. The measurement of folate status in malaria is complicated because *Plasmodium* parasites cause elevated folate concentrations in erythrocytes (see below). In addition, folate concentrations in erythrocytes are 30 times as high as in plasma, so that even a small degree of (intravascular) haemolysis can raise plasma folate values and mask cellular folate deficiency (Antony, 2008).

Chronic haemolysis in individuals with sickle cell disease results in elevated erythropoiesis rates and is believed to lead to drastically increased folate turnover and requirements (Fleming, 1989b). In 2010, 235 681 neonates were born with sickle cell disease in sub-Saharan Africa (Piel *et al*, 2013). About 50–80% of these infants will die before the age of 5 years (WHO, 2010a). Patients suffer from chronic ill health interspersed with acute anaemic, infarctive and infective crises. The disease is characterized by erythrocytes with an abnormal, rigid sickle shape that block small blood vessels, impair blood flow and reduce erythrocyte survival. The resulting chronic haemolytic anaemia results in greatly enhanced erythropoiesis. Even in the steady state, reticulocyte counts are raised up to 20% (Fleming & De Silva, 2003).

Folate status and effectiveness are affected by a mutation of the gene encoding for methylenetetrahydrofolate reductase (MTHFR), the enzyme that regulates conversion of 5,10-methylene-tetrahydrofolate to 5-methyl-THF (Figs 1,2).

Homozygosity for the 677C→T nucleotide substitution is associated with a reduction in MTHFR activity, and with reduced folate concentrations in serum and erythrocytes (Crider *et al*, 2011), but its prevalence in African populations is much lower than among Caucasians (Adjalla *et al*, 2003; Pegoraro *et al*, 2004).

Folate status can also be reduced by the use of drugs that interfere with host folate metabolism, such as some anti-malarial drugs; phenobarbital, phenytoin and carbamazepine used for first-line prevention of epileptic seizures; and methotrexate. Although widely used in developing countries, the public health importance of these drugs on folate status remains largely unknown.

Evidence of folate deficiency

With the exception of women of reproductive age, folate deficiency is defined by biochemical indicators (i.e., erythrocyte folate concentration and/or plasma/serum folate concentration, with cut-off points defining the range below which homocysteine concentrations become elevated; WHO, 2015a). The public health importance of the impact of applying these cut-off values is not fully understood (De Benoist, 2008). Elevated concentrations of circulating homocysteine were once considered as a functional indicator of folate deficiency because of their association with cardiovascular disease, but the causality of this association is now highly doubtful (Miller *et al*, 2010; Clarke *et al*, 2012).

There are additional reasons why survey reports of folate deficiency or folate deficiency anaemia should be interpreted with caution: (i) measurements of folate concentrations in erythrocytes, serum or plasma are subject to large inter-laboratory and inter-assay variability (WHO, 2015a); (ii) even though circulating folate concentrations seem unaffected by inflammation (Galloway *et al*, 2000), they can be increased by *Plasmodium* infection independently of host folate status; (iii) interventions that raise folate concentrations in erythrocytes or serum do not necessarily improve haemoglobin concentrations or reduce the risk of anaemia (see below), and; (iv) anaemia in the presence of folate deficiency is not necessarily caused by folate deficiency but can also be due to infections or other micronutrient deficiencies (Metz, 2008). Thus, at the population level, folate deficiency can be demonstrated convincingly only through randomised, placebo-controlled trials showing that folic acid results in improvements in haemoglobin concentrations or functional health outcomes.

Prevention of neural tube defects

Trials with folic acid supplementation before conception and continuing in the first 12 weeks of pregnancy have shown compelling evidence that the occurrence of neural tube defects can be reduced by 70% (De Regil *et al*, 2015). In

Sub-Saharan Africa, these defects affected 58 000 births, or 2.1 per 1000 births (2001 data in Christianson *et al*, 2006), causing disabilities, often lasting for life, and enormous costs related to surgeries, physical therapy and rehabilitation.

Anaemia control

Despite programmes for the distribution of folic acid supplementation through antenatal clinics in most countries, there is no evidence from observational studies that folate deficiency anaemia is a public health problem among infants and young children in Africa (Metz, 2007) and no evidence from randomized trials that folic acid supplementation alone in children or pregnant women leads to reduced anaemia (Fishman *et al*, 2000; Lohner *et al*, 2012). Even in children with severe anaemia (haemoglobin concentration <50 g/l), there is no evidence of folate deficiency, and severe anaemia is not associated with plasma folate concentrations (Calis *et al*, 2008).

WHO recommends folic acid supplementation to prevent anaemia in persons with sickle cell disease (Table SI). Only one quasi-randomised, placebo-controlled trial has been conducted to assess the effect of folic acid supplementation in individuals with sickle cell disease, but it failed to demonstrate that folic acid (5 mg/day) provides benefits to children as measured by haemoglobin concentrations, or rates of growth, infection or vaso-occlusive crises (Dixit *et al*, 2016). Observational studies also found no evidence that sickle cell disease was associated with folate status as assessed by folate concentrations in serum and erythrocytes, and by plasma homocysteine concentration (Rodriguez-Cortes *et al*, 1999). Overall, there is also no evidence to support folic acid interventions to prevent anaemia in sickle cell disease (Wang, 1999).

Improved pregnancy outcomes

It has been suggested that folate deficiency early in pregnancy may cause low nucleic acid synthesis and impaired growth of the placenta, leading to reduced birth weight and preterm delivery. There is some support from observational studies and trials that periconceptional supplementation with folic acid may improve fetal growth and birth weight (Rolschau *et al*, 1999; Relton *et al*, 2005; Timmermans *et al*, 2009; Furness *et al*, 2012), but the evidence remains controversial. A recent meta-analysis of randomised controlled trials (Lassi *et al*, 2013) found no conclusive evidence of benefit of antenatal supplementation with folic acid on preterm birth, stillbirth, neonatal death, mean or low birthweight, mean or low pre-delivery haemoglobin concentration, or pre-delivery folate concentrations in serum or erythrocytes. A reduction was found in the relative risk of megaloblastic anaemia, but the absolute risk reduction was small and findings were based on 4 trials with severe methodological shortcomings. The authors found that their results are probably explained

by bias due to non-random allocation, poor concealment and inadequate blinding, and concluded that there was no convincing evidence of benefit of folic acid supplementation during pregnancy on pregnancy outcomes. Similar results were found in earlier meta-analyses (Mahomed, 1997; Charles *et al*, 2005).

Folate and antifolate metabolism in *Plasmodium* parasites

Because *Plasmodium* does not incorporate exogenous thymine or thymidine, the thymidylate required for DNA synthesis must be synthesized *de novo* through the folate cycle. Protozoa and bacteria generally synthesize folates *de novo* from guanosine-triphosphate (a precursor of dihydropteroate diphosphate), *para*-aminobenzoic acid (pABA) and glutamate (Fig 3). This synthetic pathway has long been a target for drugs used against rapidly reproducing cells such as *Plasmodium* parasites and microbial pathogens.

Sulfonamides and sulfones, such as sulfadoxine, sulfamethoxazole and dapsone, are structural analogues to pABA, and act as competitive inhibitors of dihydropteroate synthetase (DHPS), an enzyme that acts by converting pABA to DHPS. These drugs have selective antiparasitic or antibacterial activity because DHPS is lacking in humans (Nzila *et al*, 2005; Nzila, 2006).

By contrast, diaminopyrimidines, such as pyrimethamine, proguanil and trimethoprim, competitively inhibit dihydrofolate reductase (DHFR), thus blocking the conversion of dihydrofolate to tetrahydrofolate, as well as the conversion of folic acid to dihydrofolate (Fig 3). Diaminopyrimidines act selectively because they bind much more strongly to bacterial and parasitic DHFR than the corresponding human enzyme (Stokstad & Jukes, 1987). In addition, selectivity of DHFR inhibitors may be based on human-parasite differences in the regulation and expression of DHFR (Zhang & Rathod, 2002).

Sulfonamides and diaminopyrimidines have a greater effect when given combined than the sum of their effects when given separately (Gregson & Plowe, 2005); the reason for this synergy may be that they block two successive steps in the same metabolic pathway.

In *Plasmodium* spp., blocking of folate synthesis by antifolates results in decreased concentrations of tetrahydrofolate, decreased conversion of serine to glycine, reduced synthesis of thymidylate, and ultimately to decreased production of parasite DNA (Gregson & Plowe, 2005). Rapidly dividing cells, such as *Plasmodium* parasites, have a high demand for nucleotide precursors for DNA synthesis, and thus are particularly sensitive to antifolates. DHPS and DHFR inhibitors act against all human malaria parasites, but *P. falciparum* is inherently more sensitive than *P. vivax*, *P. malariae* and *P. ovale* (Gregson & Plowe, 2005).

In addition to endogenous production of folate, however, many strains of *Plasmodium* parasites are also capable of utilizing exogenous folic acid or folinic acid through specialized

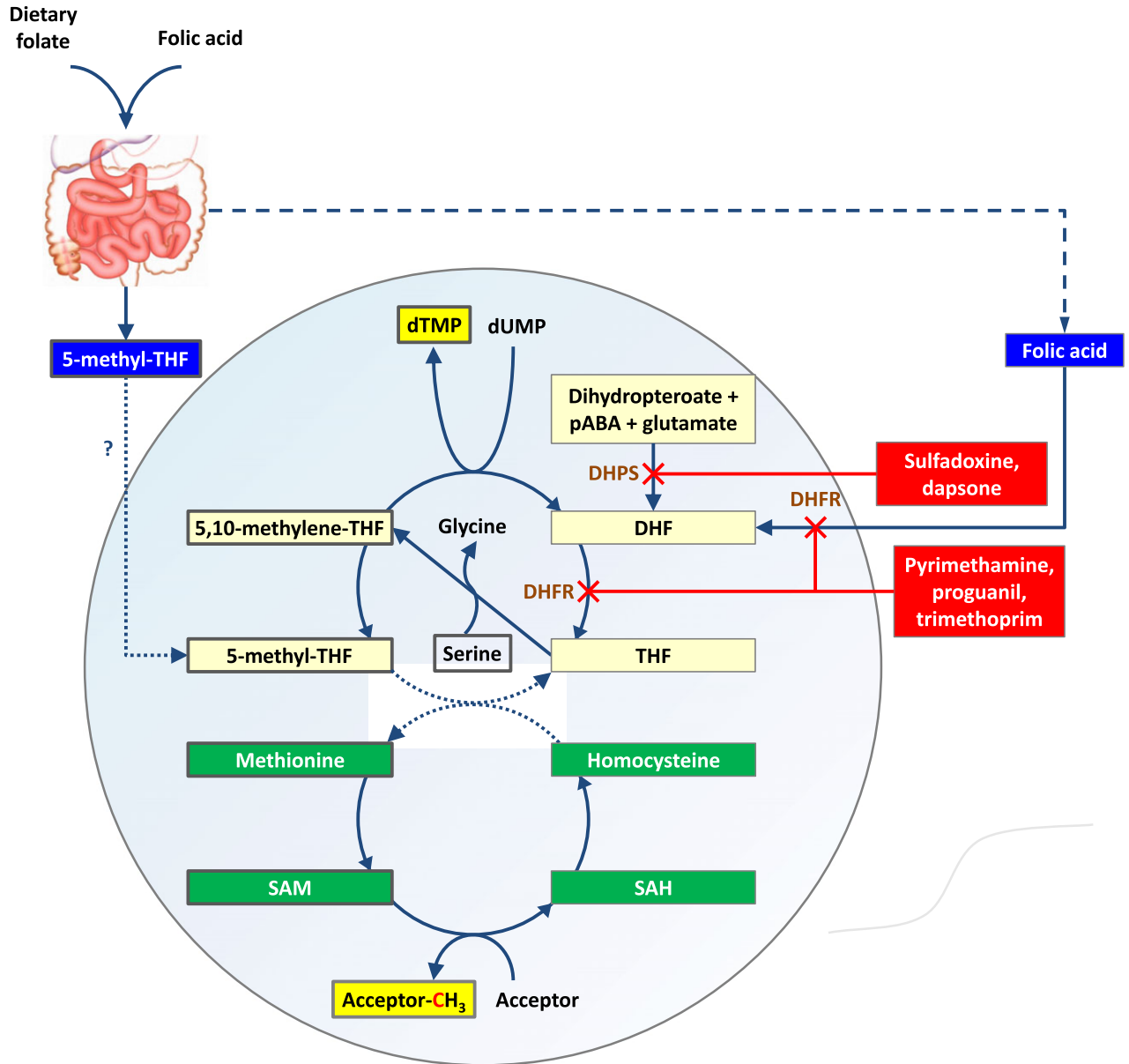


Fig 3. Folate metabolism in erythrocytic stages of *Plasmodium* parasites. Methyl donors and methyl receptors are indicated by thick and thin lines, respectively. In the endogenous pathway, *Plasmodium* can synthesize dihydrofolate and tetrahydrofolate *de novo* from dihydropteroate, pABA and glutamate moieties. Antifolate drugs (e.g. sulfadoxine, pyrimethamine) act by competitive binding to enzymes involved in this synthetic pathway. Thus DHPS is competitively inhibited by sulfadoxine and dapsone, whereas DHFR is competitively inhibited by pyrimethamine, proguanil and trimethoprim (red arrows). This antifolate activity results in inhibition of folate synthesis and, ultimately, reduced synthesis of thymidylate and parasite DNA. Ingested folic acid enters the circulation when ingested in high doses (dashed blue line). The parasite can access circulating folic acid and convert it through DHFR to dihydrofolate and tetrahydrofolate. By utilising the exogenous folic acid as a source of folates, the parasite can bypass the inhibition of the endogenous pathway and thus antagonise the activity of antifolate drugs. In theory, 5-methyl-THF is a methyl donor in the methionine cycle (lower cycle, with metabolites in green), resulting in the production of tetrahydrofolate and methionine (blue dotted lines). Recent evidence from an *in vitro* study suggests, however, that this pathway is not effectively utilised in *Plasmodium*, either because 5-methyl-THF monoglutamate from the erythrocytic pool cannot cross the membranes of the parasitophorous vacuole and the parasite (blue dotted line, with question mark) or because the parasite does not need methionine, because it derives this amino acid precursor of S-adenosylmethionine from haemoglobin degradation or human plasma (Nduati *et al*, 2008). This evidence suggests that oral 5-methyl-THF may act as a source of folates in humans without antagonising the *in vitro* activity of antifolate drugs against *P. falciparum* (Nduati *et al*, 2008; Nzila *et al*, 2014). DHF: dihydrofolate; DHFR: dihydrofolate reductase; DHPS: dihydropteroate synthase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate (a deoxynucleotide); pABA: para-aminobenzoic acid; SAM: S-adenosyl methionine; THF: tetrahydrofolate.

protein carriers across the erythrocyte membrane and across the membranes of the parasitophorous vacuole and the parasite (Krungskrai *et al*, 1990; Wang, 1999; Nzila *et al*, 2005). To avoid antagonizing the action of sulfadoxine, *in vitro* tests for sulfadoxine resistance require that human serum, which is needed to support parasite growth in culture, is depleted of residual folic acid and folinic acid (Chulay *et al*, 1984; Geary *et al*, 1985; Wang *et al*, 2007). Folic acid added to an *in vitro* *P. falciparum* culture competitively antagonizes the inhibitory action of pyrimethamine in a linear dose-relationship (Wang, 1999). Given the propensity of ingested folic acid to result in the formation of unmodified folic acid in plasma, these studies suggest that interventions with folic acid or folinic acid can reduce the antimalarial activity of antifolate drugs and thus their efficacy in a dose-dependent manner.

Erythrocytes and liver cells accumulate 5-methyl-THF in the polyglutamate form, but it is doubtful that this store can be utilised by intracellular stages of *P. falciparum*, because (i) this pool may be insufficient to sustain intracellular development; (ii) the parasite probably lacks glutamyl hydrolase, the enzyme that is required to remove the polyglutamate moieties; and (iii) antifolates would not have antimalarial activity if a source of folate is present that would bypass the action of these drugs (Ferone, 1977; Krungskrai *et al*, 1990).

There is some evidence that the parasite can utilise exogenous 5-methyl-THF to derive methionine by *de novo* synthesis (Krungskrai *et al*, 1990; Nzila *et al*, 2005). If this pathway were used to produce THF, the parasite could theoretically bypass the inhibition of folate synthesis that is induced by antifolate drugs. An *in vitro* study recently showed, however, that even at supra-physiological concentrations, 5-methyl-THF hardly antagonised the activity of pyrimethamine and chlorocycloguanil against *P. falciparum* (Nduati *et al*, 2008). This finding indicates that either 5-methyl-THF is not transported into the parasite cell or that 5-methyl-THF is present intracellularly but the *de novo* methionine pathway is not effectively utilised, perhaps because the parasite can use haemoglobin degradation or human plasma as an alternative source of methionine (Nduati *et al*, 2008).

Antifolate resistance in *P. falciparum* is due primarily to mutations in *dhps* and *dhfr* (Gregson & Plowe, 2005), resulting in altered structure and reduced drug affinity of DHPS and DHFR. This suggests that the interference of ingested folic acid with antifolate drug action is probably most pronounced in situations with high levels of antifolate resistance. In addition, such reduced antifolate efficacy is likely to depend on the dose and duration of the folic acid intervention, and on host folate status.

Policies regarding antifolate drugs against malaria

Given the enormous burden of malaria, and the important role that antifolate drugs continue to play in its prevention,

even small reductions in efficacy of these drugs are likely to have major public health repercussions. In 2015, there were an estimated 438 000 malaria deaths and 214 million episodes of malaria worldwide; of these, 395 000 deaths (90%) and 188 million cases (88%) occurred in WHO's African Region (i.e. sub-Saharan Africa except Sudan and Somalia) (WHO, 2015b). Approximately 74% of African malaria deaths occurred in children aged below 5 years (WHO, 2015b). Low birth weight associated with malaria in pregnancy may result in 100,000 infant deaths in Africa annually (Guyatt & Snow, 2004). In areas of high and moderate transmission, *P. falciparum* infection in pregnant women often occurs without symptoms but accounts for a substantial proportion of cases of maternal anaemia, low birth weight and infant deaths (Desai *et al*, 2007).

Sulfadoxine-pyrimethamine (Fansidar) is recommended by WHO for pregnant women and young children for chemoprevention in areas with a high burden of malaria (WHO, 2010b, 2014) (Table I). Chemoprevention comprises the repeated administration at curative doses of a slowly eliminated antimalarial drug, which results in periods in which subsequent new infections are inhibited by persistent but declining concentrations of the drug (post-treatment prophylactic effect). Thus pregnant women should receive therapeutic courses of antimalarial drugs on at least three occasions (regardless of the presence or absence of infection) administered at antenatal clinic visits at least 1 month apart, and starting after quickening (WHO, 2014). This strategy exploits the opportunity provided by a high proportion of pregnant women attending antenatal clinics at least once during pregnancy, and many attending at least twice. It substantially reduces the prevalence of maternal anaemia, placental parasitaemia and the occurrence of low birth weight, whilst no marked adverse events to sulfadoxine-pyrimethamine in either mother or infant have been detected (WHO, 2014). Sulfadoxine-pyrimethamine is the only drug recommended for this purpose because of its safety in pregnancy, efficacy, and because it can be delivered as a single dose under supervision to ensure adherence. By 2014, it had been adopted as a national policy in 36 countries with a high burden of malaria in Sub-Saharan Africa, Laos, Papua New Guinea and the Philippines (WHO, 2015b). In those countries for which consistent data were available, coverage varied considerably between countries, but it was estimated that half of women attending antenatal clinics received at least one therapeutic dose (WHO, 2015b).

Intermittent preventive treatment with sulfadoxine-pyrimethamine has been shown to substantially reduce malaria rates both in infants and older preschool children (Mere-mikwu *et al*, 2008; Wilson, 2011). It was endorsed as a policy in infants only through its co-administration with second and third doses of diphtheria-tetanus-pertussis vaccine (DTP2, DTP3) and measles immunization at 10, 14 weeks and 9 months of age, respectively (WHO, 2010b). In 2012, the WHO recommended that children aged 3–59 months in

Table I. Recommendations by the World Health Organization for preventive therapies against malaria with antifolate drugs.

Target group	Policy name	Definition	Intervention	Setting	Reference
Pregnant women	Intermediate Preventive Treatment for pregnant women (IPTp)	To control the effects of <i>Plasmodium</i> infection on the pregnant woman and her fetus	At least two and possibly three* therapeutic (single) doses of sulfadoxine-pyrimethamine, to be delivered after quickening. Sulfadoxine-pyrimethamine should not be given more frequently than monthly, and should not be administered concurrently with cotrimoxazole	Areas of medium-high transmission (stable)	WHO (2014)
Infants	Intermediate Preventive Treatment for infants (IPTi)	To reduce the malaria burden of targeted children	Therapeutic (single) doses of sulfadoxine-pyrimethamine, to be delivered through immunization services – usually at 10, 14 weeks and approximately 9 months of age, to infants at risk of malaria	Areas of moderate to high transmission in Sub-Saharan Africa	WHO (2010b)
Children aged 3–59 months	Seasonal malaria chemoprevention, previously referred to as Intermittent Preventive Treatment in children (IPTc)	To prevent malaria in targeted children	A treatment course of sulfadoxine-pyrimethamine (single dose) plus amodiaquine (once daily for 3 days), at monthly intervals, beginning at the start of the transmission season, to a maximum of four doses during the malaria transmission season	Areas of highly seasonal malaria transmission across the Sahel sub-region	WHO (2012)

*The World Health Organization recommends a schedule of four visits to antenatal clinics, with three visits after quickening (the moment that a pregnant woman first perceives or feels motion of the fetus in the uterus, usually at a gestational age of 18–20 weeks in primigravidae, or 15–17 weeks in multiparae; Levene *et al*, 2000).

areas of highly seasonal malaria transmission across the Sahel sub-region should receive therapeutic courses of sulfadoxine-pyrimethamine plus amodiaquine at monthly intervals, beginning at the start of the transmission season, to a maximum of four doses during the malaria transmission season, and with delivery overseen by community volunteers ('seasonal malaria chemoprevention') (WHO, 2012). By 2015, only one country (Chad) had adopted a national policy of intermittent preventive treatment for infants, whilst eight countries (Chad, Congo, The Gambia, Guinea, Mali, Niger, Senegal and Togo) have national programmes for seasonal malaria chemoprevention. Coverage may accelerate with the increasing availability of financial support (WHO, 2015b).

Sulfadoxine-pyrimethamine is also used in combination with artesunate for first-line treatment of uncomplicated malaria in Afghanistan, Azerbaijan, India, Iran, Pakistan, Saudi-Arabia, Somalia, Sudan and Yemen (WHO, 2015b). Although first-line therapy containing an artemisinin derivative is now the standard for falciparum malaria worldwide, a small but important proportion of children in sub-Saharan

Africa continue to be treated with sulfadoxine-pyrimethamine alone (WHO, 2015b).

Proguanil is used prophylactically (in combination with chloroquine) in pregnant women at risk of *P. falciparum* (Botswana, South Africa and Swaziland) and as second-line treatment for *P. falciparum* (French Guyana). It is also used with atovaquone (Malarone) both for treatment and prevention of malaria among tourists, but a donation programme initiated in 1996 to make this combination available to endemic countries was discontinued because of cost considerations (Oyediran *et al*, 2002). Chlorproguanil combined with dapsone (Lapdap) was also used to treat malaria but this was discontinued following reports that it leads to reduced haematocrit values and severe anaemia in persons with glucose-6-phosphate dehydrogenase (G6PD) deficiency, an inherited human enzyme defect that is common in African populations (Luzzatto, 2010).

Malaria is the most common precipitating cause of crises in sickle cell disease in endemic countries. To avoid such crises, national guidelines in many countries recommend that

people with sickle cell anaemia receive life-long prophylaxis with antimalarial medicines, which often include the antifolates *proguanil*, *pyrimethamine* or a combination thereof (Oniyangi & Omari, 2006).

Lastly, cotrimoxazole, a combination of the antifolate drugs *trimethoprim* and *sulfamethoxazole*, can be used to treat malaria, but it is also indicated as primary prophylaxis against human immunodeficiency virus (HIV)-related infections in children and adults with HIV/acquired immunodeficiency syndrome, including pregnant women after the first trimester; and in any infants born to HIV/infected mothers (WHO, 2013).

Increased folate intake and malaria

The Tolerable Upper Intake Limit (i.e., the highest average daily intake level of a nutrient that is unlikely to pose a potential risk of adverse effects to all apparently healthy individuals in an age- and sex-specific group) (UL) for folic acid was set to avoid neuropathy due to vitamin B₁₂ deficiency in

non-target groups (WHO/FAO, 2004), and does not take into account adverse effects due to its interference with antifolate drugs. Antagonistic effects with antifolate drugs may occur because of transient but recurrent elevation of circulating levels of 5-methyl-THF or unmetabolized folic acid following ingestion of folic acid, or because of high folate status either through long-term interventions or a diet rich in natural folates.

Supplementation

At least four randomized controlled trials in humans have shown that supplementation with folic acid can reduce the therapeutic efficacy of sulfadoxine-pyrimethamine (Table II). In Gambian children treated for uncomplicated malaria, adjunct supplementation with folic acid for 28 days resulted in an increased frequency of treatment failure with sulfadoxine-pyrimethamine. The folic acid dose in this trial was very high (5–10 mg/day dependent on body weight, as compared to an intake of 48–180 µg, dependent on age, being sufficient

Table II. Effect of folic acid on therapeutic efficacy of sulfadoxine-pyrimethamine against malaria; summary of randomized controlled trials.

Country	Study population	Comparison*	Key finding(s)	Reference
The Gambia	Children aged 6 months to 9 years with uncomplicated <i>Plasmodium falciparum</i> malaria, treated with iron	Daily folic acid (5, 7.5 and 10 mg for children weighing <15, 15–20 and >20 kg, respectively) <i>versus</i> placebo	Folic acid increased the risk of parasitological failure at day 7 (11% vs. 5%; $P = 0.25$) and day 28 (30% vs. 15%; $P = 0.04$) after the start of treatment	Boele van Hensbroek <i>et al</i> (1995)
Kenya	Patients of all ages with uncomplicated <i>P. falciparum</i> malaria and mild-moderate anaemia, treated with iron	Daily folic acid (2.5 and 5 mg for those aged <2 and ≥2 years, respectively) <i>versus</i> placebo	Folic acid increased the risk of parasitological failure ($P < 0.0001$). No evident effect on risk of clinical failure	Carter <i>et al</i> (2005)
Zambia	Children aged 6–119 months with uncomplicated <i>P. falciparum</i> malaria and haematocrit 9–21%, treated with iron	Daily folic acid (1 mg) <i>versus</i> placebo	Folic acid increased the risk of parasitological failure at days 3 ($P = 0.01$), 7 ($P = 0.12$), and 14 ($P = 0.44$) after the start of treatment	Mulenga <i>et al</i> (2006)
Kenya	Pregnant women with gestational age 17–34 weeks with <i>Plasmodium</i> infection and haemoglobin concentration >70 g/l	Daily folic acid (either 5 or 0.4 mg) <i>versus</i> placebo	Compared to placebo, folic acid (5 mg) increased parasitological failure risk at day 14 (27% vs. 14%; adjusted HR, 98.5% CI: 2.19, 1.09–4.40†; $P = 0.005$). Corresponding values for folic acid (0.4 mg): 14.5%; adjusted HR 1.07, 0.48–2.37. No evident effect on haemoglobin concentrations	Ouma <i>et al</i> (2006); Van Eijk <i>et al</i> (2008)
The Gambia	Primigravidae with gestational age >15 weeks and haemoglobin concentration >70 g/l receiving iron	Daily folic acid (1.5, 1.0 and 0.5 mg for those with haemoglobin concentration 70–90, 90–110 and ≥110 g/l) <i>versus</i> placebo	Folic acid had no evident effect on parasitological failure, but marginally improved haemoglobin concentration (1.4 g/l, 95% CI 0.1–2.7 g/l)	Mbaye <i>et al</i> (2006)

CI, confidence interval; HR, Hazard ratio.

*All study participants received treatment with sulfadoxine/pyrimethamine.

†To account for multiple comparisons and the interim analysis, Ouma *et al* (2006) reported 98.7% CIs instead of conventional 95% CIs.

to meet the dietary needs of 97.5% of children in the studied age range of 6 months to 9 years; WHO/FAO, 2004). Even in this group of children with elevated risk of folate deficiency due to malaria-induced haemolysis, folic acid did not improve haematological recovery (haemoglobin concentrations, mean cell volume or mean corpuscular haemoglobin concentration) even though it led to a marked increase in erythrocyte folate concentrations (Boele van Hensbroek *et al*, 1995).

In Kenyan patients treated for falciparum malaria with sulfadoxine-pyrimethamine, daily supplementation with folic acid (2.5 or 5 mg for those aged <2 and \geq 2 years, respectively) reduced the time to treatment failure as assessed by parasitological examination of blood slides collected at various points of follow-up (Carter *et al*, 2005). There was no evidence of an effect on recovery from anaemia.

Folic acid (1 mg/day) increased the risk of parasitological treatment failure with sulfadoxine-pyrimethamine in Zambian children even though it led to a marginal improvement of haematocrit [1.2%, 95% confidence interval (CI) 0.2–2.2%] by day 14 of follow-up. In this study, folic acid had no evident effect on the risk of parasitaemia in children treated with atovaquone-proguanil (Mulenga *et al*, 2006).

In Kenyan pregnant women with *Plasmodium* infection, daily supplementation with 5 mg folic acid doubled the risk of treatment failure with sulfadoxine-pyrimethamine at 14 days (Ouma *et al*, 2006). A lower dose of folic acid (0.4 mg/day) resulted in a marginal and statistically non-significant risk increase (14.5% vs. 13.9%; $P = 0.8$). The absence of a statistically significant effect has been interpreted as evidence that this lower dose is safe; however, the upper limit of the reported confidence interval of the hazard ratio (1.07, 98.7% CI: 0.48–2.37; adjusted for differences in baseline factors, and CI taking into account multiple comparisons and interim analysis), only excludes a 2.4-fold increase in the hazard of treatment failure. Again, there were no evident effects of folic acid on haemoglobin concentration at the end of the study.

In a trial among pregnant women in The Gambia (Mbaye *et al*, 2006), folic acid was supplemented at a daily dose of 0.5–1.5 mg, depending on initial haemoglobin concentration [its distribution (mean, range: 97, 61–158 g/l) suggests that most women in this trial received a daily dose of 0.5 or 1.0 mg]. There was no evidence that folic acid increased treatment failure risk at 14 days after administration of sulfadoxine-pyrimethamine (5.7% vs. 4.9% in the placebo group; risk difference: 0.7%, 95% CI: –2.2% to 3.7%). Caution should be exercised in extrapolating this finding over geographical areas or time. At the time that this trial was undertaken (2002–2003), *P. falciparum* was still highly susceptible to sulfadoxine-pyrimethamine in West Africa, which is reflected in the low risk of treatment failures reported. Folic acid had a statistically significant but negligible effect on haemoglobin concentration (1.4 g/l, 95% CI: 0.1–2.7 g/l).

In a small placebo-controlled trial among US soldiers in Vietnam treated with pyrimethamine, sulfisoxazole and chloroquine for *P. falciparum* malaria, folic acid (5 mg) or

folinic acid (5 mg for 5 days) improved haematocrit values, leucocyte counts and platelet counts without evidently delaying the time to clearance of parasitaemia (Tong *et al*, 1970). These findings are difficult to interpret, however, because all patients received a therapeutic regimen of chloroquine, which may have precluded detection of possible interference of the folate derivatives with antifolates.

In a randomized trial among Tanzanian children aged 1–35 months (Sazawal *et al*, 2006), daily supplementation with folic acid (50 μ g) and iron increased rates of hospital admission and all-cause mortality (combined endpoint) by 12% (95% CI: 2–23%). Because sulfadoxine-pyrimethamine was used for first-line malaria treatment, it cannot be excluded that the adverse events were attributable at least in part to supplemental folic acid.

Folate status

In Malawian children aged 0.5–12 years, high blood folate concentrations were prognostic for treatment failure with sulfadoxine-pyrimethamine (odds ratio: 1.50, 95% CI: 1.08–1.98; adjusted for other risk factors) (Dzinjalumala *et al*, 2005). Unfortunately, the cut-off value to define high blood folate concentrations was not specified in this study. In a randomized trial among Kenyan pregnant women (Van Eijk *et al*, 2008), high folate status was defined as plasma folate concentrations >15.4 μ g/l. Such high status occurred in 3% of women at baseline, and was associated with a marked risk of treatment failure with sulfadoxine-pyrimethamine (hazard ratio: 4.08, 95% CI: 1.67–10.00) (Van Eijk *et al*, 2008).

Evidence from *in vitro* studies, animal studies and observational studies in humans suggest that increasing folate status and intake is associated with increased risk and progression of malaria independent of effects on antimalarial drug efficacy (reviewed by Nzila *et al*, 2016). This effect of intake may be mediated by an increase in folate species other than 5-methyl-THF. Alternatively, and similarly to what has been shown with iron supplementation (Clark *et al*, 2014), folate can lead to a transient increase in reticulocyte numbers, which are favoured by *Plasmodium* species for invasion and reproduction.

Flour fortification

Internationally recommended levels of folic acid fortification vary by country between 1 and 5 mg/kg flour, depending on per capita flour consumption, and were set at the highest possible levels without exceeding the UL (WHO/FAO/United Nations Children's Fund [UNICEF]/Global Alliance for Improved Nutrition [GAIN]/Micronutrient Initiative [MI]/Flour Fortification Initiative [FFI], 2009; Berry *et al*, 2010). In many countries, implementation of these guidelines can be expected to result in a median daily intake of at least 200 μ g in women of reproductive age (H. Verhoef, unpublished data, available upon request).

In the USA, the level of folic acid fortification was set at 1.4 mg/kg cereal grain product, which was expected to raise the average daily intake of folic acid in women of reproductive age by 100 µg (Eichholzer *et al*, 2006). Data collected before and after the start of mandatory fortification (1998) indicate, however, that (i) typical intakes of folic acid from fortified foods were more than twice the level originally predicted (Choumenkovitch *et al*, 2002; Quinlivan & Gregory, 2003; Bailey, 2004); (ii) the median serum folate concentration in the US population more than doubled from 5.5 µg/l (12 nmol/l) in the pre-fortification period (1988–94) to 13.1 µg/l (29.7 nmol/l) in the early post-fortification period (1999–2000) (Berry *et al*, 2010; Yetley & Johnson, 2011); (iii) concentrations of unmodified folic acid in circulation increased (Kalmbach *et al*, 2008). Thus the experience with fortification in the USA strongly suggests that internationally recommended fortification levels will result in increased concentrations of 5-methyl-THF and chronic presence in circulation of unmetabolized folic acid in a substantial proportion of the population.

The WHO recommendation that erythrocyte folate concentrations in women of reproductive age should exceed 400 µg/l (906 nmol/l) to achieve the greatest reduction of neural tube defects (WHO, 2015a) is based on findings that also suggested a corresponding threshold for serum folate concentration of 7 µg/l (15.9 nmol/l) (Daly *et al*, 1995). Thus, given the variability in serum folate concentrations, it seems likely that flour fortification actually attained or targeted to prevention of neural tube defects will result in a substantial proportion of African women transiently or chronically exceeding the threshold serum folate concentration of >15.4 µg/l that has been associated with treatment failure for sulfadoxine-pyrimethamine (Van Eijk *et al*, 2008).

Discussion and conclusions

In contradiction to the long-held assumptions that have driven past recommendations on folate administration, there is no compelling evidence that folate deficiency anaemia constitutes a public health problem in children, pregnant women attending antenatal clinics, or individuals with sickle cell disease. By itself, this lack of evidence raises questions about recommendations to use folic acid in supplements, supplementary foods and point-of-use fortificants that are provided to these groups. In addition, there is substantial evidence that adherence to current guidelines about folic acid supplementation may cause failure of chemoprevention against malaria. The efficacy of antifolate drugs against *Plasmodium* is maximized in the absence of exogenous folic acid, so that there may not be a minimum safe dose.

Reports that high circulating folate concentrations predict treatment failure with sulfadoxine-pyrimethamine administered to pregnant women, and may even cause malaria without mediation by antimalarial drugs, may have far-reaching implications for the safety of current flour fortification

recommendations. Monitoring of flour fortification programmes has been recommended but will not be helpful: even a modest increase in malaria rates is likely to go undetected given the spatial and temporal variability in malaria incidence in endemic areas, and attribution will be impossible in uncontrolled conditions.

In its recent guidelines, the WHO recommends that supplements with iron and folic acid in malaria-endemic areas should be provided in conjunction with adequate public health measures to prevent, diagnose and treat malaria. Even with the progress that has been made in controlling malaria in the last decade, however, the efficacy and coverage of such measures is inadequate in most, if not all, settings because: (i) in areas of stable malaria, and under trial conditions, insecticide-impregnated mosquito nets can reduce overall child mortality by one-sixth and it can reduce the incidence of uncomplicated malarial episodes by half (Lengeler, 2004); (ii) only two-thirds of children aged below 5 years in sub-Saharan Africa sleep under insecticide-treated nets, whilst vector resistance to pyrethroids – the only class of insecticides available for mosquito net impregnation – is spreading and intensifying to an extent that they may be approaching the end of their useful lifespan; (iii) indoor residual spraying protects only 6% of the population in all of sub-Saharan Africa, with coverage declining in recent years (WHO, 2015b); and (iv) in 2014, 84% of children aged <5 years with malaria did not receive appropriate drugs, primarily because a high proportion of children with fever are not taken for care, or use the informal private sector (WHO, 2015b).

Formal risk-benefit analysis is needed to establish to what extent the prevention of neural tube defects resulting from flour fortification with folic acid outweighs the risk of malaria in the overall population.

Further studies are also needed to establish to what extent 5-methyl-THF is a safe, affordable and practicable alternative to folic acid in malaria-endemic areas (Nzila *et al*, 2014). The evidence suggesting that oral 5-methyl-THF may act as a source of folates in humans without antagonising the activity of antifolate drugs against *P. falciparum* (Nduati *et al*, 2008) is promising but it is derived from a single *in vitro* study with only two *P. falciparum* laboratory strains.

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Competing interests

The authors have declared that they have no competing interests.

Author contributions

HV prepared a first draft of the paper. All authors critiqued and approved the final manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. WHO policies to increase folate status.

Fig S1. Legislative status of flour fortification with folic

acid in African countries, as of November 2016.

Table S1. Recommendations by the World Health Organization on folic acid interventions.

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