

Review article

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Is 5-methyltetrahydrofolate an alternative to folic acid for the prevention of neural tube defects?

Abstract: Women have higher requirements for folate during pregnancy. An optimal folate status must be achieved before conception and in the first trimester when the neural tube closes. Low maternal folate status is causally related to neural tube defects (NTDs). Many NTDs can be prevented by increasing maternal folate intake in the preconceptional period. Dietary folate is protective, but recommending increasing folate intake is ineffective on a population level particularly during periods of high demands. This is because the recommendations are often not followed or because the bioavailability of food folate is variable. Supplemental folate [folic acid (FA) or 5-methyltetrahydrofolate (5-methylTHF)] can effectively increase folate concentrations to the level that is considered to be protective. FA is a synthetic compound that has no biological functions unless it is reduced to dihydrofolate and tetrahydrofolate. Unmetabolized FA appears in the circulation at doses of >200 µg. Individuals show wide variations in their ability to reduce FA. Carriers of certain polymorphisms in genes related to folate metabolism or absorption can better benefit from 5-methylTHF instead of FA. 5-MethylTHF [also known as (6S)-5-methylTHF] is the predominant natural form that is readily available for transport and metabolism. In contrast to FA, 5-methylTHF has no tolerable upper intake level and does not mask vitamin B₁₂ deficiency. Supplementation of the natural form, 5-methylTHF, is a better alternative to supplementation of FA, especially in countries not applying a fortification program. Supplemental 5-methylTHF can effectively improve folate biomarkers in young women in early pregnancy in order to prevent NTDs.

Keywords: 5-MethylTHF; neural tube defects; pregnancy.

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Introduction

Neural tube defects (NTDs) are serious congenital birth defects affecting the brain or the spinal cord. NTDs arise as a consequence of the failure of or delay in the fusion of the neural tube early in embryogenesis (days 22–28 after conception). Failure to close the neural tube can cause NTDs at levels of the body axis that undergo primary neurulation (the brain and the cervical, thoracic, lumbar, and upper sacral spine). A significant number of first occurrences [28, 45, 143] or recurrent NTDs [80] can be prevented by periconceptional supplementation of folic acid (FA) in mothers. The percentage reduction of the NTD risk by supplementing with FA depends on both the genetic background and other factors such as dietary folate intake or deficiency of other related micronutrients in a certain population. Since NTDs are multifactorial, not all cases can be prevented by supplementing with FA.

Folate functions and requirements

Folate (vitamin B₉ or vitamin B₁₁) is a water-soluble B-vitamin that functions as an acceptor or donor of one-carbon groups. 5-Methyltetrahydrofolate [5-methylTHF, (6S)-5-methyltetrahydrofolate, or L-5-methyltetrahydrofolate] is the most available folate form in plants [110], human plasma [82], and human whole blood [67]. 5-MethylTHF constitutes 95–98% of folate in serum or red blood cells (RBCs) [67].

Folates play crucial roles during cell growth and division. They are involved in the *de novo* synthesis of thymidylate and purine nucleotides and in delivering methyl groups (Figure 1). 5-MethylTHF donates a methyl group to homocysteine (Hcy) that is converted into methionine and further to S-adenosylmethionine (SAM), the main methyl donor in the cell. The methylation of Hcy is mediated by the vitamin B₁₂-dependent enzyme, methionine synthase. 5-MethylTHF is formed from the reduction of methylene-THF in a nicotinamide adenine dinucleotide phosphate-dependent reaction catalyzed by methylenetetrahydrofolate reductase (MTHFR). Folate deficiency causes a wide range of diseases like anemia, depression,

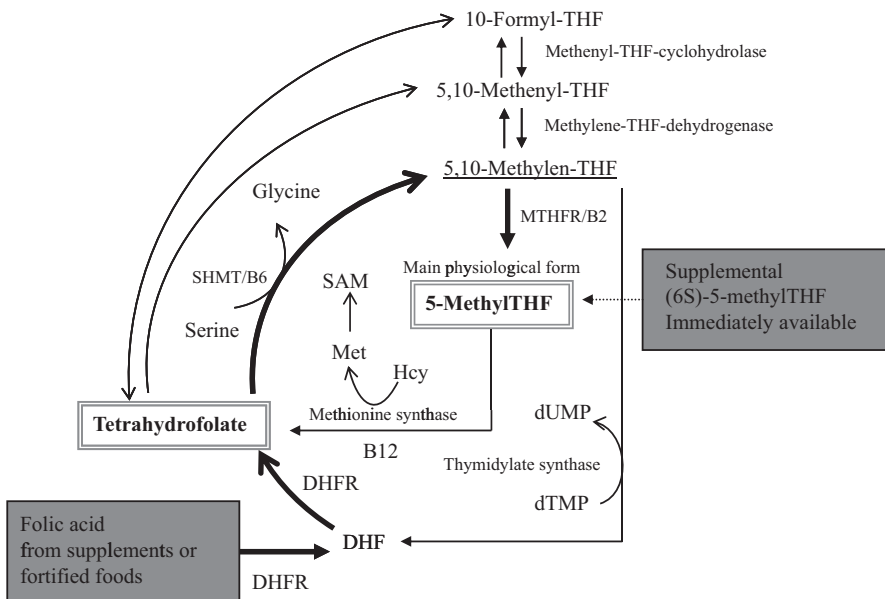


Figure 1 Folate and folic acid metabolism.

MTHFR=methylenetetrahydrofolate reductase, DHFR=dihydrofolate reductase, Hcy=homocysteine, DHF=dihydrofolate, Meth=methionine, SAM=S-adenosylmethionine, SHMT=serine hydroxymethyl transferase.

pregnancy complications, and poor pregnancy outcomes. Folate deficiency disrupts DNA synthesis and methylation, and causes hyperhomocysteinemia. Since hyperhomocysteinemia is related to pregnancy complications and poor outcome [52, 142], its concentrations should be as low as possible during pregnancy.

The dietary reference intake for folate for adults is 400 µg of dietary folate equivalents (DFEs) [86, 111]. The main sources of folate in the diet are green leafy plants. Because of the active transport to the child (via the placenta or the milk), pregnant and lactating women have higher requirements for folate.

Folates differ in the one-carbon substituent, the number of polyglutamate residues, and the oxidation state (the natural forms are reduced derivatives). In serum or plasma, folates are present as monoglutamates. Folic acid is the synthetic oxidized form of the vitamin that is not found in fresh natural foods and has no physiological function. Supplemental FA must be reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) to be able to enter the folate cycle and act as a co-factor and a source for methyl groups in the cell. In contrast to natural forms of folate in the diet, FA is more stable upon exposure to heat. These features have facilitated its use in supplements and fortified foods. The tolerable upper intake level (UL) for FA is 1 mg/day [58]. Supplemental 5-methylTHF is readily absorbed and utilized [13] and has no UL [58].

Further nutrients can support the folate/methionine cycle. For example, vitamin B₆ is a cofactor for the serine

hydroxymethyltransferase (SHMT) that supports the folate role in thymidylate synthesis. Moreover, vitamin B₂ (riboflavin) is the precursor of flavin mononucleotide and flavin adenine dinucleotide that function as cofactors for methionine synthase reductase and MTHFR, respectively. Additionally, the role of 5-methylTHF in delivering methionine and SAM is vitamin B₁₂-dependent. Moreover, choline via its oxidation product, betaine, is also a methyl donor that is required for Hcy methylation to methionine via betaine homocysteine methyl transferase. Therefore, vitamins B₆, B₁₂, and B₂, and betaine or choline are nutrients that interrelate to folate metabolism and may affect the NTD risk. Many supplements contain a combination of FA, B₁₂, B₆, and other nutrients. Multivitamins have the potential to eliminate deficiencies of nutrients that affect folate metabolism. From this point of view, multivitamins [28] can be more effective than FA alone, especially in populations with common deficiencies such as vegetarians or smokers.

Folate homeostasis

The exact mechanism of folate or FA transfer from the small intestine to the blood is poorly understood. Most of the ingested low doses of FA will be metabolized in the liver after absorption. DHFR is expressed in the liver and other tissues [3, 140]. Liver DHFR can bind FA but has a limited capacity for FA reduction [3]. Unmetabolized FA

may pass unchanged into the peripheral circulation at doses of FA >200 µg [65].

The hydrolysis of polyglutamate derivatives of THF (mainly 5-methylTHF) into monoglutamate derivatives in the gut by the brush-border enzyme glutamate carboxypeptidase II is a prerequisite for their intestinal absorption. Unlike folate absorption, folate retention in tissues depends on the ability of the cell to form polyglutamate derivatives [40].

There are three folate transporting proteins: the reduced folate carrier (RFC1), the proton-coupled folate transporter (PCFT), and folate receptor (FR). The three systems have distinct tissue distribution and affinities for the different folate forms at different pH levels. The PCFT [93] is highly expressed in the small intestine and favors 5-methylTHF over FA in physiological conditions. After a folate-rich diet, PCFT seems to be the main transporter responsible for folate absorption in the intestine. The folate receptor represents a family of three proteins (FR α , β , and γ) that are expressed on the apical side of some epithelial cells [63]. RFC1, PCFT, and FR are expressed in the placenta. The exact transport pathway of folate from the maternal to the fetal side is not known. FR seems to play a major role in folate homeostasis during early embryogenesis, since disrupting FR α causes NTDs or even lethality in the embryos [108]. The affinity of FR α for FA is much higher than for 5-methylTHF [141], but FR α contribution to tissue folate is not yet known.

Blood biomarkers of folate reflect folate status and intake

The fasting serum concentration of folate is a good marker for folate status, but it is affected by recent folate intake and may fluctuate if folate intake is not constant. Measuring the serum concentration of folate is particularly useful as an early marker that shows folate depletion or repletion (after dietary modification or supplementation). Serum folate is the strongest negative determinant of plasma total homocysteine (tHcy) concentrations in pregnant women [53]. The concentration of whole blood folate (RBC folate) is an indicator for folate storage, and this marker takes longer than serum folate to reach a steady state after folate supplementation (approximately 40 weeks) [92].

The depletion of plasma or erythrocyte folate takes at least 3 months (for plasma folate) to 9 months (for RBC folate). Compared to the levels just after 6 months of supplementation with 400 µg FA/day, 3 months of folate depletion lowered plasma folate by approximately 50%

(geometric mean changed from 34.5 to 16.6 nmol/L) [49]. Levels of plasma folate after 3 months of depletion (geometric mean 16.6 nmol/L) remained 73% higher than the starting levels (geometric mean 9.6 nmol/L) where participants received no supplements at baseline [49]. After a 3-month washout period, concentrations of RBC folate remained 20% higher than pre-supplementation levels (725 vs. 603 nmol/L) [49].

A recent meta-analysis quantified the dose-response relationship between folate intake (dietary folate plus FA) and folate biomarkers in young, pregnant, and lactating women [12]. The aim was to establish a recommended intake for optimizing folate biomarkers for women planning for pregnancy. In women aged 20–35 years, Wald et al. [127] estimated that an increase in folate intake of 100 µg/day would increase serum folate concentrations by 0.94 µg/L (95% confidence interval 0.77–1.10) (2.13 nmol/L per 100 µg/day). Berti et al. [12] estimated that a 100 µg/day total intake would increase serum folate level by 3.3 nmol/L. Lamers et al. [69] estimated that, for a 100 µg [6S]-5-MTHF supplementation over 24 weeks, plasma folate concentrations increased by 9.6 nmol/L. A doubling of total folate intake increased the folate concentration in serum and RBC by 47% and 23%, respectively, and lowered plasma tHcy concentration by 7% [12]. Berti et al. [12] observed a weaker dose-response relationship between folate intake and folate markers in pregnant and lactating women, suggesting that during these physiological periods maintaining maternal folate biomarkers at a given level is more difficult. This might be related to maternal folate depletion and higher requirements. In line with this, supplemental FA in folate-deficient women was transported to the infant via the milk in preference even to the maternal hemopoietic system [77].

Folate and B₁₂-related metabolites in cord blood

Maternal vitamin status is the main determinant of the status in neonates, suggesting that improving maternal vitamin status ensures better vitamin status in the newborns. In accordance with this, maternal and cord blood B-vitamins are strongly correlated [84]. 5-MethylTHF is the main folate form in cord blood (mean 89.4% of total folate) (Figure 2) [82]. The concentration of 5-methylTHF in cord serum is approximately two times higher than in maternal serum (mean 35.8 vs. 15.6 nmol/L) [82], suggesting that supplementing with 5-methylTHF during pregnancy can

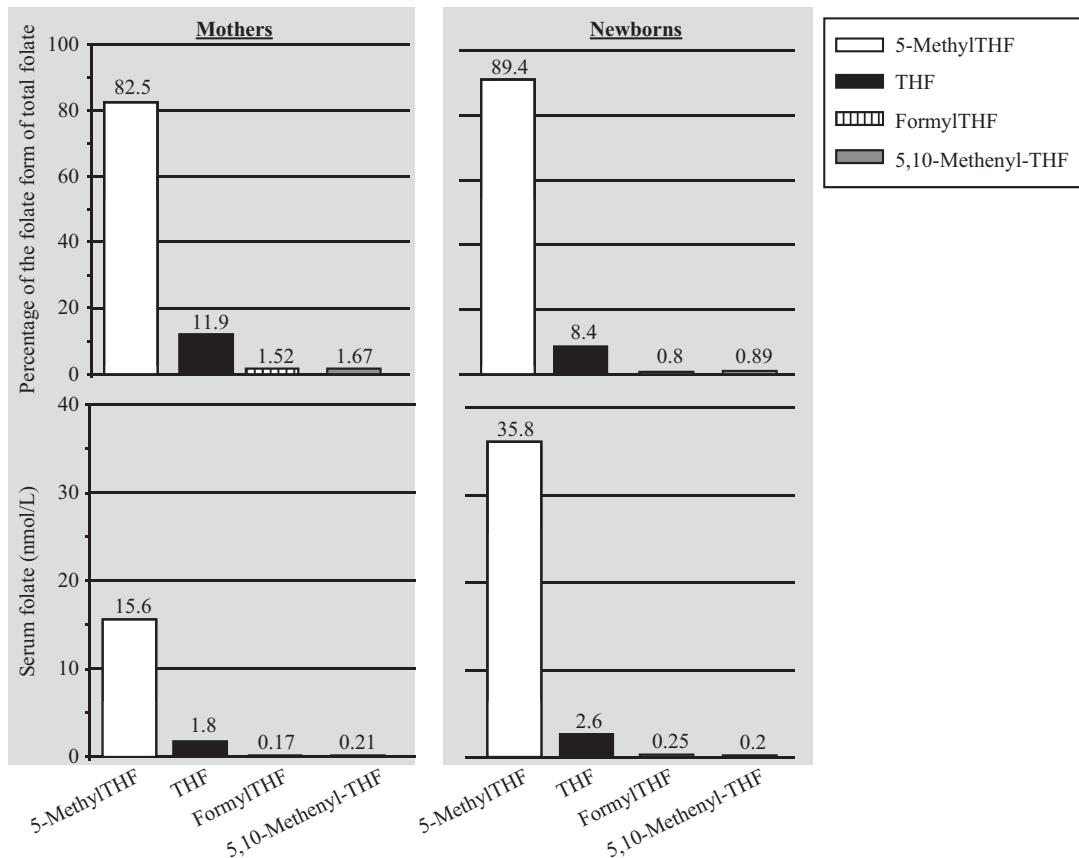


Figure 2 Concentrations of the main folate forms and their percentage of total folate in maternal serum and umbilical cord serum from neonates [82].

provide an immediate source for folate to be transported to the fetus.

The risk of neural tube defects is causally related to low maternal folate

Folate deficiency is causally related to NTDs and a few other birth defects [95]. A stepwise dose-response relationship was observed between NTD risk and low plasma or RBC folate in pregnant women [32] or low folate intake [103, 131]. Plasma folate ≥ 16.0 nmol/L (or RBC folate >906 nmol/L) was related to the lowest risk in one study on Irish pregnant women in their first trimester [32]. However, plasma and RBC folate decrease during pregnancy [53], suggesting that the preconception target level of serum folate should be higher than 16.0 nmol/L. Clinical studies showing pre-pregnancy serum folate levels necessary for NTD prevention are not available.

The mean serum folate level reached after supplementing with FA or 5-methylTHF is approximately 50 nmol/L [69]. This level can ensure optimal folate status for optimal prevention [127].

Because of the limited time window for prevention of NTDs, folate supplementation for young women is required to increase serum folate concentrations. Women of childbearing age should ensure a daily intake of at least 400 μg of folate for at least 4 weeks before and 12 weeks after conception to reduce the risk of having a child with NTD [22]. Up to June 2010, 53 countries had regulations regarding FA fortifications of wheat flour. The fortification aims to provide an estimated 200 μg FA/day in addition to food folate. The exposure of the entire population to additional FA is controversially discussed [87, 126]. There is currently an intensive discussion on optimizing the folate status of the target population (young women) without exceeding a certain intake in the population. Targeted supplementation of young women with folate in countries not applying mandatory FA fortification programs (as in Europe) has become extremely important [126].

Protective effects of folate beyond NTD prevention

Improving maternal folate status can protect against other birth defects like congenital heart defects [27, 119] and orofacial clefts [64, 116]. For example, the incidence of severe congenital heart defects decreased by 6.2% yearly after starting FA fortification in Canada [59]. The association between orofacial clefts (cleft lip and cleft palate) and maternal use of FA was not confirmed by all studies [50, 60]. The prevention of orofacial clefts may be dose-dependent, since studies using higher doses of FA (5–10 mg) found a preventive effect [31, 116], whereas studies using lower doses did not find such an effect [27]. A significant relationship between FA intake and higher birth weight was recently reported [30, 37, 88]. However, this relationship is probably dose-dependent [37] or may be related to extension of the gestational age or prevention of preterm birth [30].

Elevated plasma concentrations of tHcy or low folate concentrations during pregnancy were related to low birth weight and preterm birth [10], pregnancy complications [10, 52], or abortion [81], suggesting that folate may exert a protective effect by lowering tHcy [130]. Since FA supplementation was positively associated with birth weight [102, 115], preterm births may benefit from FA supplementation if they are born with a higher birth weight.

The association between folate status and depression, and the effect of folate administration in the treatment of depression have been addressed by several studies [72, 128]. Supplementation of FA [24] or 5-methylfolate [36] may enhance the antidepressant action of certain medications. Therefore, 5-methylTHF may support the treatment of depression during pregnancy and after delivery, but there is currently no firm evidence on a preventive effect for FA or 5-methylTHF against depression.

Genetic polymorphisms affect folate bioavailability, utilization, and requirements

The 677C→T single nucleotide polymorphism in the *MTHFR* gene is found in ≈10–22% of the European population. Individuals who are homozygous for this polymorphism have higher tHcy concentrations [104, 107] and lower folate concentrations (plasma or whole blood); they show less response to FA supplementation [25], and women have an increased risk for NTDs [121, 122]. Higher

folate status stabilizes the mutated enzyme and increases its activity by increasing the affinity of the enzyme for its flavin adenine dinucleotide coenzyme [136].

Young women with the *MTHFR* 677TT genotype are more sensitive to folate depletion in short-term studies (7 weeks) [104] and long-term studies (3 months) [25]. Individuals with the TT genotype also show less response to folate repletion compared to those with the CC genotype [25, 104]. A 7-week repletion phase (dietary folate intake 400 µg DFE/day) corrected serum and RBC folate concentrations to the baseline values [104]. The concentrations of tHcy remained higher and plasma folate lower in women with TT compared to those with CC genotype after FA supplementation [25], suggesting that the *MTHFR* genotype influences the benefit from FA supplementation.

Another important polymorphism that has been studied in relation to folate metabolism and NTD is the dihydrofolate reductase (DHFR) 19-bp deletion polymorphism [a 19-bp deletion of intron 1a (*DHFR19bpdel*); rs70991108] [90]. The association between this polymorphism and the NTD risk was inconsistent between the studies [61, 90, 120]. The DHFR in human liver-cell homogenates shows a wide range of activity between individual samples [3], suggesting large differences in an individual's ability to reduce FA. A limited ability of the mutated enzyme to reduce FA (at high FA intake ≥500 µg/day) caused higher unmetabolized FA in blood [62]. The mutated enzyme (del/del) was associated with lower whole blood folate in individuals receiving <250 µg/day FA [62]. The effects of the genotype on plasma or RBC folate or tHcy are currently controversial [42, 109].

The *MTHFD1* catalyzes the biosynthesis of 10-formylTHF in the cytoplasm. The 1958G→A polymorphism in *MTHFD1* causes lowered enzyme activity and impaired *de novo* synthesis of purine [23]. The mutation has been linked to several birth defects including NTDs [17, 89]. In an animal model, maternal *MTHFD1*gt/+ genotype showed a 50% decrease in 10-formylTHF synthesis and impaired fetal growth, disrupted folate metabolism, and purine biosynthesis in the fetus, but did not cause NTDs [8].

Serine hydroxymethyltransferase SHMT1 is involved in thymidylate biosynthesis primarily in the cytoplasm and the nucleus [1]. Beaudin et al. [7] showed that mice embryos with disrupted *Shmt1* exhibited failure of neural tube closure especially under maternal folate and choline deficiency. In contrast, maternal disruption of *Shmt1* did not cause low RBC folate level, elevated tHcy level, or NTD phenotype in the embryo. In this study, maternal folate and choline-deficient diet rather than maternal *Shmt1* genotype were related to NTD [7].

Taken together, polymorphisms in the folate cycle predispose to a higher risk of birth defects when the maternal folate status is limited. The effect of the polymorphisms is probably insignificant at a higher maternal folate status. Available evidence strongly suggests that folate deficiency and FA supplementation may have different metabolic effects in a genetically susceptible subset of the population (for example, MTHFR TT carriers) [25, 104]. This effect can be related to the limited availability of the active folate. The direct administration of (6S)-5-methylTHF offers advantages, since it is directly available and does not need to be metabolized.

Strategies to prevent NTDs by improving maternal folate status

Low dietary folate intake is related to low consumption of folate-rich foods, long storage of the folate-containing foods, and a reduction in vitamin content during food processing [57]. Mean folate intake in European populations ranges from 180 to 280 $\mu\text{g}/\text{day}$ [5, 29, 139], which is not sufficient to prevent folate-responsive NTD cases. In addition, factors like smoking, very young age, and lack of knowledge about the importance of folate supplementation before pregnancy can adversely affect the folate status and increase the risk of having a child with a NTD.

It is recommended that all women of childbearing age receive 0.4–0.8 mg of FA per day in order to prevent NTDs. Because of the limited time window for prevention and the low compliance, the recommendation for young women failed to achieve the goal in most countries [6, 35]. Moreover, the optimal dose of FA depends on baseline folate, the folate intake, the MTHFR polymorphism, smoking, and the time window available to reach preventive serum levels. The fortification of grain products with FA (in the USA 100 $\mu\text{g}/100$ g grain products) was thought to increase folate intake by approximately 200 $\mu\text{g}/\text{day}$ [94]. Fortification with FA increased folate status in the whole population, but this effect seems not to be sufficient in the target group [4, 91]. Moreover, increasing FA intake caused the appearance of unmetabolized FA in the blood [83, 113]. Unmetabolized FA was detected in newborns and in 4-day-old formula-fed infants [112]. FA may affect the immune system. For example, unmetabolized FA was associated with reduced natural killer (NK) cell toxicity [117]. NK cells are regarded to be the first line of defense in the prevention of carcinogenesis and viral infections. Furthermore, FA supplementation during pregnancy has also been discussed in relation to an increased risk of

respiratory illness in children [132], although this association has not been confirmed by other studies [74, 75].

There is currently no firm evidence indicating that FA is transported or accumulated in the fetus, nor of a firm relationship with disease phenotypes after birth. However, no studies are currently available on concentrations of unmetabolized FA in cord blood from women taking higher doses of FA (4–5 mg/day) for the prevention of recurrent NTDs. The appearance of unmetabolized FA in cord blood can be avoided by supplementing with the natural folate form, 5-methylTHF.

Another concern in people with high intakes of supplemental FA is the masking of vitamin B₁₂ deficiency. This is not the case when supplementing with 5-methylTHF. Since vitamin B₁₂ is required for folate metabolism, vitamin B₁₂ deficiency can cause a folate trap. High doses of FA can correct hematological signs of vitamin B₁₂ deficiency and can delay the diagnosis of B₁₂ deficiency, thus increasing the risk of developing neurological complications. In contrast, 5-methylTHF supplementation given to B₁₂-deficient individuals cannot be utilized for methionine or folate cycles and cannot mask vitamin B₁₂ deficiency.

The purpose of the food fortification programs is to increase folate intake in young women, but the intake has been increased in the whole population [87]. Targeted administration of vitamin supplements is the most effective way to specifically increase folate intake in women of child-bearing age before and during pregnancy when folate requirements are high. This is particularly important in countries that do not apply the fortification.

Natural folate can prevent folate-responsive neural tube defects

Poor maternal diet has been related to the occurrence [96, 106] or recurrence [71] of NTDs. In particular, low dietary folate was related to a higher NTD risk [41]. Folate intake in the first 6 weeks of pregnancy was particularly protective [16]. This relationship has been shown to be dose-dependent [131]. The lowest risk was found in women with total folate intake >350 $\mu\text{g}/\text{day}$ [16]. Moreover, the dietary folate intake and supplement usage early in the second trimester were related to the pregnancy outcome in a study of >23,000 women [79]. The risk reduction of NTDs was 0.78 for every 500 μg increase in total folate intake (dietary plus supplemental) [79].

Low dietary folate intake is the main determinant of low serum or RBC folate concentrations and of high plasma concentrations of tHcy. In one prospective study

of 56,049 Irish pregnant women, maternal blood samples were available from 81 women with NTD-affected pregnancies and 247 pregnant women as controls [66]. Compared to the controls, lower concentrations of plasma folate (7.9 vs. 10.4 nmol/L; $P=0.002$) and RBC folate (609 vs. 766 nmol/L; $P<0.001$) were found in mothers of cases compared with mothers of controls [66]. A continuous dose-response relationship between NTD risk lowering and maternal RBC folate was observed in the following analyses of results from the same population [32].

The differences in the incidence of NTDs and folate-responsive NTDs are partly attributed to differences in dietary folate intake between populations [11]. For example, geographical differences have been reported in the incidence of NTDs in China [11]. After promoting FA supplementation prenatally, the NTD incidence decreased in north and south China by approximately 75% and 40%, respectively [11, 51]. In line with this, strong differences between south and north China in serum folate levels (in women aged 35–44 years: 19.9 vs. 9.7 nmol/L) and RBC folate levels (in women aged 35–44 years: 911 vs. 508 nmol/L) were later reported [48].

Therefore, dietary folate is associated with higher serum folate concentrations and a lower NTD risk. The risk reduction after FA supplementation is probably due to 5-methylTHF, the active natural form and the dominant folate form in plasma or RBCs. The fact that 5-methylTHF is the major folate form in many foods can explain the association between a higher dietary folate supply and the NTD risk reduction [69].

Handling natural folates versus folic acid

Folates from natural sources are polyglutamated derivatives (mainly 5-methylTHF) [18] that must be hydrolyzed before absorption in the ileum can take place. The food matrix affects folate absorption from natural foods [43, 76]. The bioavailability of food folates showed large variability in short-term studies (12–24 h) [44, 85]. Other physiological factors (genetic polymorphisms, baseline folate status, status of the related nutrients) can affect folate bioavailability (both dietary folate and FA) [19, 46, 99, 134].

Consumption of folate-rich foods over 4–6 weeks improves serum and blood folate concentrations (by up to 50%) and decreases plasma concentrations of tHcy by up to 29% [18, 56, 97, 105]. Young women who were consuming 800 DFE/day from natural sources for 12 weeks showed a 67% increase in serum folate concentrations and a 33%

increase in RBC folate concentrations compared with women consuming 400 DFE/day [56]. Similarly, Brown et al. [20] found that RBC folate concentrations in the range considered to be protective against NTD (>906 nmol/L) were primarily found in women who took approximately 400 μg FA. Individuals not receiving supplemental FA require 500–600 $\mu\text{g}/\text{day}$ of total folate to achieve a plasma tHcy level of 10.0 $\mu\text{mol}/\text{L}$ [118], but serum folate level under these conditions (15 nmol/L) seems below the optimal range for NTD prevention [118]. In one study in a Dutch population, the dietary intake in the majority of women (200 $\mu\text{g}/\text{day}$) was not sufficient to reach plasma tHcy concentrations of below 10.0 $\mu\text{mol}/\text{L}$ [34], suggesting that current folate intake does not provide maximum protection against NTDs in this European population.

Individuals with the MTHFR 677 TT genotype require a higher intake of folate in order to achieve similar tHcy concentrations as those in individuals with the MTHFR 677 CC genotype [2, 25, 78]. One study has shown that a total folate intake of approximately 660 μg DFE/day derived mainly from fortified cereals was necessary to achieve near-normal plasma tHcy concentrations in adults with the MTHFR 677TT genotype [2].

In a 6-month placebo-controlled study, the effect of FA (100, 200, or 400 μg daily) on RBC folate concentrations was tested in 121 women [33]. RBC folate levels increased in all supplementation groups. The median (95% confidence interval) of post-treatment RBC folate level was 1293 (1098–1481) nmol/L in the group with 400 $\mu\text{g}/\text{day}$ of FA and was 1076 (978–1139) nmol/L in the group that received 200 $\mu\text{g}/\text{day}$ [33].

Long-term supplementation with 200 μg FA/day, a dose similar to the current intake from fortified foods [94], was as effective as 400 μg in lowering tHcy concentrations [114]. Ashfield-Watt et al. [2] found that supplementation of 400 μg of FA was able to lower tHcy concentrations to a similar extent as those of food folate. After 4 months, plasma concentrations of folate were higher in the FA group compared with the dietary folate group [2]. The effectiveness of increasing RBC folate concentrations by means of increasing folate intake from natural sources versus fortified foods or supplemental FA has been tested in a small study on young women [26]. After 12 weeks, changes in RBC folate concentrations were highest in the supplement and fortified food groups [26], whereas 10 women showed no changes in RBC folate after dietary modifications [26]. Very low doses of FA (50 and 100 $\mu\text{g}/\text{day}$) were tested [98, 123]. In short-term studies (6 weeks) [98], 400 μg FA/day was defined as the minimum dose for adequate tHcy lowering. A meta-analysis of previous dose-finding studies concluded that

800 µg/day was the optimal dose [73]. Studies on genetic modifications in the folate cycle confirmed that the role of folate in preventing NTDs may go beyond a tHcy-lowering effect [8].

The different bioavailability and metabolism of FA and dietary folate cause imprecise estimation of folate requirements especially from foods fortified with FA [100]. The estimated bioavailability for food folate is 50–98% of that for FA [47, 101, 133, 137]. Studies on bioavailability applied different time intervals and depended on different tracing approaches like using isotopes [101] and measuring blood or serum folate, or metabolic markers [18]. Furthermore, unmetabolized FA may cause overestimation of the bioavailability of FA, since many methods cannot distinguish between folate forms [18]. The recovery of FA by some methods is higher than that for 5-methylTHF, thus causing overestimation of active folate [138]. Recently, the validity of the conventional approach used in bioavailability studies comparing FA with dietary food folate has been questioned [135]. Using whole diets rather than single foods has been suggested in order to test the post-absorptive metabolism of the different folate forms (including free FA) [100].

In summary, the bioavailability of food folates depends on pre-absorptive, absorptive, and post-absorptive processes. The estimated relative bioavailability of food folate is lower when compared with the supplemental FA. However, some evidence suggests that FA is not a proper reference material for the bioavailability studies and should be replaced by 5-methylTHF. After all, increasing food folate consumption is currently not an effective strategy for optimizing folate status in young women [21].

Handling methyl folate versus folic acid

The dose, form, and duration of folate intake for NTD prevention have been central topics for several years. For example, the US FDA has recently approved oral contraceptives combined with (6S)-5-methylTHF in order to reduce the risk of NTDs in women who conceive while using the pill or shortly thereafter (reviewed in Ref. [54]).

The response of folate status parameters (plasma and RBC folate) was tested after supplementing with 453 nmol/day of dietary folate and an equimolar dose of supplemental FA or the bioactive diastereoisomer (6S)-5-methylTHF for 16 weeks [135]. The increase in serum and RBC folate concentrations observed after increasing

dietary folate was less than that observed after FA or (6S)-5-methylTHF supplementation over 16 weeks [135]. Unmetabolized FA was detected in the plasma of subjects who received FA (mean 0.2 nmol/L) but not in those who received 5-methylTHF [39]. Using (6S)-5-methylTHF rather than FA was recommended as the reference folate to estimate dietary (food) folate bioavailability [135]. Accordingly, (6S)-5-methylTHF (416 µg/day) can improve [69] or maintain [55] RBC folate concentrations to a significantly greater extent than FA.

Lamers et al. [70] conducted a trial in 144 young females using 400 µg FA, 416 µg (6S)-5-methylTHF, 208 µg (6S)-5-methylTHF, or placebo for 24 weeks. Concentrations of plasma tHcy and folate were tested in 4-week intervals. An increase in plasma total folate levels and a decrease in plasma tHcy levels were seen 4 weeks after starting the trial [70]. Plasma folate continued to increase at 8 weeks, but tHcy did not continue to decline. The decline in plasma tHcy in the groups receiving 208 µg (6S)-5-methylTHF or 400 µg FA was similar. The percentage changes of tHcy after supplementation of 400 µg of FA or 416 µg of (6S)-5-methylTHF relative to the placebo group after adjustment for baseline concentration were –15% vs. –19%, respectively, ($P > 0.05$) [70]. However, the study used an immunological assay that cannot rule out unmetabolized FA measured as total folate.

In one study on lactating women, (6S)-5-methylTHF (416 µg/day, 906 nmol/day), FA (400 µg/day, 906 nmol/day), or placebo was administered for 16 weeks [55]. The mean RBC folate concentration after supplementation with (6S)-5-methylTHF (2178 nmol/L) was higher than after supplementation with FA (1967 nmol/L; $P < 0.05$) or placebo (1390 nmol/L; $P < 0.002$). Women in this study were pre-saturated with 1 mg FA/day during pregnancy [55], thus explaining the high concentrations of RBC. Studies using the plasma area under the curve (AUC) after pre-saturation of the participants may show a higher AUC [85]. In addition, after delivery, women in all three study arms seemed to be in the depletion phase [55]. The extent of the depletion as shown by lowered RBC folate after 4 weeks (8.2% vs. 11.1%) and 16 weeks (21% vs. 39%) seemed to be approximately 50% lower in the (6S)-5-methylTHF compared to the FA group [55]. Plasma tHcy was relatively stable in the three groups over 16 weeks. However, the mean plasma folate concentrations seemed to be maintained over 16 weeks only in the (6S)-5-methylTHF group. The mean plasma folate concentrations declined slightly over 16 weeks in the FA group [55]. (6S)-5-MethylTHF seemed to be slightly better than FA in maintaining RBC and plasma folate after delivery.

The effects of daily FA (100 µg) and (6S)-5-methylTHF (113 µg) were compared in a 24-week supplementation trial in middle-aged male and female subjects [124]. Plasma concentrations of tHcy decreased by 9.3% and 14.6% in the FA and the (6S)-5-methylTHF groups, respectively, compared to the placebo group [124]. In contrast, FA supplementation increased the concentrations of plasma folate to 34.5 nmol/L (52% higher than baseline) and RBC folate to 1137 nmol/L (31% higher than baseline). This increase was higher than that caused by (6S)-5-methylTHF (plasma folate 25.6 nmol/L, 34% increase; RBC folate 984 nmol/L, 23% increase) [124]. At 24 weeks, the increases in plasma and RBC folate concentrations did not differ significantly between the two supplemented groups [124]. In a study of 104 young female subjects (18–49 years), FA and (6S)-5-methylTHF increased plasma and blood folate concentrations to a similar extent [125]. Neither supplement group showed differences in the curves and did not reach a steady state after 24 weeks [125].

The estimate of the dose-response relationship showed that the association between folate status biomarkers and 5-methylTHF intake was stronger than for FA intake [12], suggesting that 5-methylTHF may be more effective in improving folate biomarkers. Despite the fact that the administered doses, sampling intervals, number of sampling occasions, and pre-saturation of volunteers

vary between trials, equimolar doses of FA or reduced folate concentrations resulted in at least equivalent metabolic response (lowering tHcy) or increasing plasma or RBC folate concentrations. The advantages and limitations of food folate, 5-methylTHF, and FA are shown in Table 1. Efficacy studies on the effect of 5-methylTHF in improving folate blood markers are available and encouraging. However, the role of 5-methylTHF in preventing NTDs or other birth defects has not been tested in clinical studies.

Conclusion

A mean serum folate level of approximately 50 nmol/L was achieved by supplementing with 400 µg of 5-methylTHF or FA for 12 weeks [69]. An optimal serum folate level for NTD prevention should be reached before conception. Dietary folate intake is low in the general population, and dietary modifications are unlikely to improve blood folate status in the target group within a short time. Moreover, women with polymorphisms in the folate cycle have higher requirements that cannot be achieved by increasing dietary folate intake over a few weeks. Despite mandatory FA fortification, the optimal folate level for prevention of NTDs could not be achieved, especially in

Table 1 Summary of advantages and limitations of food folate, 5-methylTHF, and folic acid.

	Limitations	Advantages
Food folate	Bioavailability affected by food matrix, food preparation, and co-nutrients; bioavailability affected by hydrolyzation of the polyglutamate residues; storage, washing, and cooking destroy the vitamin; normal B ₁₂ and B ₆ status is required for optimal utilization	Natural form; no upper tolerable limit It does not mask vitamin B ₁₂ deficiency
5-MethylTHF	More expensive than FA; for optimal utilization and metabolism, 5-methylTHF supplementation should be accompanied by normal B ₁₂ and B ₆ status; 5-methylTHF is currently used in some pregnancy-related supplements [68]	High bioavailability; main folate form in blood (serum or RBC) and cord serum; no need to be activated; it can directly enter the folate cycle; no upper tolerable limit in US dietary reference intakes; it does not mask vitamin B ₁₂ deficiency
Folic acid	<ol style="list-style-type: none"> 1. Causes unmetabolized FA in blood at doses >200 µg/day 2. FA is itself not active, but must be metabolized through several steps in order to enter the folate cycle 3. FA may be less reduced in carriers of the DHFR (19 bp del/del) polymorphism and can appear unmetabolized in blood 4. Upper tolerable limit is 1 mg/day 5. Controversial role of unmetabolized vitamin in plasma 6. Higher doses of FA can mask vitamin B₁₂ deficiency and delay its diagnosis by correcting hematological signs 7. Controversy about the roles of chronic high intake in cancer promotion 8. For optimal metabolism, FA supplementation should be accompanied by normal B₁₂ and B₆ status 	Low costs, heat insensitive

Table 2 Why 5-methyltetrahydrofolate is an alternative to FA.

High bioavailability	Metabolic effects of (6S)-5-methylTHF and FA on lowering tHcy are comparable in most studies. (6S)-5-methylTHF was either comparable to FA or more effective in maintaining or increasing serum or plasma concentrations of folate. Studies using the dual-label stable isotope protocol depend on measuring the urinary excretion of [¹³ C]-labeled folate or labeled reference dose of [³ H]-folic acid. The dual-label stable isotope methods produced values of 37–153% [43, 129]. These studies also showed that reduced food folates and FA are handled differently by the body, and (6S)-5-methylTHF was recommended as the reference dose [38, 85]. (6S)-5-MethylTHF can increase serum folate that reaches the range of protection for NTDs within a short time
Avoiding unmetabolized FA in blood	Unmetabolized FA in blood is found after chronic consumption of >200 µg/day and may have negative effects on the human body (NK activity, immune system). Unmetabolized FA has no biological function. Supplementation of (6S)-5-methylTHF cannot lead to the occurrence of unmetabolized FA in the blood
Women with polymorphisms show a better response with plasma folate markers to (6S)-5-methylTHF	(6S)-5-methylTHF is favored in women with polymorphisms in folate-related enzymes (especially MTHFR and DHFR). (6S)-5-MethylTHF is highly relevant in countries that do not apply fortification with folic acid
Stability of (6S)-5-methylTHF	(6S)-5-MethylTHF is stable in processed foods (cereals, white bread). (6S)-5-MethylTHF is commercially available in a stabilized form as Ca-salt (Metafolin® used in pharmaceutical preparations and food supplements) (Merck & Cie, Schaffhausen, Switzerland). For food fortification, further stability tests should be performed for different kinds of processed foods
Pregnancy and postnatal depression	(6S)-5-methylTHF may be more effective than FA
Safety of (6S)-5-methylTHF	Safety of (6S)-5-methylTHF has been confirmed by several studies: for example, Bostom et al. [15] investigated daily supplementation with 17 mg (6S)-5-methylTHF over 12 weeks with respect to reducing tHcy in hemodialysis patients. Bentley et al. [9] utilized 1.13 mg/methylTHF during pregnancy in an open-label, non-randomized design. No side effects were reported. Fava et al. [36] used 7.5 and 15 mg methylTHF/day in patients with depression
(6S)-5-methylTHF for prevention of NTDs?	Randomized controlled trials on the efficacy of (6S)-5-methylTHF in NTD prevention are not available. However, NTD prevention is related to serum or blood folate concentrations, and (6S)-5-methylTHF is at least as effective as FA in improving folate-related markers in blood

low-income and less educated women [14]. Supplementation in the preconceptional period seems to be the best effective way to improve folate status within a short time (4–12 weeks). This is particularly difficult for unplanned pregnancies.

Supplementation studies showed comparable effects for 5-methylTHF and FA in increasing serum or RBC folate concentrations. The natural form of folate, 5-methylTHF, offers several advantages compared to FA (Table 2): it does not mask B₁₂ deficiency, it is already a biologically active form, it does not cause unmetabolized FA in blood, and it is absorbed and utilized at least as well as FA. Although there are no clinical trials on the effectiveness of 5-methylTHF in preventing NTDs, metabolic studies have shown that 5-methylTHF is a biologically active form of the vitamin and it seems to be at least as effective as FA in improving folate biomarkers. The literature clearly shows that a better food folate intake

is associated with better folate markers and that food folate can prevent NTDs (by increasing folate status). FA can prevent NTDs by increasing serum or blood folate level. 5-MethylTHF can effectively increase serum or blood folate markers. Therefore, supplementing with 5-methylTHF for NTD prevention seems to be rational. In contrast to the MRC study [80], testing the efficacy of 5-methylTHF against a placebo would be unethical. Comparing the preventive effect of FA with a proposed better alternative (5-methylTHF) will require following several thousands of pregnancies over a long time. The costs of testing 5-methylTHF against FA would be extremely high. We do not have any reason to assume that a randomized controlled trial is justified before recommending 5-methylTHF.

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